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Short Communication

Rangelia vitalii and *Hepatozoon canis* coinfection in pampas fox *Lycalopex gymnocercus* from Santa Catarina State, Brazil

Infecção por *Rangelia vitalii* e *Hepatozoon canis* em *Lycalopex gymnocercus* proveniente do Estado de Santa Catarina, Brasil

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Abstract

Rangelia vitalii is a haemoparasite that infects erythrocytes, white blood cells and the cytoplasm of endothelial cells of blood capillaries of canids in South America, and has been detected in both domestic dogs and sylvatic canids. *Hepatozoon canis* is a parasite that infects neutrophils and monocytes of many mammalian hosts. This study reports the infection of *Lycalopex gymnocercus* from Santa Catarina, Brazil, with *R. vitalii* and *H. canis*. The piroplasm was observed on both blood smears and molecular tests. Many large piroplasms were detected inside the erythrocytes, with round, oval, or teardrop-shaped organism, that occurred singly or in pairs. They had an abundant, pale blue cytoplasm and decentral dark red small nucleus. The animal was also infected with *H. canis* that was detected only by molecular tests. The majority of haematological and biochemistry parameters were within the reference values for domestic dog and wild canids.

Keywords: Haemoparasites, molecular characterization, piroplasm, sylvatic canid.

Resumo

Rangelia vitalii é um hemoparasita que infecta eritrócitos, macrófagos e células endoteliais de canídeos na América do Sul, e vem sendo detectado tanto em cães domésticos quanto em canídeos silvestres. *Hepatozoon canis* é um parasita que infecta monócitos e neutrófilos de mamíferos. No presente estudo, é descrita a infecção de *Lycalopex gymnocercus*, proveniente de Santa Catarina, Brasil, por *R. vitalii* e *H. canis*. O piroplasma foi diagnosticado nos esfregaços sanguíneos e por técnicas moleculares. Nos eritrócitos foram observados vários merozoítos grandes, ovais, arredondados ou em forma de gota, ocorrendo isoladamente ou em pares. Estes piroplasmas apresentavam citoplasma abundante, corado em azul claro, com núcleo pequeno, avermelhado e descentralizado. O animal apresentou coinfecção com *H. canis*, que foi diagnosticado somente pelos testes moleculares. A maior parte dos parâmetros hematológicos e bioquímicos do animal estava dentro dos valores de referência para cães domésticos e canídeos silvestres.

Palavras-chave: Hemoparasitas, caracterização molecular, piroplasmas, canídeos silvestres.

Introduction

Rangelia vitalii was first described by Pestana (1910a, b) as a previously unknown piroplasm from dogs, named *Piroplasma vitalii*. This parasite was later named *Rangelia vitalii* by Carini & Maciel (1914). Despite the fact that *R. vitalii* has been described since the early 1900s, the species status was only recently confirmed by molecular and transmission studies (SOARES et al., 2011; LEMOS et al., 2012). This protozoan is transmitted by the ixodid tick *Amblyomma aureolatum* (FRANÇA et al., 2010) and cannot be distinguished from *Babesia* sp. infection in erythrocytes.

Canine rangeliosis has been reported in South America in domestic dogs in the south eastern and southern regions of Brazil (LORETTI

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& BARROS, 2005; FRANÇA et al., 2010; LEMOS et al., 2012; MOREIRA et al., 2013), Argentina (EIRAS et al., 2014), and Uruguay (SOARES et al., 2015), and was also found to infect sylvatic canids. For example, Soares et al. (2014) detected the infection in *Cerdocyon thous* (crab-eating foxes) from Rio Grande do Sul and São Paulo using polymerase chain reaction (PCR), as no piroplasms were found by microscopic examination. In the same study, none of the four *Lycalopex gymnocercus* (pampas foxes) examined were found to be positive for *R.vitalii* (SOARES et al., 2014). Quadros et al. (2015) detected *R. vitalii* and *Hepatozoon canis* in *L. gymnocercus* from Santa Catarina; however, these parasites were not detected in blood smears. Recently, in Minas Gerais state, the parasite was found to infect the cytoplasm of endothelial cells from a free-ranging *Chrysocyon brachyurus* (maned wolf) that was co-infected with many parasites, including *Hepatozoon* sp. (SILVEIRA et al., 2016).

Hepatozoon species are blood parasites that infect a wide range of intermediate vertebrate hosts and its gamonts are observed inside neutrophils and monocytes of mammalian hosts (SMITH, 1996). In Brazil, *Hepatozoon* spp. have been reported to infect a variety of Carnivora species, including domestic dogs and wild canids (ALENCAR et al., 1997; CRIADO- FORNELIO et al., 2006; ANDRÉ et al., 2010; GIANNITTI et al., 2012; ALMEIDA et al., 2013; SILVEIRA et al., 2016).

In this study, we describe the detection of *R. vitalii* on blood smears, with molecular confirmation, and molecular detection of *H. canis* in *L. gymnocercus* from Santa Catarina, Brazil. We also present haematological data for the animal.

Materials and Methods

A free ranging *L. gymnocercus*, adult male, was found injured after being hit by a car in Lages, Santa Catarina, Brazil, in July 2014. The animal was taken to the Veterinary Hospital of the Agroveterinary Sciences Center (CAV), Santa Catarina State University (UDESC), where it was examined. The animal showed decreased consciousness (somnolence) and had moderate dehydration.

Approximately 10 mL blood was collected from the jugular vein after physical examination. Blood smears were prepared immediately after blood sample collection and were stained using both the Diff-Quik Staining System (Laborclin) and Giemsa 10%. Diagnosis was made based on the examination of slides under a light microscope, at 1000 × magnification. The remaining blood was frozen at –20 °C for molecular examination. Parasitaemia was calculated by counting at least 500 red blood cells (number of infected red blood cells / total red blood cells counted × 100) (CONRAD et al., 2006). The detected parasites were identified, and their length and width were measured in a computerized image analysis system using Qwin Lite 2.5 software (Leica). The animal died of the injuries sustained from being hit by the car, but the necropsy was not done.

Red blood cell (RBC) and white blood cell (WBC) counts and haemoglobin concentrations were analysed in an automatic counter (CC510-Celm; Barueri) using the impedance method. The packed cell volume (PCV) was measured by the microhaematocrit procedure (JAIN, 1986). Differential leukocyte counts as well as search for blood parasites were performed on blood smears. The mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) were calculated. The platelet count was analysed using a haemocytometer chamber (Neubauer chamber) with 1% ammonium oxalate solution as the diluent. Total plasma protein (TPP) and specific gravity (urine) were measured by refractometry (Digit-Biosystems).

To analyse the biochemical parameters (urea, creatinine, serum total protein [TP], albumin [Alb], and globulin [Glob]), blood (kept in a glass bottle without anticoagulant) was centrifuged at 1,710 × *g*, and the serum was obtained. The serum was used at several biochemical dosages prepared using a semi-automatic device (TP Analyzer Plus; Thermo Plate, São Paulo, Brazil) with the support of commercial kits (Labtest; Minas Gerais, Brazil).

To confirm the identity of the fox species, as some doubts could occur about the morphological characteristics between *L. gymnocercus* and *Lycalopex vetulus*, we performed molecular for species barcoding. For that purpose, genomic DNA was extracted from blood using the standard phenol/chloroform protocol (SAMBROOK et al., 1989). The 5′ portion of the mitochondrial DNA (mtDNA) control region was amplified by PCR (SAIKI et al., 1985) using primers MTLPRO2 and CCR-DR1 primers (TCHAICKA et al., 2007). Products were examined on a 1% agarose gels stained with ethidium bromide, purified using polyethylene glycol, sequenced with ABI chemistry, and analysed with an ABI-PRISM 3100 automated sequencer (Applied Biosystems, Foster City, CA, USA).

DNA extraction for haemoparasite detection was performed from 200-µL aliquots of blood using a GFX Genomic Blood DNA Purification kit (GE Healthcare, Buckinghamshire, UK), according to the manufacturer's instructions. Each DNA sample was dissolved in 100 µL elution buffer.

Molecular identification of *R. vitalii* through nested PCR was based on amplification of 18S rDNA using the primers BT18SF1/BT18SR1 and BT18SF2/BT18SR2 (PAPARINI et al., 2012). Nested PCR was performed in a total volume of 25 µL containing 12.5 µL GoTaq Colorless Master Mix (Promega Corporation, WI, USA), 10 pmol of each primer, 1 µL DNA or primary PCR amplicon, and 9.5 µL ultrapure sterile water. The PCR conditions for the primary PCR (primers BT18SF1/BT18SR1) consisted of a pre-PCR step of 95 °C for 5 min; followed by 40 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 2 min; and a final extension of 72 °C for 7 min. The PCR conditions of the secondary PCR (primers BT18SF2/BT18SR2) consisted of a pre-PCR step of 95 °C for 5 min; followed by 40 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 80 s; and a final extension of 72 °C for 7 min (PAPARINI et al., 2014).

DNA samples were also screened for the presence of *Hepatozoon*-specific 18S rDNA by PCR using the 4558 and 2733 primers pair, which amplifies 1120 bp (MATHEW et al., 2000). Conventional PCR were also performed in a total volume of 25 µL containing 12.5 µL GoTaq Colorless Master Mix (Promega Corporation), 12.5 pmol of each primer, 5 µL DNA, and 5 µL ultrapure sterile water. The cycling conditions for the primers 4558 and 2733 were as follows: 94 °C for 3 min; followed by 40 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 90 s; and a final extension for 7 min at 72 °C, with a 4 °C hold.

In each PCR assay, a negative control (distilled sterile water) was used for both reactions. For positive control for *R. vitalii*, a *Babesia vogeli* DNA isolated from a naturally infected dog was used, and for *H. canis* was used a DNA isolated from another *H. canis* naturally infected dog.

Aliquots of 3 µL of amplified products were analysed on 1% agarose gels with gel Red (Uniscience) by electrophoresis at

80 V for 60 min in TAE buffer and visualized under an ultraviolet transilluminator. PCR fragments were estimated by comparison with known amounts of eletrophoretic standards using a 1 kb plus DNA ladder (Invitrogen, Carlsbad, CA, USA).

The total remaining reaction products were purified using Illustra ExoProStar 1-Step (GE Healthcare) according to the manufacturer's recommendations and sequenced using a BigDye v.3.1 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) with an automated Applied Biosystems ABI 3500 DNA genetic analyser.

The obtained sequences were edited using BioEdit software, version 7.2.5 (HALL, 1999) and compared for similarity to the sequences deposited in *GenBank* using BLAST (ALTSCHUL et al., 1990). CLUSTAL X (LARKIN et al., 2007) was used to align the sequences obtained in this study with sequences retrieved from *GenBank*. The jModelTest v.2.1.10 (DARRIBA et al., 2012) was used to identify the best evolutionary model, according to the Akaike information criterion. GTR+I+G and GTR+G were the models chosen for phylogenetic reconstruction of piroplasm and *Hepatozoon* spp. sequences, respectively. Phylogenetic trees were construed by Bayesian Inference using MrBayes 3.1.2 (RONQUIST & HUELSENBECK, 2003). Markov chain Monte Carlo (MCMC) simulations were run for 10^7 generations in two parallel runs, with sampling of trees at 1000-generation intervals and a burn-in of 25%. Phylogenetic trees were visualized in FigTree v.1.4.3 (TREE BIO, 2016).

Results

Phylogenetic analysis of the fox mtDNA generated a tree topology in which our sample was highly supported (bootstrap value: 100) as *L. gymnocercus*. The sequence generated for this study has been deposited in NCBI *GenBank* database (accession number KX618693). In addition to this sequence, previously published sequences of *Lycalopex* genera (NCBI *GenBank* accession numbers JX890309–JX890389) and sequences of *C. thous* (used as outgroup) (TCHAICKA et al., 2007) were included in the analyses. Eighty-one individuals representing all known species of this genus were included. Phylogenetic analysis excluded the possibility of misidentification with *L. vetulus* or *C. thous*, other Brazilian canids showing similar morphology. These species were grouped in different clades with confidence (bootstrap values > 98) (data not demonstrated).

Many large piroplasms were detected inside the erythrocytes (Figures 1A, B) and were initially confounded with *B. vogeli*. The parasitaemia was calculated as 1.2% of infected cells. The intra-erythrocytic merozoites of this piroplasm were round, oval, or teardrop-shaped organisms occurring singly or in pairs. The organisms had an abundant, pale blue cytoplasm and decentral dark red small nucleus. The oval shapes measured 3.4 ± 0.4 µm (minimum: 2.79; maximum: 4.58) long and 3.1 ± 0.35 µm (minimum: 2.48; maximum: 4.1) wide. The teardrop shapes were less common and measured 3.0 ± 0.3 µm (minimum: 2.3; maximum: 3.36) long and 2.1 ± 0.25 µm (minimum: 1.7; maximum: 2.5) wide. Gamonts of *H. canis* were not found on blood smears.

The blood and urine evaluations demonstrated a low platelet count (50 x 10^9/L) (normal range 200-500 x 10^9/L), total protein (41.2 g/L) (normal range 54-71 g/L), and albumin (21.1 g/L) (normal range 26-33 g/L) and a high urea concentration (41.91 mmol/L) (normal range 7.64-21.41 mmol/L). Reference range refers to domestic dogs due to lack of normal values for *L. gymnocercus*. The other parameters were in accordance with the normal values to domestic dogs (JAIN, 1993; KANEKO et al., 1997) and wild canids (MATTOSO et al., 2012).

According to the PCR results, the blood sample was positive for piroplasm and *Hepatozoon* spp. The nucleotide sequence of the piroplasm isolate from the blood of *L. gymnocercus* showed 99% genetic similarity to *R. vitalii* (KF218605 and KF218606) from *Canis familiaris* of Argentina by BLASTn analysis and grouped in the same clade with *R. vitalii* sequences obtained from dogs and wild animals in the phylogenetic tree (Figure 2). The *Hepatozoon* sequence showed 100% similarity to *H. canis* (AY150067) isolated from a fox of Spain and 100% to *H. canis* (KU569158) isolated from a Brazilian dog. In addition, phylogenetic analysis showed that the *H. canis* sequence detected in this study grouped in the clade composed of *H. canis* parasites with 97% probability (Figure 3). *Rangelia vitalii* and *H. canis* sequences were deposited in *GenBank* (KX816959 and KX816958).

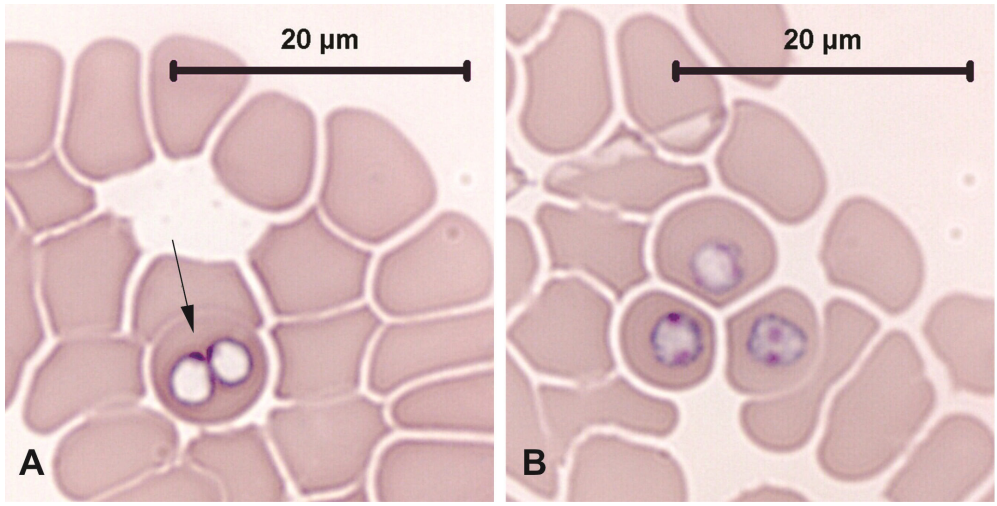

Figure 1. *Rangelia vitalii* on blood smear of *Lycalopex gymnocercus* from Santa Catarina state, Brazil. (A) teardrop forms; (B) three oval dividing form, with two nuclei. Scale bars = 20 µm. (Blood smear, Giemsa).

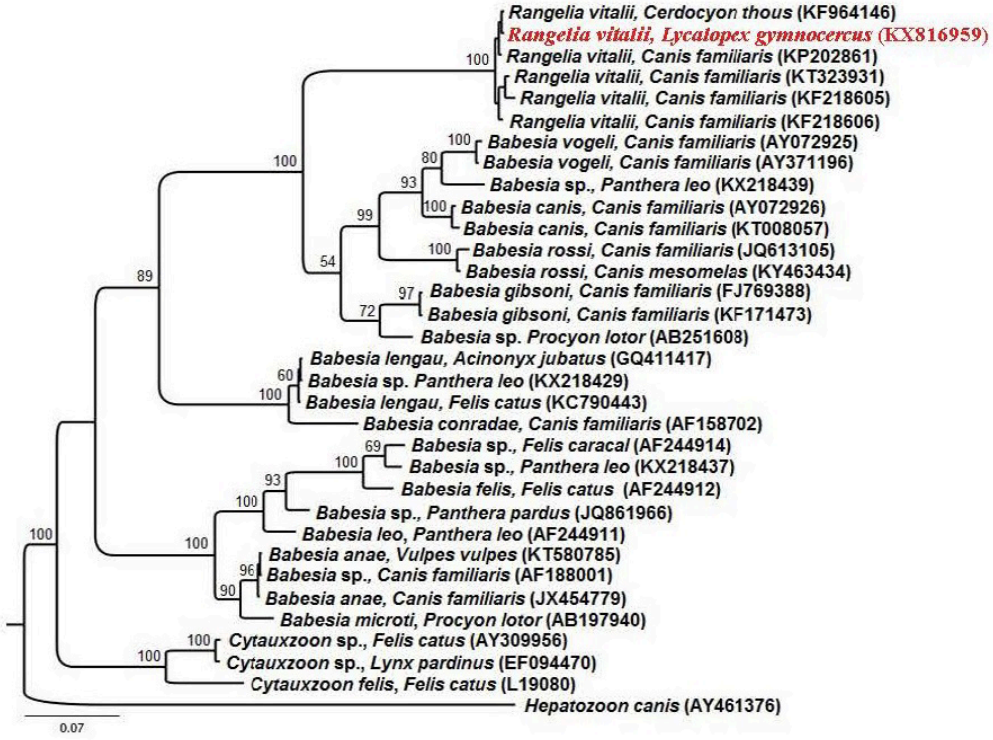

Figure 2. Bayesian Inference (BI) tree based on the 18S rRNA gene partial sequences (528bp) of *Rangelia vitalii*, isolates from Brazilian *Lycalopex gymnocercus*, and other hemoparasites, using GTR + I + G evolutionary model *Hepatozoon canis* was chosen as outgroup. Numbers at the nodes indicate posterior probabilities under BI. Posterior probabilities lower than 50 are not shown. The sequence obtained in this study is in red.

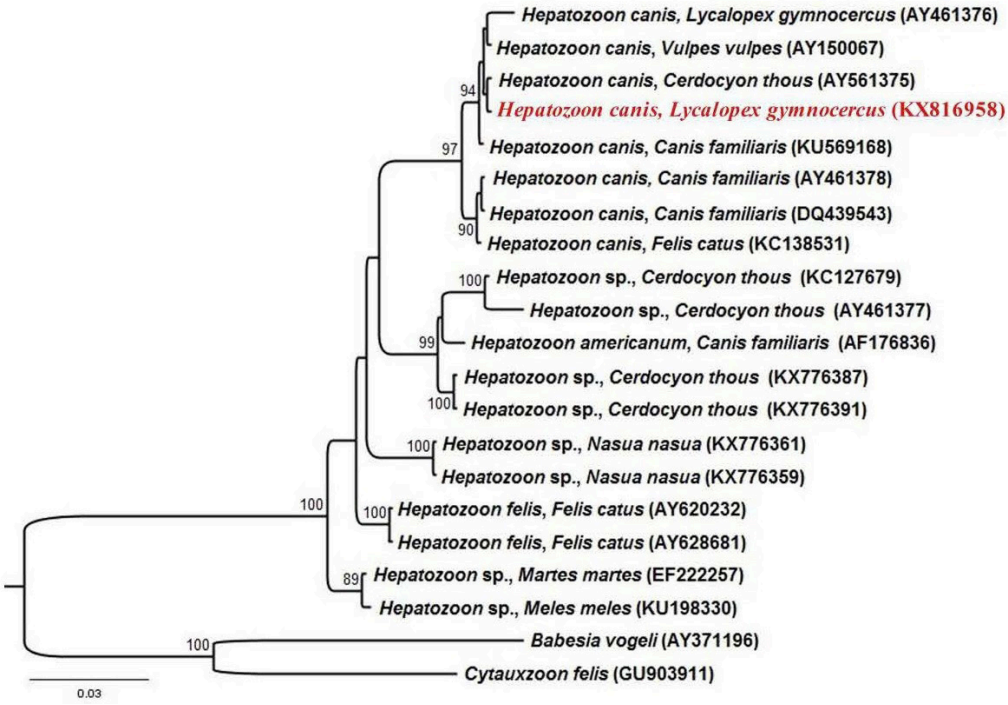

Figure 3. Bayesian Inference (BI) tree based on the 18S rRNA gene partial sequences (629bp) of *Hepatozoon canis*, isolates from Brazilian *Lycalopex gymnocercus*, and other *Hepatozoon* spp., using GTR + G evolutionary model. *Cytauxzoon felis* and *Babesia vogeli* were chosen as outgroups. Numbers at the nodes indicate posterior probabilities under BI. Posterior probabilities lower than 50 are not shown. The sequence obtained in this study is in red.

Discussion

Rangelia vitalii has been detected in sylvatic Brazilian canids since 2014 when Soares et al. (2014) detected, by PCR, nine *C. thous* positive for *R. vitalii*. Subsequently, this parasite was also found in *L. gymnocercus* (QUADROS et al., 2015; FREDO et al., 2015). The first detection of this protozoan in wild canids was described by Ruas et al. (2003), who initially identified the parasite as *Babesia* sp. in *L. gymnocercus* from southern Brazil. The authors showed that the only tick species found to infest the infected animal was *A. aureolatum*, the natural vector of *R. vitalii*; besides, the known vector of *Babesia vogeli*, *Rhipicephalus sanguineus*, was not observed parasitizing the examined animals (RUAS et al., 2003). Nevertheless, at that time, there was no molecular confirmation of the piroplasm identity.

In this study, we showed, for the first time, the intraerythrocytic stages of *R. vitalii* in a wild canid from Brazil. Those stages were similar in form and size to those described in natural and experimentallly infected dogs (SILVA et al., 2011; FRANÇA et al., 2014). The observation of *R. vitalii* in erythrocytes is a rare event, and parasitaemia is typically low when it is detected (SILVA et al., 2011). In experimentally infected dogs, *R. vitalii* merozoites were first detected in blood smears within 5 days of infection, with the peak of parasitaemia from days 9 and 11 post infection. The parasites were then decreased in negative smears until 21 days after infection (SILVA et al., 2011). Our canid was probably in the acute stage of infection as parasitaemia was high (1.2%), and many different forms were observed in the erythrocytes, but not in leucocytes.

Laboratory findings of natural cases of canine rangeliosis are similar to those of extravascular immune-mediated haemolytic anaemia (KRAUSPENHAR et al., 2003; FIGHERA et al., 2010; FRANÇA et al., 2010). The complete blood count values of *L. gymnocercus* were within the reference values for domestic dogs and wild canids (JAIN, 1993; MATTOSO et al., 2012). The animal was slightly dehydrated, which could have masked the anaemia (RANDOLPH et al., 2010). On blood smears, regeneration indicators were not observed, although in *R. vitalii* experimentally infected dogs, the degree of anaemia varies, and reticulocytosis is often observed (SILVA et al., 2011). None of these signs were found in the *L. gymnocercus*. Low platelet count is a common sign in natural (FRANÇA et al., 2010) and experimental (SILVA et al., 2011) cases of canine rangeliosis. However, in our case, the low platelet count could also be related to trauma due to the injury sustained by the animal (MISCHKE, 2005). The animal had low total protein and albumin concentrations and high urea concentrations. Significant hypoproteinaemia associated with low albumin levels was detected in *R. vitalii* experimentally infected dogs (PAIM et al., 2013). However, because albumin is the most abundant protein in the serum, any reduction in this protein would result in a reduction in total protein (KANEKO et al., 1997). Because poor nutrition is common in wild animals, hypoproteinaemia may be related to malnutrition (KANEKO et al., 1997). The higher urea level without a concomitant rise in creatinine could be justified by dehydration of the animal (STOCKHAM & SCOTT, 2011). The urine density was normal, suggesting a lack of renal damage. In experimentally infected dogs, the levels of urea and creatinine did not differ from those in normal dogs (SILVA et al., 2011; COSTA et al., 2012), reinforcing the suspicion that dehydration was responsible for the elevation of urea. Soares et al. (2014) observed that in *C. thous* infected with *R. vitalii*, haematological and biochemical parameters were normal, with only a slight increase in serum total protein.

The other haemoprotozoan detected only by PCR, *H. canis*, is usually found in wild canids (CRIADO-FORNELIO et al., 2006; GIANNITTI et al., 2012), including those with concomitant rangeliosis (QUADROS et al., 2015). Criado-Fornelio et al. (2006) detected different *Hepatozoon* genotypes on wild canids from Brazil, including a *H. americanum*-related organism. Giannitti et al. (2012) studied a specimen of *P. gymnocercus* (i.e., *L. gymnocercus*) in southern Argentina and observed a genotype of *Hepatozoon* closely related to *H. felis,* including the presence of several cysts, resembling the "onion skin" cysts of *H. americanum*, in the skeletal and cardiac muscle of this animal. On the other hand, Quadros et al. (2015) diagnosed *Hepatozoon* sp. as 100% identical to a corresponding sequence of *H. canis* from Rio Grande do Sul, Brazil.

The animal died as a consequence of injuries caused by being hit by a car and not as consequence of the parasitism. The results of the haematological exams, in addition with the presence of blood stages, allowed us to conclude that the animal was in the acute phase of infection. The few blood alterations could not be attributed to the infection, and further studies, like long term monitoring of infected animals, are needed to determine the impact of blood parasite infections on the health of wild canids. Quadros et al. (2015) reported clinical signs in *L. gymnocercus* naturally infected with *R. vitalii*. Thus, we speculate that an animal showing clinical signs of infection may be more prone to being hit by a car or captured. Moreover, in addition to their participation as disease-causing agents in endangered carnivores, Alvarado-Rybak et al. (2016) highlighted the importance of epidemiological studies of piroplasmid infections in wild carnivores and their roles as reservoirs of piroplasmids for domestic animals.

Further studies are needed to assess the epidemiology and pathogenic effects of these haemoparasites in the health of wild canids, and their role as reservoirs. There are few and isolated reports on *R. vitalii* infection of sylvatic canids. When it comes to the occurrence of this piroplasmid species in *L. gymnocercus,* the present study represents the third report, but the first to show the intraerythrocytic stages in the blood of wild canids. The prevalence of *R. vitalii* infection at the population level should be investigated, extending epidemiological studies to others Brazilian regions, with a higher number of animals. Although we could not determine the consequences of the infection on the animal health, long term monitoring infected animals, aiming at determining the effect of the parasites on their health, would be enlightening.

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