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Gastrointestinal, skin and blood parasites in *Didelphis* spp. from urban and sylvatic areas in São Paulo state, Brazil

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SUMMARY

Didelphis (Marsupialia, Didelphimorphia) are synanthropic mammals, whose omnivorous diet predisposes them to infections caused by endoparasites. Their higher frequency in urban areas makes them potential carriers of zoonotic protozoans and helminths, enhancing potential transmission to humans. Our purpose was to study two common species, *Didelphis albiventris* (54 individuals) and *D. aurita* (2 individuals), which were screened for blood, skin and intestinal parasites in animals captured in urban areas and in riparian forest regions associated with the Capivari River Basin, in Monte Mor's municipality, São Paulo state (SP), Brazil. Blood and tissue samples were collected for DNA extraction and PCR. Fecal samples were collected and submitted to two sedimentation and two flotation methods. 77.6% of fecal samples were positive for nematode eggs, 34.5% for trematode eggs and 32.7% for protozoans. Two *D. aurita* specimens were naturally infected by *Trypanosoma cruzi*. Molecular analysis in a *D. albiventris* captured on a forested rural area was positive for *Leishmania* sp. DNA. Several parasites were found infecting *Didelphis* sp., demonstrating that this group of animals can harbor important zoonotic parasites, potentially playing a role as sylvatic reservoirs for human and domestic animal pathogens.

1. Introduction

The genus *Didelphis* sp. (Didelphimorphia, Didelphidae, Didelphinae) includes mid sized are marsupials across the Americas (Quintao e Silva and Costa, 1999), being represented by four species in Brazil: *Didelphis marsupialis, Didelphis imperfecta, D. aurita* and *D. albiventris* (Malta and Luppi, 2006), of which, the last two are found in the State of São Paulo (SP) (Bovendorp and Villar, 2017; Cerqueira, 1985; Lima et al., 2017). *D. albiventris* is found in South America subtropical and temperate regions, in riparian forests and are active mainly during twilight and at night (Quintao e Silva and Costa, 1999). *D. aurita* occurs in Atlantic rain forest regions and has semi arboreal habits, being more active during twilight and at night. Both species are adapted and can be found in anthropic areas (*D. albiventris* is the most commonly found) both in the ground level and the trees' canopy (Cerqueira and Tribe, 2007). In general opossums have opportunist feeding habits with a diet of fruits, seeds, insects, mollusks, nestling birds, amphibians, reptiles and small mammals, showing a high degree of synanthropy and excellent adaptation for environments created or modified by humans (Ceotto et al., 2009). Their omnivorous diet exposes opossums to more endoparasites, turning them into hosts and reservoirs of protozoan and helminthic parasites (Elsheikha et al., 2004).

Several helminth species from the Strongyloidea and Trichuroidea superfamily, Ascaridae, Oxyuridae and Spiruridae family, among others, have been found in *D. albiventris* (Quintao e Silva and Costa, 1999). Some sporadic records for these helminths have also been recorded for *D. aurita* (Chagas-Moutinho et al., 2014; de Castro et al., 2017; Gomes et al., 2003; Travassos, 1934) Several protozoan parasites have also been found in the *Didelphis* genus, such as *Eimeria* sp., *Cryptosporidium* sp., *Giardia* sp., *Toxoplasma gondii* and *Sarcocystis neurona.* They are also known reservoirs of *Trypanosoma cruzi* and *Leishmania infantum chagasi* (da Silva et al., 2016; Donalisio and Paiz, 2017; Herrera et al., 2005; Legey et al., 2003; Silva et al., 2016). Sympatry and exposure to the same parasites has been identified as a determining factor for helminth infections in marsupials (Jiménez et al., 2011). As a consequence we expect to find similar parasites in the specimens

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examined.

Canine and human cases of cutaneous leishmaniasis caused by *Leishmania braziliensis* have been described in the study area (Cutolo et al., 2014), therefore investigating *Leishmania* sp. presence on biopsied/necropsied skin samples of the captured opossums is also important.

Considering the opossums role as hosts and carriers of parasites, as well as their presence in urban environments, our purpose was to inventory the parasites in blood, skin, and intestines of two species of marsupials occurring in urban areas and in riparian forest regions associated with the Capivari River Basin, in Monte Mor's municipality, São Paulo state (SP), Brazil.

2. Material and methods

2.1. Study area

Monte Mor's municipality is located in the administrative region of Campinas (SP, Brazil) at latitude 22°56′48 "south, longitude 47°18'57" west and 560 m altitude. It has an estimated population of 48.592, distributed in an area of 241 km^2. It has a humid subtropical climate (Cwa - Köppen climate classification), and the city territory is crossed by the Capivari River (IBGE, 2008, 2010; SEADE, 2009).

The vegetation of the studied area is characterized by secondary riparian forests and shrubby areas with natural vegetation corresponding to only 3.8% of the total municipality area (SIFESP).

2.2. Animal capture

Animals were captured in two distinct ways: through routine captures by the municipality Zoonosis Control Service (Serviço de Controle de Zoonoses) and actively using traps in riparian regions associated with the Capivari River. Captures made by the zoonosis control service were performed after popular notification from residents, which eventually found the animals alive, wounded or dead in public or private urban or rural areas.

Captures made in the forest were performed from June 2011 to May 2012 using galvanized wire traps (45 × 16 × 16cm), laid on the floor, baited with a feed that combined banana, corn meal, vanilla essence and apple. Each capture period took two consecutive nights per month.

2.3. Animal tagging and biometric records

Captured specimens were measured (head-body length, tail length, ear and posterior paw without nail), weight (dynamometer Pesola®), photographed, clinically evaluated, and identified to avoid capturing and registering the same animal twice. Males and females were differentiated through the presence/absence of scrotum or marsupium. Adults were differentiated from infants and juveniles. Adults had characteristic fur pattern, full teething and reached full maturity (± 8 months). Infants and juveniles were differentiated by whether they lived in their mother marsupium (up to ± 100 days) or were independent. Identification was performed using different combinations of up to three holes in the auricular pavilion associated with numbered ear tags of predefined numeration.

After marking and collecting samples, the animals were observed to ensure full recovery from anesthesia and then released in the same place they were captured or relocated to other regions when found in urban areas.

2.4. Blood and tissue sample collection and analysis

Blood samples were collected through tail vein puncture (Casagrande et al., 2009). Two to 3 ml of blood were collected per animal, and used for blood smears and to obtain serum. Blood smears were fixated with methanol and stained with Giemsa or rapid panoptic®

Table 1
Fecal analysis results from *Didelphis* spp. captured in Monte Mor's municipality from January 2010 to January 2013. Material obtained on recaptures was counted separately.

	D. albiventris		*D. aurita*	
	Positive	Negative	Positive	Negative
Cruzia tentaculata	41	15	2	0
Trichuridae	17	39	1	1
Trichostrongylidae	4	52	2	0
Singamidae	2	54	0	2
Spiruroidea	15	41	0	2
Acylostomatidae	23	33	2	0
Coccidiida oocyst	23	33	2	0
Eimeria spp oocyst	1	55	0	2
Octosporella spp	1	55	0	2
Absence of parasites[a]	13		0	

[a] Number of animal where no parasite was found.

and archived in the animal biology department from Campinas State University, for screening and photographic registry of the findings. Serum samples were stored in plastic microtubes properly identified and kept at −20 °C to build a serum bank.

Auricular skin fragments were obtained from animal tagging. Other potential samples such as skin, broken nails, and eventual biopsy tissue were also collected, kept at −20 °C and forward to the Adolfo Lutz Institute (São Paulo, Brazil) for DNA extraction and PCR molecular testing for the presence of *T. cruzi* and *Leishmania* spp.

DNA extraction was performed using QIAamp DNA mini kit (QIAGEN®) with a QIAcube extractor (QIAGEN®), according to the manufacturer instructions and DNA concentration and purity were determined by the ratio of O.D. at 260 and 280 nm in a NanoDrop ND1000 (Thermo Scientific). PCR was performed using primers TCZ1 (5′-CGAGCTCTTGCCCACACGGGTGCT-3′) and TCZ2 (5′-CCTCCAAGCA GCGGATAGTTCAGG-3′), which amplifies 188 bp of a 195-bp repetitive nuclear sequence for *T. cruzi* detection (Kirchhoff et al., 1996). For *Leishmania* spp. detection, primers 150 (5′-GGG(G/T)AGGGGCGT TCT(C/G)CGAA-3′) and 152 (5′-(C/G)(C/G)(C/G)(A/T)CTAT(A/T)TTA CACCAACCCC-3′) were used to amplify 120 bp from the conserved region of *Leishmania* minicircle kDNA (Gomes et al., 2007; Passos et al., 1999). PCR were carried out with GoTaq®Green Master Mix – Promega (7 µl of water, 12,5 µl of mix solution, 1 µM of each primer and 5 µl of DNA) in a Veriti® 96-Well thermal cycler (Applied Biosystems). A blank control (without DNA) and a positive control (DNA extracted from parasite culture) were used. Agar gel electrophoresis was performed in a 2% agarose gel in TBE buffer (0,045 M de Tris-Borato; 0,001 M de EDTA, pH 8) stained with 0,5 µl/ml ethidium bromide and PCR products were visualized under UV illumination.

All animals found dead were necropsied.

2.5. Fecal collection and analysis

Fecal samples were collected from the traps and/or directly from the animal anuses, using a swab, stored individually in plastic vials and kept refrigerated at 4 °C until analysis. Fecal samples were evaluated macroscopically for the presence or absence of proglottids and/or adult worms. For microscopic evaluation, fecal samples were submitted to two sedimentation methods (Hoffman et al., 1934; Rugai et al., 1954), along with two flotation methods (Faust et al., 1938; Willis, 1921). Parasite fecal culture was performed using granulated animal charcoal for larvae search according to Looss (1911). When possible, dichromate potassium solution ($K_2Cr_2O_7$) at 2.5% was added for coccidia oocyst sporulation. Fecal samples were processed in the Helminthology Laboratory (Animal Biology Department) at UNICAMP, where the eggs and larvae observed were photographed, measured and fixated in Railliet-Henry solution or in MIF. Adult parasites were submitted to the

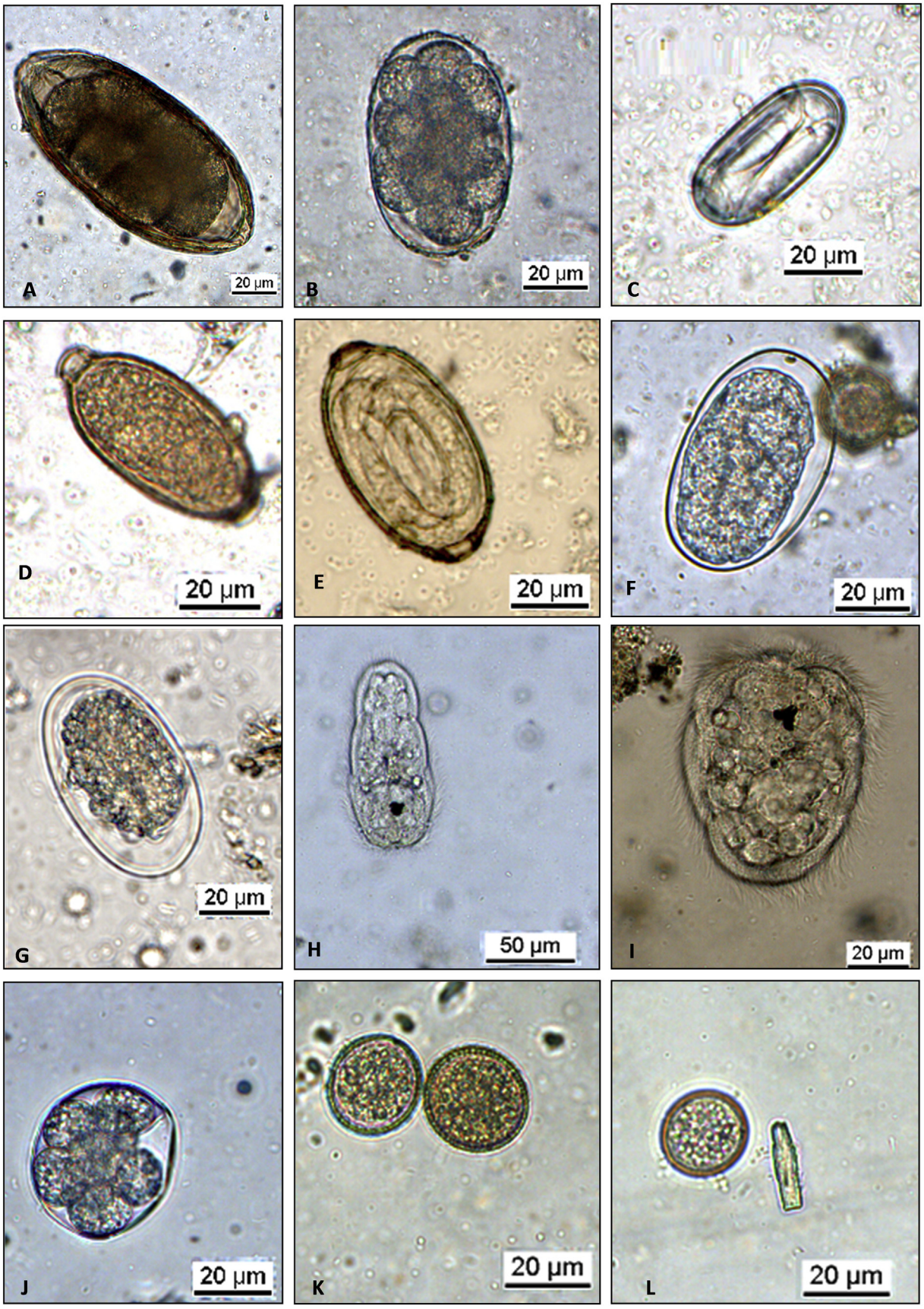

Fig. 1. Main findings obtained during fecal analysis. A – *Cruzia tentaculata* egg; B –Ancylostomatidae egg; C - Spiruroidea eggs; D – Trichuridae egg; E – Singamidae egg; F-G – Trichostrongylidae eggs; H – Miracidium larvae hatched from an unidentified Trematoda egg; I – Miracidium larvae hatched from an unidentified Trematoda egg; J - *Octosporella* sp. Like oocyst; K-L – Coccidiida oocyst.

Table 2
Helminth specimens collectedfrom the digestive tract of necropsied opossums (*D. albiventris*) found in the Monte Mor's municipality from January 2010 to January 2013. Monte Mor, SP, Brazil.

RG	Identification		Necropsy	
	Sex	Age	Parasites found	Location
S22/10	M	Ad	*Turgida turgida*	Stomach
			Cruzia tentaculata	Cecum and Colon
			Aspidodera sp.	Cecum and Colon
			Viannaia sp.	Duodenum
			Rhopalias coronatus	Small Intestine
S43/10	F	Ad	*C. tentaculata*	Cecum
S44/10	M	J	NDT	–
S45/10	F	Ad	*T. turgida*	Stomach
			C. tentaculata	Cecum
			Viannaia sp.	Duodenum
			Travassostrongylus spp.	Duodenum
S46/10	F	I	–	–
S47/10	F	I	–	–
S48/10	F	I	–	–
S49/10	F	I	–	–
S50/10	F	I	–	–
S52/10	F	J	*T. turgida*	Stomach
			Viannaia sp.	Duodenum
			Aspidodera sp.	Colon
S53/10	M	I	–	–
SR	M	Ad	*C. tentaculata.*	Large Intestine
S59/10	M	I	–	–
S12/11	M	Ad	*T. turgida*	Stomach
			Viannaia sp.	Duodenum
			C. tentaculata	Cecum
			Aspidodera sp.	Cecum and Colon
S13/12	F	I	*Cruzia* sp.[a]	Cecum
S14/12	M	Ad	*T. turgida*	Stomach
			C. tentaculata	Cecum
S15/12	M	I	–	–

RG - registry; F- female; M – male; Ad – adult; J – juvenile; I – Infant.
[a] Specie identification was not possible since only two worms were found

same process and identified using optic microscopy.

2.6. Ethic statement

This Project was approved by the Ethics Committee for Animal Use – CEUA/Unicamp (Protocol: 2546–1) and by the Brazilian Institute of the Environment and Renewable Natural Resources – IBAMA (Number: 31724–1).

3. Results

3.1. Animal captures

From January 2010 to January 2013, 46 animals were captured by Monte Mor's municipality Zoonosis Control Service. All animals belonged to the species *D. albiventris*, of which 17 (36.9%) were found dead and then necropsied. During this period two animals were recaptured (one recaptured twice), making 49 captures performed by the zoonosis control service during this study. From June 2011 to May 2012 ten different specimens were captured using traps (two *D. aurita* (20.0%) and eight *D. albiventris* (80.0%)). One *D. albiventris* specimen was captured twice, totalizing 11 captures.

Among the 46 animals captured by the Zoonosis Control Service, 19 (41.3%) were females and 27 (58.7%) were males, 24 (52.2%) were adults, 9 (41.3%) were juveniles and 13 (28.3%) were infants. Regarding capture locations, 24 (52.2%) were captured in private residencies, 10 (21.7%) were captured in public streets, 2 (4.3%) in commercial buildings, 2 (4.3%) in public buildings, 7 (15.2%) in private crofts and 1 (2.2%) in a highway.

Among the ten animals captured using traps, five were males and five were females, among those, nine were adults and one was a juvenile.

3.2. Fecal sample analysis

A total of 58 fecal samples from 54 animals were analyzed. Forty-five were positive for nematode eggs, 20 for trematode eggs and 19 for protozoan parasites (Table 1, Fig. 1). One animal was positive for trematodes in the first capture and after being recaptured it was also positive for protozoan parasites, therefore captures and recaptures were considered independently. Eggs and protozoans are represented in Fig. 1.

3.3. Necropsy

From the 17 necropsied animals (Table 2), we found parasites in 8 (47%), through macroscopic visualization of the gastrointestinal tract, mainly in the stomach and intestine. One specimen (6%) did not have viscera to analyze due to a dog attack. Parasites are represented in Fig. 2.

All specimens were deposited in the Zoology Museum (ZUEC) specimen collection at Unicamp (registry: ZUEC NMA 26; ZUEC NMA 27; ZUEC NMA 28; ZUEC NMA 29; ZUEC PLA 161; ZUEC NMA 30) and are available for consultation.

3.4. Blood and tissue sample analysis

Blood smear analyses revealed two *D. aurita* positive for *Trypanosoma* sp. with trypomastigote cells identified through microscopic direct examination (Fig. 3).

Among the 56 captured animals, 37 had biological samples (skin) collected and processed by PCR analysis targeting *Leishmania* sp. and *T. cruzi* DNA. Three animals out of 37 had positive reactions on PCR analysis. Two for *T. cruzi* and one animal for *Leishmania* sp.. *T. cruzi* was identified on the same two animals that had presented *Trypanosoma* tripomastigotes in the blood smears.

Presence of *Leishmania* sp. DNA on a skin sample demonstrates that *D. albiventris* can be harboring this parasite on the sylvatic and urban environment of Monte Mor's municipality.

T. cruzi blood smears were deposited at the Zoology Museum (ZUEC) specimen collection at Unicamp (registry: ZUEC EUG 06) and are available for consultation.

4. Discussion

Didelphis spp. have high synanthropy, favored by their omnivorous diet and excellent adaptation to the environment created or changed by humans which turns their presence more frequent in urban areas (Silva et al., 2016). As reported in this paper, 46 *D. albiventris* were captured from January 2010 to January 2013. This is the species most commonly found in local urban areas and the only one captured during this period (Cerqueira and Tribe, 2007).

Among captured animals, a female carrying five infants inside her marsupium, was found inside a deactivated wood oven in an urban area, showing that these animals can indeed adapt and reproduce in anthropic environments. Phlebotomine sandflies were found inside the oven. They were collected and identified as *Evandromyia cortelezzii* and *Evandromyia lenti* (Cutolo et al., 2014), illustrating the role such mammals may play on the epidemiology of Leishmaniasis, behaving as a potential link between sylvatic and urban transmission cycles, bringing not only the *Leishmania* parasite to the backyards of houses, but also acting as a potential source of blood meal to sandflies, enhancing the chance of a domiciliary transmission to humans and/or dogs living on it vicinity. Donalisio et al. (2017) also found *Pi. monticola* and *Ex. firmatoi* naturally infected with *L.* (*L.*) *infantum*, emphasizing the existence of the parasite sylvatic cycle. Also, in this same area,

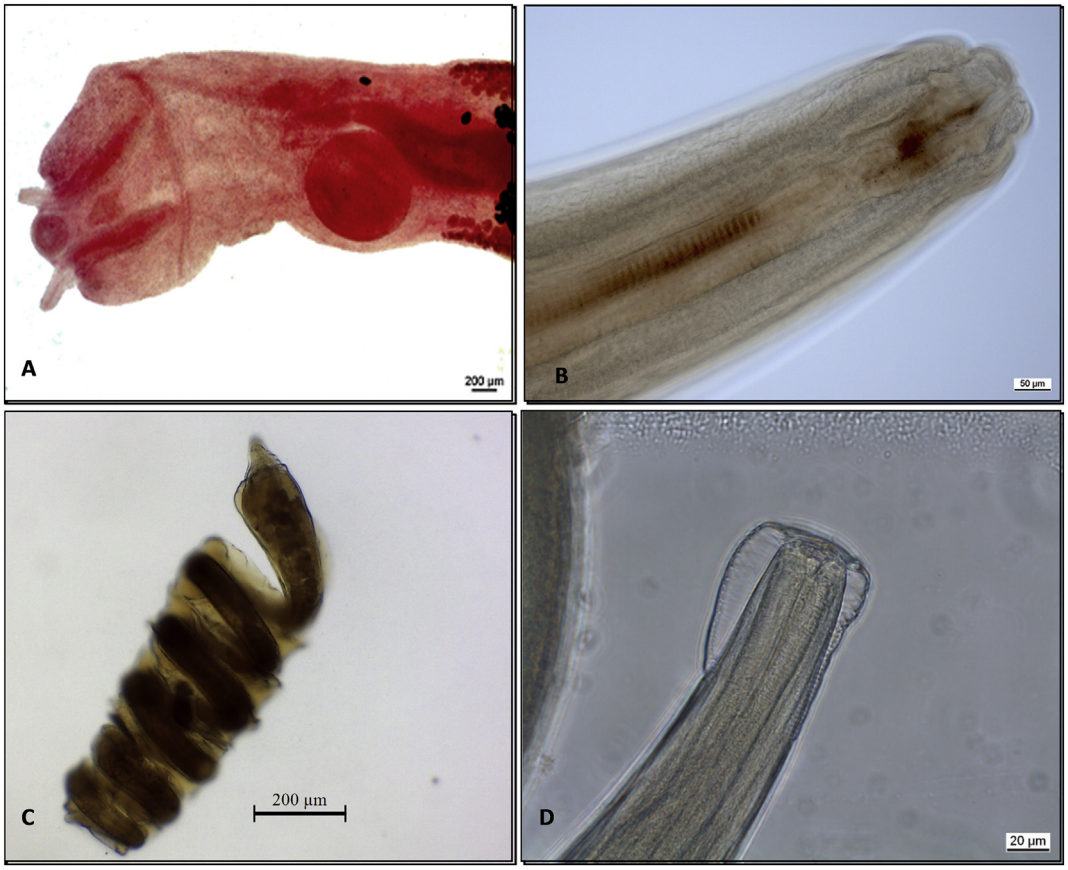

Fig. 2. Images of adult parasites recovered from necropsied opossums from Monte Mor's municipality from January 2010 to January 2013. A – *Rhopalias coronatus* (anterior section); B – *Cruzia tentaculata* (anterior section); C – *Viannaia* sp.; D – *Travassontrongylus* sp. (anterior section).

canine leishmaniasis seroprevalences of 1.5% and 1.2% in 2013 and 2015, respectively, suggesting that the disease is expanding to urban areas.

Out of 58 fecal samples, 45 (77,6%) were positive for nematode eggs, 20 (34.5%) for trematode and 19 (32.7%) for protozoan oocysts. The main parasite found during fecal analysis was *Cruzia tentaculata*, being found in 41 (70.7%) samples, which is consistent with the available literature that states that this is the main parasite infecting opossums (Adnet et al., 2009; Gomes et al., 2003; Santos-Rondon et al., 2012). *C. tentaculata* adult parasites were also found in the large intestine of 41% of necropsied animals. The parasites were found in the cecum in large quantities except in one animal were only two specimens were found, most likely due to its age since it was an infant. The animal had its intestinal content submitted to coproparasitologic evaluation, however no helminthic eggs were found, leading us to believe that the infection was still recent.

Ancilostomatidae-like eggs were found in 40% of the analyzed fecal samples, with no further differentiation possible due to the similarity of eggs among different species of this family. These type of eggs were also found in *D. albiventris* feces by Quintao e Silva and Costa (1999) when evaluating animals from Pampulha (Belo Horizonte, Brazil). We also identified Trichuridae type eggs in 17 samples, although adults were not found during necropsy. The same applied to Singamidae eggs detected in fecal samples of two animals.

Trichostrongylidae eggs were found in four fecal samples. Only two genera have been described in South American opossums, *Travassostrongylus* sp. and *Viannaia* sp. (Quintao e Silva and Costa, 1999). During necropsy we found four animals that were infected with *Viannaia* sp. and only one with *Travassostrongylus* sp.

In 15 fecal samples analyzed, Spiruroidea type eggs were found, also in five necropsied animals, *Turgida turgida* adults were found in the stomach.

Coccidiida type oocysts were detected in 23 fecal samples and in one sample an *Eimeria* spp. oocyst was also found while in another *Octosporella* spp. oocysts were found.

Didelphis spp. are important *T. cruzi* reservoirs (Herrera et al., 2005; Legey et al., 2003). Blood smears showed trypomastigotes forms of *Trypanosoma* sp. in two *D. aurita* specimens actively captured through traps. Natural *T. cruzi* infection was confirmed on those two animals through PCR analysis using biopsied skin ear fragments. To our knowledge, this was the first report of the parasite in opossums from Monte Mor's municipality, which is important since the presence of *Panstrongylus megistus* (*T. cruzi* vector) has been confirmed in the area by the municipality Zoonosis Control Service (data not published). This finding shows that biological vector, sylvatic reservoir and *T. cruzi* are registered in the area and a natural focus of the disease occurs in Monte Mor's municipality.

One *D. albiventris* specimen found in a private rural property was positive for *Leishmania* spp. DNA. The presence of *Leishmania* DNA suggests that these marsupials could be acting as natural reservoirs for this parasite in the area.

Our findings reported a *D. albiventris* specimen naturally harboring *Leishmania* DNA as well as two specimens of *D. aurita* infected by *T. cruzi* tripomastigotes. These finding associated with the presence of these diseases vectors suggests that natural foci of transmission of the causing agents of Cutaneous Leishmaniasis and Chagas Disease are occurring in Monte Mor's municipality, with *Didelphis* sp. marsupials acting as potential sylvatic reservoirs for such zoonotic agents.

5. Conclusions

In this work, we tried to access the parasitological status of

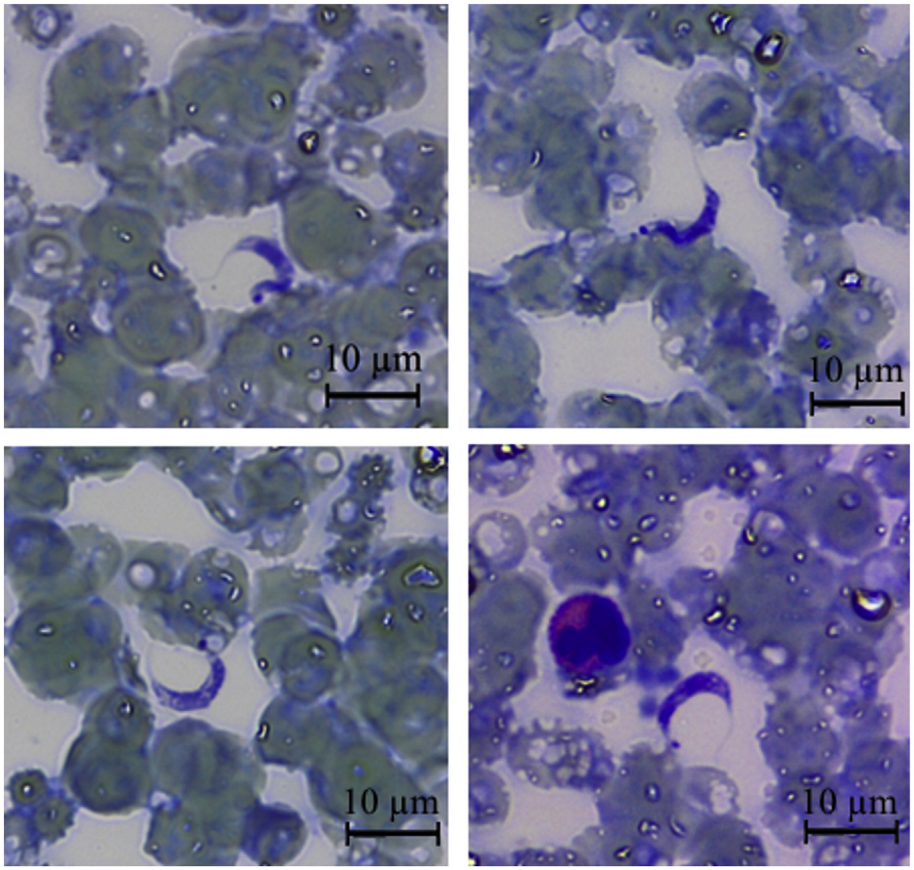

Fig. 3. *Trypanosoma cruzi* parasites found in *D. aurita* blood smears.

opossums in the Monte Mor's municipality (SP, Brazil). *D. albiventris* was the only species captured in urban areas and it was also the main captured species in the riparian forest. Different helminthes and protozoan parasite species were found, being *Cruzia tentaculata* the most commonly found parasite, which is also in accordance to data found by other authors (Adnet et al., 2009; Gomes et al., 2003; Santos-Rondon et al., 2012).

To our knowledge this was the first report of *D. aurita* naturally infected with *T. cruzi* in Monte Mor's municipality, which reflects the need for further investigations, especially since the vector *P. megistus* is present in the region and surrounding areas. The same can be said to leishmaniasis, since we found an opossum infected and there are further reports of canine and human cases in the municipality (Cutolo et al., 2014). These findings highlights the existence of natural foci of cutaneous leishmaniasis and Chagas disease in the studied area, with *Didelphis* opossums may act as sylvatic reservoirs of these important zoonotic causing agents.

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Conflict of interest

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no financial support for this work that could have influenced its outcome.

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