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Apicomplexa parasites in the brains of road-killed wild animals in the State of Paraná, Brazil

Parasitas apicomplexas em cérebro de animais silvestres mortos em estradas do Estado do Paraná, Brasil

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Highlights

Detection of *Sarcocystis* spp, *N. caninum* and *T. gondii* in brain fragments of wild animals.
The presence of important protozoan has been demonstrated in wild animals.
This information is essential for the public health and conservation of the environment.

Abstract

The use of run-over wild animals is an efficient strategy for scientific research of pathogens. The aim of this study was to detect DNA from phylum Apicomplexa in the brain of road-killed wild animals from the North-Central and North Pioneer mesoregions of Paraná, Brazil. Pre-established transects were run weekly; when found, animals were packed into individual packages and sent for autopsy. The brain fragments were collected and kept at -20 ° C until processing. The DNA extracted from the samples was amplified by *nested*-PCR for the 18S rDNA gene from the phylum Apicomplexa. All positive samples were submitted to DNA sequencing to define the species. A total of 90 animals were collected, however, only 68 animals (75.6%) that had integrity of the brain were included in the study. It was possible to identify the species by DNA sequencing in four samples: *Sarcocystis* spp. was identified in one *Colaptes melanochloros* (Green-barred woodpecker) and one *Mazama gouazoubira* (Gray brocket). *Neospora caninum* was observed in a

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Leopardus pardalis (Ocelot) and *T. gondii* was present in *Didelphis albiventris* (white-eared opossum). The results indicated that parasites with economic and public health relevance were present in wild animals, which may favor infection of humans and animals.
Key words: *Neospora caninum.* Road-killed. *Sarcocystis* spp.. *Toxoplasma gondii.* Wild animals.

Resumo

O uso de animais silvestres atropelados é uma estratégia eficiente para a pesquisa científica de patógenos. O objetivo deste estudo foi detectar DNA de parasitas do filo Apicomplexa em amostras de cérebro de animais silvestres atropelados nas mesorregiões Centro-Norte e Pioneira do Norte do Paraná, Brasil. Os transectos pré-estabelecidos foram percorridos semanalmente; quando encontrados, os animais foram armazenados em embalagens individuais e enviados para autópsia. Os fragmentos cerebrais foram coletados e mantidos a -20 ° C até o processamento. O DNA extraído das amostras foi amplificado por *nested*-PCR para o gene 18S rDNA do filo Apicomplexa. Todas as amostras positivas foram submetidas ao sequenciamento de DNA para definição da espécie. Um total de 90 animais foram coletados, no entanto, foram incluídos no estudo apenas 68 animais (75,6%) que apresentavam encéfalo. No sequenciamento foi possível identificar parasitos apicomplexos pelo sequenciamento de DNA em quatro amostras: *Sarcocystis* spp. em *Colaptes melanochloros* (pica-pau-verde-barrado) e em *Mazama gouazoubira* (veado-catingueiro); *Neospora caninum* em *Leopardus pardalis* (jaguatirica); e *T. gondii* em *Didelphis albiventris* (gambá-de-orelha-branca). Os resultados demonstraram que protozoários com relevância econômica e de saúde pública estavam presentes em animais silvestres, o que pode favorecer a infecção de humanos e animais.
Palavras-chave: Animais selvagens. Atropelamento. *Neospora caninum. Sarcocystis* spp.. *Toxoplasma gondii.*

Over the past few years, greater proximity of wild fauna and humans has been observed as a result of urban development. This aspect is accompanied by the accelerated adaptation of wild animal populations to specific urban conditions, called synurbization (Luniak, 2004). The current sharing of the environment between animals and humans has caused an ecological imbalance. Mortality from the collision with vehicles is the most visible impact roads cause in wildlife. It is estimated that 15 wild animals die every second by being run over in Brazil, representing almost 500 million per year, of which 1% corresponds to medium to large vertebrates such as anteaters, jaguars, crab-eating raccoons, and bush dogs. The South and Southeast Regions of Brazil have a higher frequency of roadkill incidents, which are distributed mainly in the states of São Paulo, Paraná, and Santa Catarina (Centro Brasileiro de Ecologia de Estradas [CBEE], 2020).

With the high number of road-killed animals, researchers have become interested in using these animals as study material. This type of sample facilitates relevant knowledge about the wild environment without the need to capture wild animals, which favors research without causing any type of imbalance for the studied populations (Richini-Pereira, Marson, Silva, & Langoni, 2010). Wild animals are extremely important in the epidemiological

chain of infections by parasites of the phylum Apicomplexa, and species such as *Neospora caninum, Toxoplasma gondii,* and *Sarcocystis* spp. present great occurrence among them (Wells et al., 2015).

Neospora caninum, Toxoplasma gondii, and *Sarcocystis* spp. belong to the phylum Apicomplexa requires two hosts: an intermediate host (usually a prey); and a definitive host (usually a predator), further forms tissue cysts in the intermediate host. In addition, wild animals act as intermediate hosts and maintainers by parasite in the environment (Dubey, Calero-Bernal, Rosenthal, Speer, & Fayer, 2016).

The central nervous system (CNS) is one of the tissues that apicomplexans affect in intermediate hosts (Konradt et al., 2016), including in brain of wild animals (Nascimento et al., 2015). In addition, Cavalcante et al. (2011) demonstrated that feeding dogs with brains is more effective for excretion of *N. caninum* oocysts, than other organs, which make the brain a good choice for postmortem search of parasites. The present study aimed to detect parasites DNA of the phylum Apicomplexa in brain samples in road-killed wild animals, by polymerase chain reaction (PCR) and DNA sequencing.

The project was approved by the Animal Use Ethics Committee (CEUA) of the State University of Londrina on March 10, 2017 (number 30/2017), and it was also approved by the Biodiversity Authorization and Information System (SISBIO) on December 19 October 2016 (number 55384-1).

The animals were collected during the years 2016 and 2018. Four transects were investigated weekly to active searching: Transect 1 (Tr1): municipality of Londrina; Transecto 2 (Tr2): municipality of Cornélio Procópio; Transecto 3 (Tr3): municipality of Alvorada do Sul; and Transecto 4 (Tr4): municipality of Maringá (Figure 1). In addition, road-killed animals founded or reported to the 2nd Environmental Police Company, 2nd Highway Police Company, or members of the project were also collect the animals.

The searches were carried out with the presence of at least two researchers in a car, with each inspecting one side of the highway. To visualize small species, an average speed of 50 km/h was standardized. Only animals that were in good condition (minimal evisceration and/or low degree of autolysis) were collected and sent to the Laboratory of Animal Pathology, State University of Londrina, for the autopsy. The identification of wild animals was according dos Reis, Peracchi, Pedro and Lima (2006) for mammalian animals and Ridgely, Gwynne, Tudor and Argel (2015) and Wiki Aves - A Enciclopédia das Aves do Brasil (2008). Animals presenting integrity of the encephalon (some animals had a destruction of the cranium) were submitted. Fragments of the brain and cerebellum were collected and stored in microtubes at -20°C until processing.

Brain DNA extraction was performed with the commercial PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's recommendations, using 20mg of duplicate samples. A negative control (ultrapure water) was added to each set of extractions. The extracted DNA was aliquoted into calibrated 1.5 ml tubes and stored at -20°C until polymerase chain reaction (PCR) was performed. The extracted DNA was quantified using L-QUANT (Loccus Biotechnology, Cotia, Brazil). The samples used in this study had a minimum DNA concentration of 25 ng/µL.

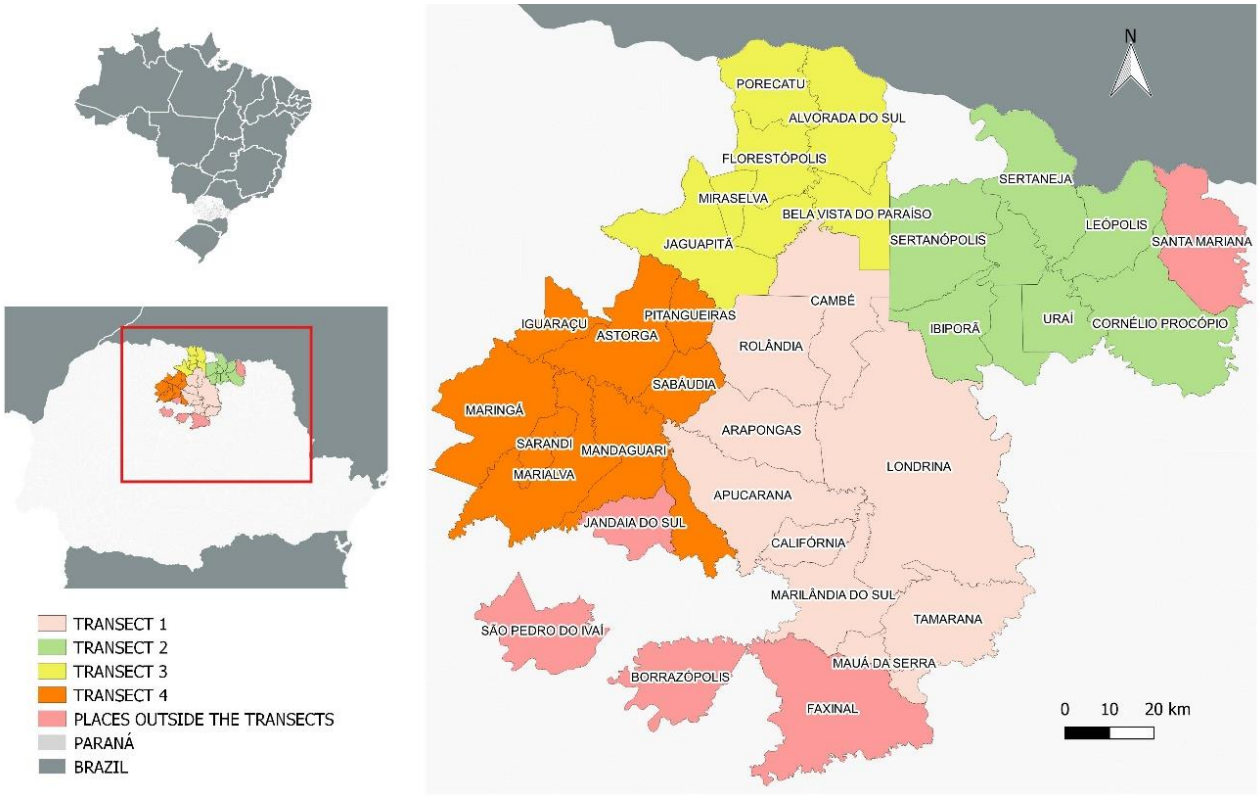

Figure 1. Place of study and transects traveled weekly in the active search for wild animals killed by trampling on North-Central and North Pioneer mesoregions from November 2016 to October 2018

Nested-PCR for the 18S rDNA gene of the phylum Apicomplexa was performed using primers described by Vitaliano et al. (2014). The amplified products generated in the PCRs were visualized after electrophoresis in a 1.5% agarose gel stained with syBr safe DNA stain (Invitrogen, Carlsbad, California, USA).

The positive samples were purified with PureLink Gel Extraction Kit (Invitrogen, Molecular Probes,130 Eugene, OR, USA) and quantified using Picodrop. After that, they were submitted to Sanger DNA sequencing. Direct sequencing was performed using a BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Carlsbad, USA) on the 3500 Genetic Analyzer (Applied Biosystems, Carlsbad, USA), according to the manufacturer's instructions. The obtained sequences were examined for quality using PHRED software (http://asparagin.cenargen.embrapa.br/phph). Consensus sequences were determined using CAP3 software (http://asparagin.cenargen.embrapa.br/cgi-bin/phph/cap3.pl). the sequence identity, information on species and query code (QC) was obtained by comparison with all sequences deposited in GenBank using BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The study covered a period of two years, in which a total of 90 road-killed wild animals were collected; however, only 68 (75.6%) had brain viable for analysis, 76.5% (52/68) of the animals collected belonged to the class Mammalia, 22.1% (15/68) to the class Aves, and 1.5% (1/68) to the class Reptilia. In this study, only sample from mammalian and avian species were included.

Within the class Mammalia was represented by species *Leopardus pardalis* (1/68), *Leopardus wiedii* (1/68), *Procyon cancrivorus* (1/68), *Galictis cuja* (1/68), *Mazama gouazoubira* (1/68), *Lepus europeus* (1/68), *Hydrochoerus hydrochaeris* (2/68), *Puma concolor* (2/68), *Dasypus novemcinctus* (3/68), *Leopardus guttulus* (3/68), T*amandua tetradactyla* (3/68), *Sapajus apella* (3/68), *Cavia aperea* (3/68), *Cerdocyon thous* (5/68), *Nasua nasua* (5/68) and *Didelphis albiventris* wich was the most frequently collected in the study, representing 25.5% (17/68) of the total.

In relation to the class Aves, was identified the species of *Vanellus chilensis* (1/68), *Coragyps atratus* (1/68), *Patagioenas picazuro* (1/68), *Guira guira* (1/68), *Caracara plancus* (1/68), *Pitangus sulphuratus* (1/68), *Tangara sayaca* (1/68), *Colaptes melanochloros* (1/68), *Megascops choliba* (1/68), *Crypturellus tataupa* (1/68), *Columbina picui* (2/68) and *Athene cunicularia* (2/68). As for the class Repitilia, the specie *Salvator merianae* (1/68).

Twenty-eight animals were found in Tr1, nine on Tr2, four on Tr3, and five on Tr4. The other animals (32.4%; 22/68) were collected outside of the transects through notification from partner institutions or by members of the project team (Figure 1).

Thirteen samples were positive in PCR but, sequencing was not possible in nine of due to the low amount of DNA in the tested samples. From the sequencing analyses of the 18S rDNA gene, positive animals were observed for *Sarcocystis* spp. (n = 2), *N. caninum* (n = 1), and *T. gondii* (n = 1) according to comparison performed in BLAST (Table 1).

Table 1
Species of parasites of the phylum Apicomplexa found in road-killed animals on the highways of the North-Central and North Pioneiro mesoregions of the State of Paraná, southern Brazil, between 2016 and 2018

PARASITE	SPECIES	MUNICIPALITY	QC	Identity (%)
Sarcocystis spp	*Colaptes melanochloros*	Califórnia	99%	98.82%
Sarcocystis spp	*Mazama gouazoubira*	Cornélio Procópio	100%	99.12%
T. gondii	*Didelphis albiventris*	Londrina	100%	99.14%
N. caninum	*Leopardus pardalis*	Alvorada do Sul	98%	99.45%

QC= Query cover (percentage of the query sequence length that is included in the alignment).

The animals positive to *Sarcocystis* spp. It was observed 98.82% of identify in a Green-barred Woodpecker (*Colaptes melanochloros*) in California city and 99.12% of identify in a Gray Brocket (*Mazama gouazoubira*) in Cornelio Procopio city. The positive sample to *T. gondii* from a White-eared opossum (*D. albiventris*), Londrina city, presented 99.14% of identify. With regarding *N. caninum,* it was observed 99.45% of identify in a Ocelot, (*L. pardalis*), Alvorada do Sul.

According to the literature, positivity for *N. caninum* was observed from serological tests performed on captive and free-living felids (Ebani, Nardoni, Maestrini, Perrucci, & Mancianti, 2021). In the present study, we identified *N. caninum* in the brain of *L. pardalis* through molecular testing.

Descriptions of *Sarcocystis* in the brain of birds are not very common. Infection in the central nervous system by *S. neurona* was observed in a Straw-necked ibis (*Threskiornis spinicollis*) that was hospitalized presenting ataxia, proprioceptive deficit, and inappetence (Dubey, Johnson, Bermudez, Suedmeyer, & Fritz, 2001). Another study demonstrated by morphological and molecular tests that Brown-headed cowbirds (*Molothrus ater*) can act as an intermediate host of Sarcocystis spp. (Mansfield et al., 2008). In the present study, *Sarcocystis* spp. was found in the brain of Colaptes melanochloros; however, it was not possible to evaluated which species of Sarcocysts infected the animal.

Another interesting fact was the detection of *Sarcocystis* spp. in *M. gouazoubira.* A similar study occurred with wild animals run over in the state of São Paulo; however, no positive results were observed for samples of M. gouazoubira (Richini-Pereira et al., 2016). It is known that herbivorous animals act as intermediate hosts of the parasite, and there are descriptions of infection in armadillos, cats, horses, raccoons, birds, skunks, and sea otters (Dubey et al., 2001; Konradt et al., 2016).

Dildelphis albiventris was the species most found during the search. It has nocturnal or crepuscular habits and are omnivores, feeding of leaves, fruits and seeds as well as insects, small mammals and birds. This type of diet favors infection by *T. gondii* since it facilitates contact with both the bradyzoites present in the muscles of animals and the oocysts in the environment. In addition, *D. albiventris* has synanthropic habits, living in urban areas in close contact with domestic felines and humans (Reis et al., 2006); therefore, this species can be a good indicator of environmental contamination by protozoans. Furthermore, hunting and consumption of wild animals, including opossum, are common in some Brazilian cities, becoming an important source of infection (Silva et al., 2008).

Road-killed wild animals have a great importance, as they can be used as resource for epidemiological studies without causing damage to wild fauna (Richini-Pereira et al., 2010). During the present study, a large number of road-killed animals was observed in the city of Londrina (47.65%; 32/68) when compared to the other cities in the North-Central and North Pioneiro mesoregions. This was probably because Londrina was the starting and ending point of the four transects covered during the searches. In addition, many animals were found by notifications from project participants residing in this municipality.

This study described the molecular detection of *Sarcocystis* spp. in *Colaptes melanochloros* and *Mazama gouazoubira* as

well as *N. caninum* in the brain of L. pardalis. In addition, Didelphis albiventris showed DNA fragments compatible with *T. gondii.* These results demonstrated the presence of protozoa in wild animals in the North-Central and North Pioneiro mesoregions of Paraná of importance in animal and human health. In addition, studies such these are essential for understanding environmental quality, for public health, and species conservation.

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