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Rangelia vitalii in a free-ranging maned wolf (*Chrysocyon brachyurus*) and co-infections

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ABSTRACT

An adult free-ranged female maned wolf was rescued from a periurban area subject to anthropogenic disturbances in the Minas Gerais, Brazil. The animal presented poor body condition and anemia. The clinical condition rapidly deteriorated culminating in dead and a necropsy was performed. The main gross lesions were marked anemia and blood content in the intestines accompanied by many types of parasites. The protozoa *Rangelia vitalii* was identified by histopathological analysis predominantly within the cytoplasm of endothelial cells of capillaries of the small intestine. The lymph nodes, spleen, bone marrow, dermis, lungs and kidney had similar protozoal forms but with mild or moderate intensity. *Rangelia vitalii* was confirmed by molecular assays. *Hepatozoon* sp., *Leishmania* sp., and *Entamoeba* spp., apparently not related to the clinical signs were also detected. The myriad parasites found in the intestines included nematodes (*Ancylostoma caninum*, *A. braziliensis*,, *Molineus* sp., *Pterygodermatites* sp., and *Trichuris* sp.), cestodes (*Spirometra* sp.) and (acanthocephalans. To our knowledge, *R. vitalii* was identified in *C. brachyurus* for the first time. These findings emphasize the fragility of Brazilian ecosystems, especially in disturbed areas, reinforcing the necessity of efforts to preserve these areas and wild carnivores, some of which are threatened with extinction, such as the maned wolf.

1. Introduction

The maned wolf (*Chrysocyon brachyurus*) is the largest canid of South America and inhabits the Cerrado biome, some areas in the Pantanal, and in the southeast region of Brazil (de Paula and Gambarini, 2013). The animal also is found in some provinces of Argentina, Paraguay, Peru, Bolivia and Uruguay (possibly extinct). Currently, the species is listed as near threatened in the vulnerable categories of the Red List of the International Union for Conservation of Nature (IUCN) (de Paula and de Matteo, 2015).

Significant threats to maned wolf populations include the drastic reduction of its habitats, especially due to the advancement of agriculture and livestock, the disorderly growth of urban areas, the increased contact in disturbed environments, the abundance of roads in proximity and the close contact with humans and domestic animals (Mangini et al., 2006; Lopes et al., 2008; de Paula and Gambarini, 2013). In free ranging wolves, mortalities due to diseases assume an important role, emerging recently as the major threat for carnivore conservation (Funk et al., 2001).

Studies about pathogens in free-ranging maned wolves are scarce. Despite that, parasites and other pathogens represent an elementary component of ecosystems and their biodiversity, their presence even in any natural hosts can be a threat in areas disturbed by habitat degradation and reduction (Mangini et al., 2006).

Rangelia vitalii, a member of the protozoan phylum Apicomplexa, order Piroplasmorida, causes a tick-borne disease in dogs referred to as “nambiuvú” (=blood dribbling down from the ear margins) or “peste de sangue” (=bleeding plague) (Krauspenhar

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et al., 2003). Currently, canine rangeliosis is confirmed to occur in Brazil (Krauspenhar et al., 2003), Argentina (Eiras et al., 2014), and Uruguay (Carvalho et al., 2015). Recently, in south and southeast Brazil, infection by *R. vitalii* was reported in wild Pampas fox (*Lycalopex gymnocercus*) (de Quadros et al., 2015) and crab-eating fox (*Cerdocyon thous*) (Soares et al., 2014; de Quadros et al., 2015; Fredo et al., 2015). However, there are no reports of infection and/or disease related to this protozoan in *C. brachyurus*. Due to the importance of conservation of *C. brachyurus* in Brazil, this investigation was performed to check for parasitism caused by *R. vitalii* and co-infections in one free-ranging maned female wolf from a periurban area subject to anthropogenic disturbances.

2. Material and methods

2.1. History

In November 2013, one adult maned wolf female weighting 18 kg was rescued from a periurban house located in the Rio Acima municipality (20°5′44.732″ S and 43°47′24.263″ W) in the state of Minas Gerais (MG), southeast of Brazil. The animal had physical debilitation and pain in the right foreleg. After its rescue by the Brazilian Federal Environmental Agency (IBAMA), it was transported to a veterinary clinic. Clinical evaluation showed right elbow luxation and tick infestation. Fipronil spray 0.25% was administered. Blood sample were collected in an ethylene diamine tetraacetic acid (EDTA) tube for further molecular analyses and for preparation of blood smears. Eleven days later, the animal died and necropsy was performed at the Pathology Sector, School of Veterinary Medicine, Universidade Federal de Minas Gerais (UFMG).

The study was approved by Ethics Committee for Animal Research of the UFMG under protocol 332/2013 and by the Brazilian Institute for Environment and Natural Renewable Resources (IBAMA, Belo Horizonte, MG, Brazil) under license 34633-5.

2.2. Necropsy and sampling

Necropsy was performed and the animal was grossly evaluated. Samples of brain, lung, trachea, heart, esophagus, stomach, intestine, kidney, bone marrow, spleen, and lymph nodes were collected in duplicate: one portion of each tissue was fixed in 10% neutral-buffered formalin for histopathology; and the other portion of the same tissue was frozen at −20 °C and stored for subsequent DNA extraction. For search for the parasites in the intestines, anatomical segments were separated by double ligatures, the contents removed and the mucosae scraped. The collected material was washed in strainers and the solid portion was fixed and preserved in acetic formaldehyde. For histopathological analysis, tissues were dehydrated using a series of increasing ethanol concentrations, cleared in xylene, and embedded in paraffin. Sections of 4 μm were obtained and stained with hematoxylin and eosin (HE) for routine histological evaluation under light microscopy (Luna, 1968). Parasite identification in histological sections was performed according to Gardiner and Poynton (1999).

2.3. Parasitological analysis

Blood and organ samples (spleen, mesenteric lymph node and intestines) were evaluated for hemoparasites in Laboratory of Veterinary Protozoology, Biology Institute, UFMG. Blood smears were subjected to quick Romanowsky staining (Panótico Rápido; Laborclin, Pinhais, PR, Brazil) and examined under immersion oil. Helminths recovered were examined at the Laboratory of Veterinary Helminthology, Biology Institute, UFMG. Images of the parasites were made with a digital camera Carl Zeiss (Oberkochen, Germany) and the measures of the parasites were performed using AxioVision 4.8 (Carl Zeiss). All collected specimens were identified according to Yamaguti (1961), Travassos et al. (1969), Vicente et al. (1997), and Anderson et al. (2009). Samples of cestodes were stored in tagged vials containing 70% ethanol for DNA extraction.

2.4. DNA extraction and PCR amplification

DNA was extracted from helminths, organs and blood samples using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA), according to the manufacturer's instructions for animal tissue and whole blood, respectively.

In all PCR assays, the reaction mixture in the first round contained 7.5 μL of GoTaq®Green Master Mix (Promega, Madison, WI, USA), 0.6 μL of a solution containing the mixed primers (10 mM) and 5.4 μL of nuclease free water. A 1.5 μL of total DNA was added to the reaction mixture to obtain a final volume of 15 μL. The reaction mixtures in the second round assays were similar, except the templates were the products from the first round PCR reactions (1.5 μL). Sets of primers used to detect the parasite species and amplifications were performed using a touchdown PCR programmed as previously (Gasser et al., 1996; Noyes et al., 1999; Soares et al., 2011; Spolidorio et al., 2011) (Table 1). Positive and negative controls were used for all PCR assays. The PCR amplicons were separated by electrophoresis on 1% agarose gels (40 min, 100 V), stained with GelRed™ (Biotium, Hayward, CA, USA), and visualized under ultraviolet light.

For sequencing, positive products from PCR reactions were purified using a QIAquick PCR Purification Kit (Qiagen Biotecnologia Brasil, São Paulo, Brazil) according to manufacturer's instructions. The purified amplicons were sequencing using an Applied Biosystems model ABI3130 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA) and the Applied Biosystems BigDye® Direct Cycle Sequencing Kit (v. 3.1), with the POP-7™ polymer as the separating matrix, using primers employed in the PCR reaction. Sequences obtained were aligned, edited, and analyzed using MEGA 6.0 software at the URL http://asparagin.cenargen.embrapa.br/phph (Tamura et al., 2013). The identity of each sequence was confirmed using alignment, via homology to sequences available at GenBank using BLAST software (Altschul et al., 1990).

The phylogenetic tree was constructed by analyzing the ITS gene from Diphyllobothriidae strains deposited in GenBank. Nucleotide sequences were aligned with MUSCLE from de MEGA 6.0 package (Tamura et al., 2013). Each alignment was analyzed using the Maximum Likelihood in MEGA 6.0 software. Internal branch confidence was assessed by the bootstrapping method using 1000 bootstrap replicates.

3. Results

3.1. Clinical history

Clinical examination revealed weakness, poor body condition, and anemia when the animal was admitted. Immobilization for traumatic right elbow luxation was performed. The clinical condition progressively worsened and ten days later, the animal died. Blood smear analysis revealed intracellular gamonts in the cytoplasm of neutrophils, suggesting *Hepatozoon* infection.

3.2. Gross and histopathology

The maned wolf had poor corporal condition and, surrounding the perineal area, there was dark-red material consistent with digested blood. There was also mild subcutaneous edema, and the peripheral lymph nodes were brown with multifocal to coalescing

Table 1
Specific primers used for the detection of parasites.

Specificity	Primer sequence (5′- **3**′)	Target	Name	Product size (bp)	Reference
Piroplasmida	CATGAAGCACTGGCCHTTCAA	hsp70	hsp 70 F1	740	Soares et al., 2011
1st reaction	GCNCKGCTGATGGTGGTGTTGTA		hsp 70 R1		
2nd reaction	GGATCAACAAYGGMAAGAAC	hsp70	hsp 70 F2	720	Soares et al., 2011
	GBAGGTTGTTGTCCTTVGTCAT		hsp 70 R2		
Hepatozoon spp.	GGTAATTCTAGAGCTAATA	18SrRNA	HEP144-169	574	Spolidorio et al., 2011
	ACAATAAAGTAAAAAACA		HEP743-718		
Kinetoplastida	CAGAAACGAAACACGGGAG	ssrRNA gene	TRY816F	1500–900	Noyes et al., 1999
1st reaction	CCTACTGGGCAGCTTGGA		TRY816R		
2nd reaction	TGGGATAACAAAGGAGCA	ssrRNA	SSU450F	450	Noyes et al., 1999
	CTGAGACTGTAACCTCAAAGC	gene	SSU450R		
Helminths	GTAGGTGAACCTGCGGAAGGATCATT	ITS1, 5.8	NC5	–	Gasser et al., 1996
	TTAGTTTCTTTTCCTCCGCT	S, ITS2	NC2		

white foci. The musculature and parenchymatous organs were markedly pale red, indicating anemia. The mucosa of the middle and distal parts of the trachea and primary bronchi had 0.3 cm whitish nodules, with intralesional parasites suggestive of *Oslerus* sp. The spleen had moderate enlargement of the red pulp. The lumen of small and large intestines were filled with red hemorrhagic content. In addition, there were many acanthocephala and cestodes with characteristics of the order Pseudophyllidea, as well as nematodes (especially Ancylostomatidae) in the small intestine. The mucosa was thickened and with many petechial hemorrhages, especially in the small intestine. The mesenteric lymph nodes had drainage hemorrhages.

Histologically, there were multifocal lymphoplasmacytic tracheitis and bronchitis associated with *O. osleri* infection (Avelar et al., 2013). The small intestine had mild lymphoplasmacytic infiltration in the mucosa. Within the cytoplasm of endothelial cells of capillaries and venules, there were numerous round 3 μm protozoa with pale cytoplasm and basophilic nucleus, compatible with *R. vitalii*. This infection was particularly intense in the lamina propria and submucosa of the small intestine (Fig. 1). Similar protozoa but with moderate intensity were found in the lymph nodes, spleen, and bone marrow of femur. In spleen, bone marrow and lymph nodes, there were moderate plasmacytosis, erythrophagocytosis and hemosiderosis. In the dermis, lungs, heart, and kidneys, protozoa in small vessels were found without inflammatory reaction. Multiple foci of several round to ovoid 30–50 μm parasitic organisms were found in the cytoplasm of intestinal epithelial cells lying in direct proximity to the lamina propria (Fig. 2). Morphologically, these organisms were characterized by a basophilic eccentric nucleus with a large karyosome, vacuolated, granular and slight basophilic cytoplasm with smooth, thin and eosinophilic wall compatible with an amoeba form.

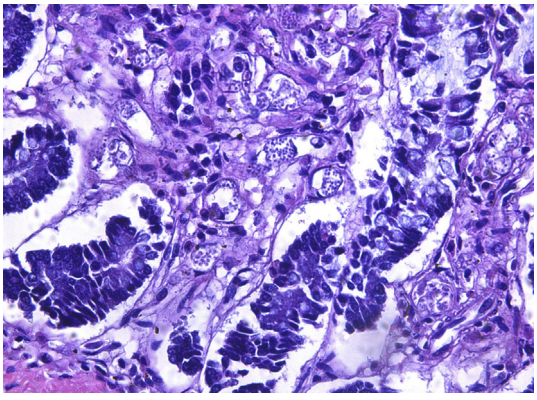

Fig. 1. Small intestine of *C. brachyurus*. There are numerous round protozoa within cytoplasm of endothelial cells of the lamina propria compatible with *Rangelia vitalli*. H.E. 40X.

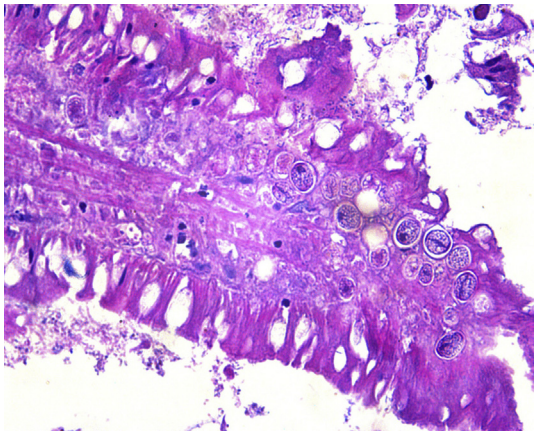

Fig. 2. Small intestine of *C. brachyurus*. There are several round to ovoid parasitic organisms measuring of 30–50 μm of diameter in the cytoplasm of intestinal epithelial cells and in the lamina propria. H.E. 40X.

Intestinal lumen had many cestodes. These parasites did not have a body cavity, the organs were surrounded by parenchymatous matrix, and were enclosed by tegument. The digestive tube was not present but many calcareous bodies were seen. The testis and uterus were located centrally and the eggs were operculated and embryonated. Kidneys had mild membranous glomerulonephritis, multifocal interstitial lymphoplasmacytic nephritis, and mild tubular protein casts.

3.3. Parasitology

Even though initial veterinary report stated tick infestation, the identity of the tick species could not be ascertained due to subsequent administration of fipronil by the veterinarian. A total of 33 *O. osleri* (21 females and 16 males) were found in the trachea and bronchi. Ancylostomatidae were detected in the small intestine (503 females and 316 males). In addition, there were nematodes of genus *Molineus* (209 females and 198 males) and *Pterygodermatites* sp. (36 females and 26 males), 12 acantocephalus and 34 cestodes (Order Pseudophyllidea). Length of Pseudophyllidea specimens ranged from 12 cm to 48 cm, and morphological and morphometrical characteristics were compatible with the Family Diphyllobothriidae, according to Yamaguti (1961). *Trichuris* specimens (2 females and 4 males) were detected in the large intestine.

3.4. Molecular assays

All the positive results of PCR assays were confirmed for

nucleotide sequences analysis by Blastn. Infection by *R. vitalii* was confirmed by PCR using DNA from mesenteric lymph node. A Blastn sequence analysis revealed that the sample was 99% identical to *R. vitalii* sequences obtained from a dog in Brazil (GenBank JF279603.1). Whole blood was PCR positive for *Hepatozoon* and sequencing showed 98% identity to *H. americanum* isolated from *Amblyomma maculatum* in USA (GenBank AF176836.1) and *Hepatozoon* spp. isolated from *C. thous* in Brazil (GenBank AY461377.2). Blood sample was also PCR positive for Kinetoplastid protozoa and sequencing showed 98% sequence identity with *Leishmania infantum* (GenBank XR_001203206.1) and *L. donovani* (GenBank FR799614.1; GQ332356.1). Molecular assays and analysis also revealed that sequences obtained from a gene of diphyllobothriid of this maned wolf were 88% identical to *Spirometra erinaceieuropaei* from dogs (GenBank FJ886746.1) and cats (GenBank KC561781.1). This proximity was reinforced by phylogenetic analysis, which revealed the formation of a cluster with a high percentage of internal branches among diphyllobothriid of this study (C129), *Spirometra* spp., and *S. erinaceieuropaei* (Fig. 3). The nucleotide sequences amplified from parasites were deposited in GenBank under the following accession numbers: KU500888 (*Leishmania* sp.); KU500889 (*Spirometra* sp.); KU507416 (*Hepatozoon* spp.); KU507417 (*R. vitalii*).

4. Discussion

The maned wolf of this study was intensely infected with multiple species of parasites. This multiparasitism resulted in a set of systemic changes, especially severe anemia. *R. vitalii* was detected in endothelial cells of various organs, but myriad organisms were observed especially in small intestine. Intense infection by *R. vitalii*, Ancylostomatidae, and perhaps the pseudophyllids, acted together resulting in anemia.

There has been an gradual increase in the occurrence of *R. vitalii* infections in Brazilian wild canids in the recent years, with the first reported infection in *C. thous* from the southeast region (São Paulo) and from the south (Rio Grande do Sul) in the absence of clinical signs (Soares et al., 2014). Subsequently, the protozoan was reported in pampas fox (*L. gymnocercus*) in another state in South (Santa Catarina). This animal developed a fatal and classical form of this disease with anemia and jaundice (de Quadros et al., 2015). In *C. thous* and *L. gymnocercus*, infections by *R. vitalii* concomitant to tick infestation by *A. aureolatum* and with anemia have been reported (Fredo et al., 2015). To the best of the authors' knowledge, this is the first report of infection by *R. vitalii* in a maned wolf, with severe anemia without jaundice and intense parasitism predominantly within the cytoplasm of endothelial cells of the small intestine. These findings differ from the lesions often found in domestic dogs (Krauspenhar et al., 2003; da Silva et al., 2011). In dogs, jaundice and persistent hemorrhages through the external surface of the ears and nose are the most common lesions. In these cases, *R. vitalii* were most numerous in the cytoplasm of endothelial cells of peripheral lymph nodes, bone marrow, kidneys, and choroid plexus (Loretti and Barros, 2005). In dogs, the anemia is regenerative and probably caused by extravascular immune-mediated hemolysis (França et al., 2013). *Amblyoma aureolatum* and *Rhipicephalus sanguineus* were suggested as vectors of *R. vitalii* in rural domestic dogs (Loretti and Barros, 2005; Soares et al., 2011) and probably the primary hosts of *R. vitalii* are the South American wild canids and their endemic ticks. Soares et al. (2014) detected this protozoa in wild canids without clinical signs of rangeliosis and suggested that *C. thous* is a natural reservoir of *R. vitalii* in Brazil.

Molecular analyzes revealed that the *Hepatozoon* found in this maned wolf was closely related to *H. americanum*, as found in leukocytes of whole blood smears from native *C. thous* in Brazil (Criado-Fornelio et al., 2006; André et al., 2010; Almeida et al., 2013). *Hepatozoon* species closely related to *H. canis* were detected in a maned wolf in captivity in São Paulo (André et al., 2010).

Leishmania closely related to the species responsible for visceral leishmaniasis (VL) was molecularly detected in whole blood of the *C. brachyurus* analyzed in this study. Canine visceral leishmaniasis is an endemic zoonosis in the metropolitan region of Belo Horizonte (Silva et al., 2001). Antibodies to this parasite have been detected in several Brazilian wild canids, including free maned wolf of the state of Minas Gerais (Curi et al., 2012) and a maned wolf kept in captivity in the Zoo of Belo Horizonte (Luppi et al., 2008). In the present study, there were no infected macrophages with intracytoplasmic amastigotes; however, the animal had membranous glomerulonephritis, which is frequently associated with *Leishmania* spp. infections (Luppi et al., 2008).

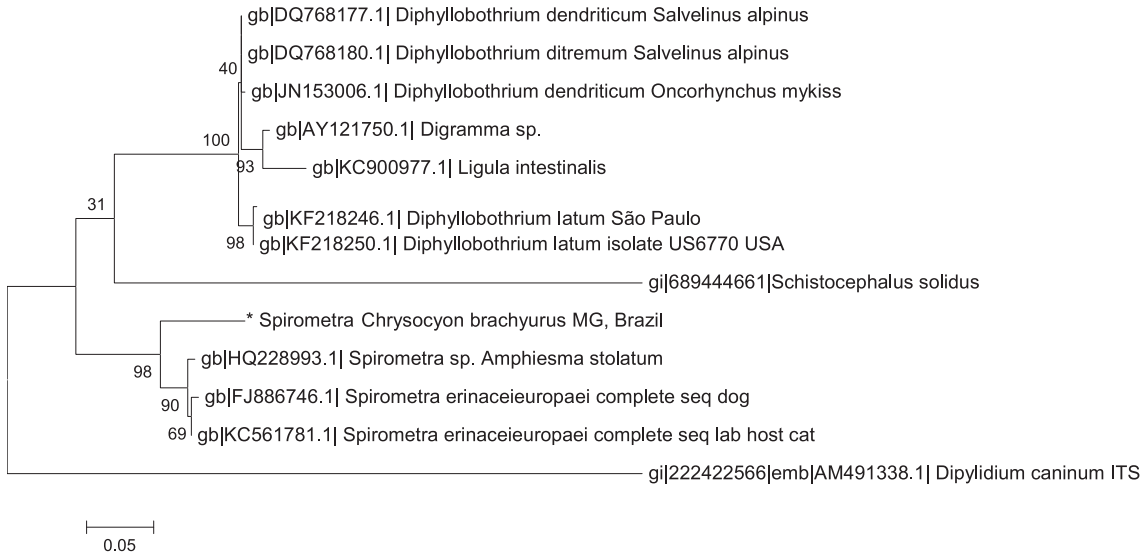

Fig. 3. Maximum Likelihood tree based on ITS genes of helminths. *Dipylidium caninum* sample were used as outgroup. Scale bar represents the nucleotide substitutions per position. Branch lengths represent the amount of genetic distance change between the strains. * *Spirometra - Chrysocyon brachyurus* MG, Brazil denotes the sequences obtained from this study. The accession numbers of the publicly available reference sequences are indicated.

Based on fecal antigen ELISA, Alam et al. (2015), showed a relatively low prevalence rate of *E. histolytica* infection in dogs from Pakistan without clinical signs. In the present study, the histological characteristics of the parasite in the small intestine suggested trophozoites of *Entamoeba* sp. (Stedmen et al., 2003), but the infection was not related with mucosal necrosis, an unusual finding, both with respect to the anatomical location of infection as well as the infected species. There are no published reports of *Entamoeba* infections in wild canids.

A wide variety of helminths including hookworm, *Molineus*, *Pterygodermatites*, *Trichuris*, *Spirometra* and acanthocephalans were found in intestines. Hookworms cause blood spoliation and hemorrhages in the intestinal mucosa, leading to blood loss and anemia. Ancylostomatidae found in this study are common in domestic carnivores (Freitas, 1977), which reinforces the contact between this maned wolf with domestic species and their pathogens in periurban areas.

The organisms of the genus *Molineus* are known to parasitize carnivores worldwide (Durette-Desset and Chabaud, 1981). *Molineus brachiurus* was reported in maned wolf (Vieira et al., 2008). *Pterygodermatites pluripectinata* and *P. affinis* were found in high prevalence in *C. thous* of northeast region (Lima et al., 2013) but not in maned wolf.

Cestodes of *Spirometra* spp. and *S. mansonoides* were reported in *C. thous* (Ruas et al., 2008; Lima et al., 2013) and in *L. gymnocercus* (Ruas et al., 2008). Report of *Spirometra* infection in maned-wolf was not found. According to molecular analysis, specimens found in the present study were closely related to *S. erinaceieuropaei*. The definitive hosts are carnivores and their plerocercoid larvae are responsible for the sparganosis zoonosis (Dybing et al., 2013). Infections in humans in Brazil by *Spirometra* sp. have been described (Gomes et al., 1996; Mentz et al., 2011). Massive adult infections by Pseudophyllidea in the small intestine of humans can lead to megaloblastic anemia due to the deficiency caused by vitamin B12 (Scholz et al., 2009) and folic acid consumption by the parasite (Jimenez et al., 2012).

The maned wolf evaluated in this study was found in a periurban area. Anthropogenic interventions are considered primary factors for the emergence of infectious agents (Aguirre and Tabor, 2008). Parasites that affect wild and domestic carnivores can circulate between sympatric populations of animals, facilitating distribution of infections to humans (Polley, 2005). Wild carnivores require large territorial areas for maintenance (Pastoret and Brochier, 1999) which also facilitates the contact with other species of wild animals, human beings, domestic dogs, and their pathogens.

According to our knowledge, this is the first report in a maned wolf that includes parasites of all phyla (Arthropoda, Protozoa, Platyhelminthes, Nematoda and Acanthocephala), including some with zoonotic potential. These include *Leishmania*, *Entamoeba* and *Spirometra*. These findings emphasize the fragility of Brazilian ecosystems, especially in disturbed areas, reinforcing the necessity for efforts to preserve these areas and wild carnivores, some of which are threatened with extinction, including the maned wolf.

5. Conclusion

Myriads of *Rangelia vitalii* were identified in *C. brachyurus* for the first time, allowing us to relate the clinical pathological findings to the host's susceptibility to this protozoan. The multiparasitism indicated also the susceptibility of the animal to pathogens that cause disease in dogs. Probably, the infectious agents are acquired when wild animals access periurban zones. The parasites with zoonotic character found in this animal reveal the importance of the maned wolf for public health.

Conflicts of interest

There are no conflicts of interest in this work.

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