



The role of freshwater fish in the life cycle of *Diectophyme renale* in Southern Brazil

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ABSTRACT

Brazil stands out by the diverse records of *Diectophyme renale* in different hosts; however, there is little information about the life cycle of the nematode in the region. This study aims to report on third-stage larvae infections in fish in southern Brazil. In this context, 324 fish of different species belonging to Characiformes, Cyprinodontiformes and Siluriformes were collected in an urban area of Rio Grande do Sul State, where domestic and wild hosts were reported with the nematode. Of the total fish examined, 25(7.7%) were found to be parasitized by third-stage larvae of *D. renale* which were found encysted in the stomach serous membrane and in the celoma cavity of *Hoplosternum littorale* (Siluriformes) with a prevalence of 53.2% (25/47) and mean intensity of infection of 4.4 larvae/host (1 to 13 larvae). The occurrence of larvae in *H. littorale* indicates the presence of parasitosis in the region; however, the contribution of this fish species as a source of infection for dogs in urban areas must be considered with caution given the difficulties these dogs may face in the capture and predation of the fish to the point of effectively maintaining the urban cycle of *D. renale*. In addition, the low level of larvae registered in the total sample of fish examined indicates that these hosts are unlikely to play an important role in the transmission of *D. renale* to domestic animals in the region of the study.

1. Introduction

Diectophyme renale (Goeze, 1782) (Enoplida: Diectophymatidae) parasitizes domestic cats and dogs as well as wild carnivores (Canids and Mustelids) in the American, European and Asian continents. The preferred site of infection is the right kidney, although there are several reports of helminths found in the peritoneal cavity and with less frequency in both kidneys as well as other sites of infection (Anderson, 2000; Measures, 2001).

The nematode life cycle involves the participation of freshwater organisms such as oligochaetes (intermediary hosts) and fish and anurans (paratenic hosts) within which the infective third-stage larva can be lodged and transmitted to the definitive hosts (mammals) through the trophic chain. In the definitive hosts the parasite reaches the kidney where it completes the life cycle laying eggs which are excreted via the urine and contaminate the environment (Mace and Anderson, 1975; Measures and Anderson, 1985).

The participation of fish as paratenic hosts was verified by Measures and Anderson (1985) through experimental studies performed in Canada. In Brazil, larvae of *D. renale* in *Acestrorhynchus lacustris* (Lütken, 1875) (Characiformes), *Gymnotus sylvius* Albert & Fernandes-Matioli, 1999 (Gymnotiformes) (Abdallah et al., 2012) and *Hoplosternum littorale* (Hancock, 1828) (Siluriformes) (Mascarenhas et al., 2016) were reported. Also, there are registers of third-stage larvae in the frog *Rhinella icterica* (Spix, 1824) (*Chaunus ictericus*) (Bufonidae) (Pedrassani et al., 2009), freshwater turtles *Trachemys dorbigni* (Duméril & Bibron, 1835) (Emydidae) (Mascarenhas and Müller, 2015) and *Phrynops hilarii* (Duméril & Bibron, 1835) (Chelidae) (Mascarenhas et al., 2017), and the snake *Philodryas patagoniensis* (Girard, 1857) (Dipsadidae) (Mascarenhas et al., 2018).

In Brazil the nematode has been mainly diagnosed in domestic dogs and only rarely in domestic cats (Amato et al., 1976; Neves et al., 1983; Miranda et al., 1992; Kommers et al., 1999; Monteiro et al., 2002; Kano et al., 2003; Pereira et al., 2006; Colpo et al., 2007; Verocai et al., 2009;

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Sousa et al., 2011; Cottar et al., 2012; Eicke et al., 2014; Pedrassani et al., 2014; Silveira et al., 2015; Bach et al., 2016; Rappeti et al., 2017). In wild mammals there are registers of *D. renale* mainly in carnivores such as: *Galictis cuja* (Molina, 1782) (Barros et al., 1990; Pesenti et al., 2012; Zabott et al., 2012); *Lontra longicaudis* (Olfers, 1818) (Mustelidae) (Echeniqueet al., 2018); *Speothos venaticus* (Lund, 1842) (Proença, 1935); *Chrysocyon brachyurus* (Illiger, 1815) (Varzone et al., 2008; Duarte et al., 2013; Vulcani et al., 2015); *Cerdocyon thous* (Linnaeus, 1766) (Canidae) (Ribeiro et al., 2009); *Nasua nasua* (Linnaeus, 1766) (Procyonidae) (Milanelo et al., 2009); *Leopardus geoffroyi* (Felidae) (Trindade et al., 2018); and with single reports in Pilosa *Choloepus didactylus* (Linnaeus, 1758) (Rocha et al., 1965) and the primate *Cebus apella* (Linnaeus, 1758) (Ishizaki et al., 2010).

Despite the diversity of vertebrate hosts reported in Brazil, there is a gap in the knowledge of the epidemiologic aspects involved in the transmission and maintenance of the parasitosis since there is little knowledge about the life cycle and biology of *D. renale* in the South American continent. In this context, this study aimed to assess the occurrence of *D. renale* larvae in fish from an urban area in southern Brazil, thus contributing to the epidemiological studies in the region where several domestic and wild hosts have been registered with the nematode.

2. Material and methods

2.1. Area of study

The study was conducted in an urban area of Pelotas, Rio Grande do Sul State, Brazil (31°45'50.6"S – 52°18'53.5"W) in which there is an intense urbanization process (Fig. 1). Fish were collected from three locations: urban pluvial canal, São Gonçalo canal, and a swamp area adjacent to these canals (Fig. 1). The São Gonçalo canal is 76 km long and connects the Patos of Lagoon to Mirim Lagoon (Agência da Lagoa Mirim, 2018). The floodplain of the São Gonçalo canal is characterized by the presence of plains and fields with different physiognomies (Sosinski, 2009). The regional climate may be classified as humid subtropical with a yearly maximum temperature average of 28 °C in January and February and minimal temperature average of 8.8 °C in June and July. The annual average precipitation in the city is 1439.5 mm with rain regularly distributed throughout the year (Wrege et al., 2012).

2.2. Host collecting

Three hundred twenty-four specimens were examined: *Cyphocharax voga* (Hensel, 1870) ($n = 39$), *Hyphessobrycon igneus* Miquelarena, Menni, López & Casciotta, 1980 ($n = 13$), *Astyanax* spp. ($n = 8$), *Hoplias malabaricus* (Bloch, 1794) ($n = 2$), *Cheirodon interruptus* (Jenyns, 1842) ($n = 2$), *Cheirodon ibicuiensis* ($n = 2$), *Hyphessobrycon luetkenii* (Boulenger, 1887) ($n = 1$) (Characiformes); *Phalloceros caudimaculatus* (Hensel, 1868) ($n = 100$), *Cnesterodon decemmaculatus* (Jenyns, 1842) ($n = 68$), *Austrolebias nigrofasciatus* Costa & Cheffe, 200 ($n = 18$), *Cynopoeilus melanotaenia* (Regan, 1912) ($n = 1$), (Cyprinodontiformes); *Hoplosternum littorale* (Hancock, 1828) ($n = 47$), *Loricariichthys anus* (Valenciennes, 1835) ($n = 11$), *Corydoras paleatus* (Jenyns, 1842) ($n = 5$), *Rhamdia quelen* (Quoy&Gaimard, 1824) ($n = 3$), *Callichthys callichthys* (Linnaeus, 1758) ($n = 3$), and *Pimelodus maculatus* Lacepède, 1803 ($n = 1$) (Siluriformes). The fish were identified according to Corrêa et al. (2015).

The collections were made between February 2015 and December 2016 with the help of a trawl (5 mm mesh), gill net (35 mm mesh), and dip net (5 mm mesh). The study was licensed by Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio/n° 47,397) and approved by the Ethical Commission of Animal Experimentation of UFPel (CEEA – 1859).

2.3. Collection and morphological identification of the larvae

The fish were necropsied and the stomach, kidneys, heart, liver, eyes, swim bladder, gills, muscles and celoma cavity were examined. The larvae were fixed in AFA, conserved in ethanol 70% and clarified with Amann's lactophenol for morphological identification according to Mace and Anderson (1975) and Measures and Anderson (1985). The infection indices were estimated according to Bush et al. (1997). The photomicrographs were made in an Olympus® BX 41 microscope with a camera system attached and the plates were prepared in Adobe Photoshop®CS5. Vouchers were deposited in the Coleção de Helmintos do Laboratório de Parasitologia de Animais Silvestres (CHLAPASIL/UFPel) (Numbers 617 – 622), Rio Grande do Sul, Brazil.

2.4. Molecular identification of the larvae

The larvae were washed in a 0.9% saline solution and stored at –20 °C until DNA extraction. The Genomic DNA was extracted following the K/phenol-chloroform protocol of Sambrook et al. (1989). The PCR reactions were performed in a total volume of 25 µl containing 100–150 ng of DNA, 12.5 µl of GoTaq Green Master Mix, 2× (Promega), 0.5 µl of each primer and PCR water to complete the total volume of 25 µl. A pair of ribosomal 18S sub-unity primers were used: Nem18SF, 5'-CGCGAATRGCTCATTACAACAGC-3' and Nem18SR, 5'-GGGCGGTATCTGATCGCC-3' (Floyd et al., 2005). The program used for the PCR was a cycle of 5 min at 95 °C, 35 cycles of 1 min at 95 °C, 1 min at 50 °C and 1 min at 72 °C. In the end an extension was made of 8 min at 72 °C. The amplicons were seen by electrophoresis in agarose gel at 1.2%, dyed with Blue Green Loading Dye I (LGC Bio). The amplicons were sent to Macrogen (<http://www.macrogen.com>) for purifying and sequencing. The sequences obtained were validated by comparison between fragments forward and reverse and grouped with Mega 6.0 (Tamura et al., 2013) software and submitted after that for identification in the NCBI/Genbank database using the Basic Local Alignment Search Tool (BLAST – <https://www.ncbi.nlm.nih.gov/BLAST/>).

3. Results

Twenty-five (7.7%) fish were found parasitized by third-stage larvae of *D. renale* (Fig. 2), all of which were *H. littorale*, determining a prevalence of 53.2% (25/47). The mean intensity of infection was 4.4 larvae/host (1–13 larvae), which were encysted in the stomach serous membrane and in the celoma cavity. Table 1 shows the morphometry of these larvae.

Fragments of approximately 900pb were generated in observation to the agarose gel. The resulting sequences were of 790pb (deposited in the Genbank under MH445970) and when analyzed in the Genbank database matched 100% identity with the sequence AB595139.2 of *Diocetophyme renale*, isolated in Japan.

4. Discussion

The third-stage larvae found in *H. littorale* showed a smaller size than those larvae found in other fish (Measures and Anderson, 1985), anurans (Mace and Anderson, 1975; Pedrassani et al., 2009), freshwater turtles (Mascarenhas and Müller, 2015) and snakes (Mascarenhas et al., 2018). According to Measures and Anderson (1985), the size of the larvae may vary depending on the host species. As to the site of infection, Measures and Anderson (1985) reported that most of the cysts were collected in fish muscles, different from the fish in the present study, in which cysts were not found in this site of infection. In other hosts, such as freshwater turtles and anurans, there are reports of larvae encysted in the musculature, celoma cavity and stomach serous membrane (Mace and Anderson, 1975; Pedrassani et al., 2009; Mascarenhas and Müller, 2015) corroborating the present study.

Ichthyoparasitology studies have a great importance due to the

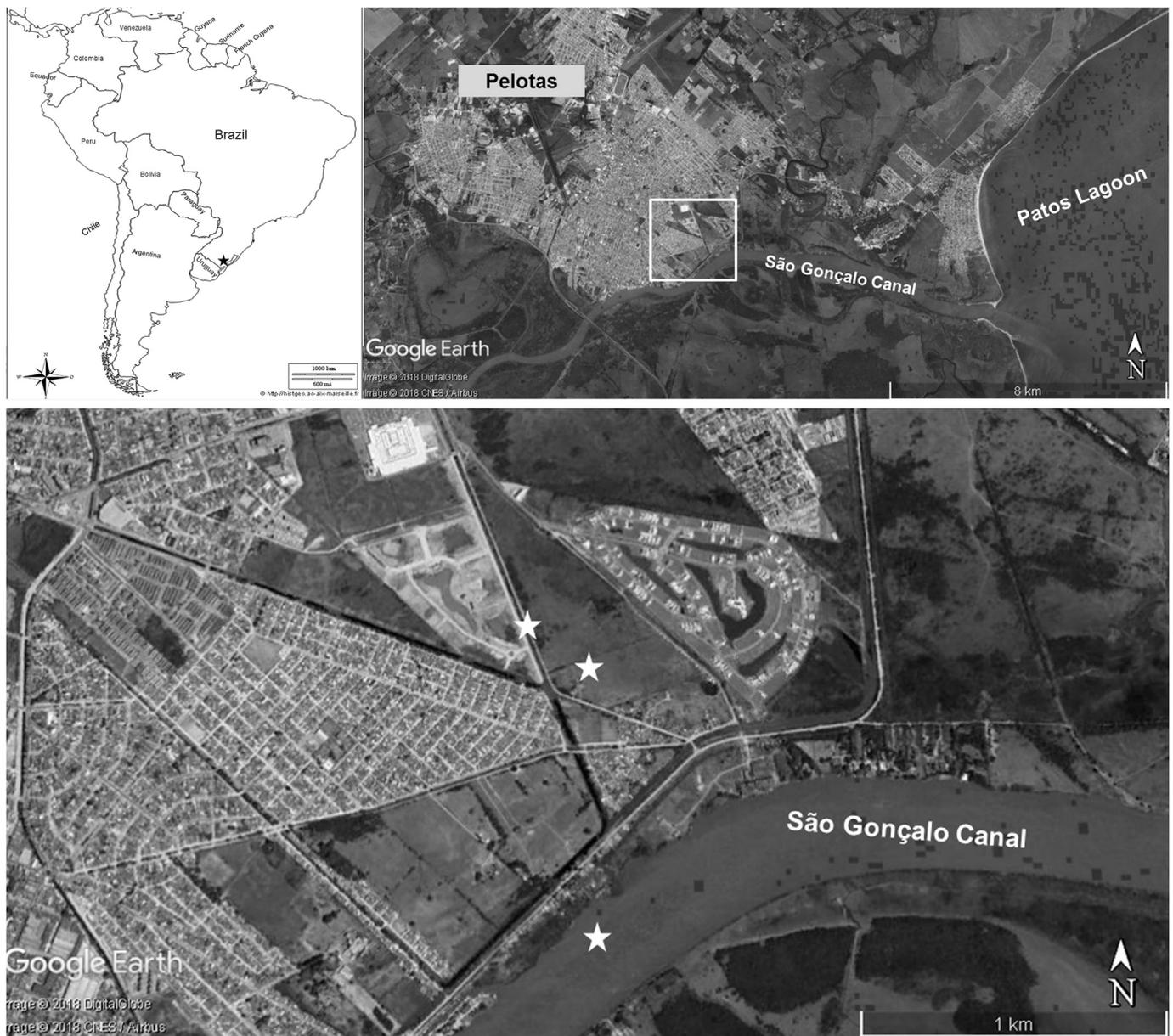


Fig. 1. Fish collection sites in the urban area of the municipality of Pelotas, Rio Grande do Sul, Brazil. Source: Extracted and modified the site Google® Earth (©2018 Google – Images ©2018 Digital Globe).

utilization of fish as food for human populations worldwide. In Brazil, although there are several ichthyoparasitology studies (Pavanelli et al., 2013), few registers of *D. renale* larvae were reported, raising questions regarding the importance of these hosts in the transmission and maintenance of diotrophmatosis in domestic animals. Measures and Anderson (1985) examined 279 fish belonging to *Lepomis gibbosus* (Linnaeus, 1758) (Centrarchidae) in Canada and reported 1–4 third-stage larvae in 14% of the hosts. In Brazil, Pedrassani (2009) examined 100 fish of different species of Siluriformes, Characiformes, and Perciformes in an endemic area for canine diotrophmatosis in the State of Santa Catarina; however, all the host were negative for *D. renale* larvae. Abdallah et al. (2012) examined 448 fish belonging to nine species and found 2.9% to contain *D. renale* larvae. Larvae were found in *Gymnotus silvius* (Gymnotiformes) ($n = 51$ examined) and *Acestrorhynchus lacustris* (Characiformes) ($n = 62$ examined) with a prevalence and mean intensity of infection of 20% and 1.5 helminth/host and 5% and 1 helminth/host, respectively. The prevalence reported by Measures and Anderson (1985) and Abdallah et al. (2012) is similar to that found in

the present study, considering the total number of fish examined, independent of species.

In helminthological studies with *H. littorale* in Rio de Janeiro (Abdallah et al., 2006) and São Paulo (Abdallah et al., 2012; Dias et al., 2017), where 100 and 120 fish were examined respectively, *D. renale* larvae were not reported. The presence of larvae in *H. littorale*, in the study region, may be related to the sum of three factors: (1) the presence of definitive hosts, such as domestic dogs which shed the parasite eggs in the environment; (2) the presence of susceptible intermediate hosts; and (3) feeding and behavior of *H. littorale*.

In the study area, over 70 cases of canine diotrophmatosis have been diagnosed between 2010 and 2015 (Rappeti et al., 2017) and recent data from UFPEL have shown that over 80 domestic dogs were diagnosed in the region between 2016 and 2017 (Veterinary Doctor Soliane Carra Perera, personal communication). The dog population in the city of Pelotas is approximately 67,000 (47,000 semi-domesticated, 13,000 domesticated and 7000 strays) (Municipal Administration, 2012), which makes the situation of the city relating to the infection

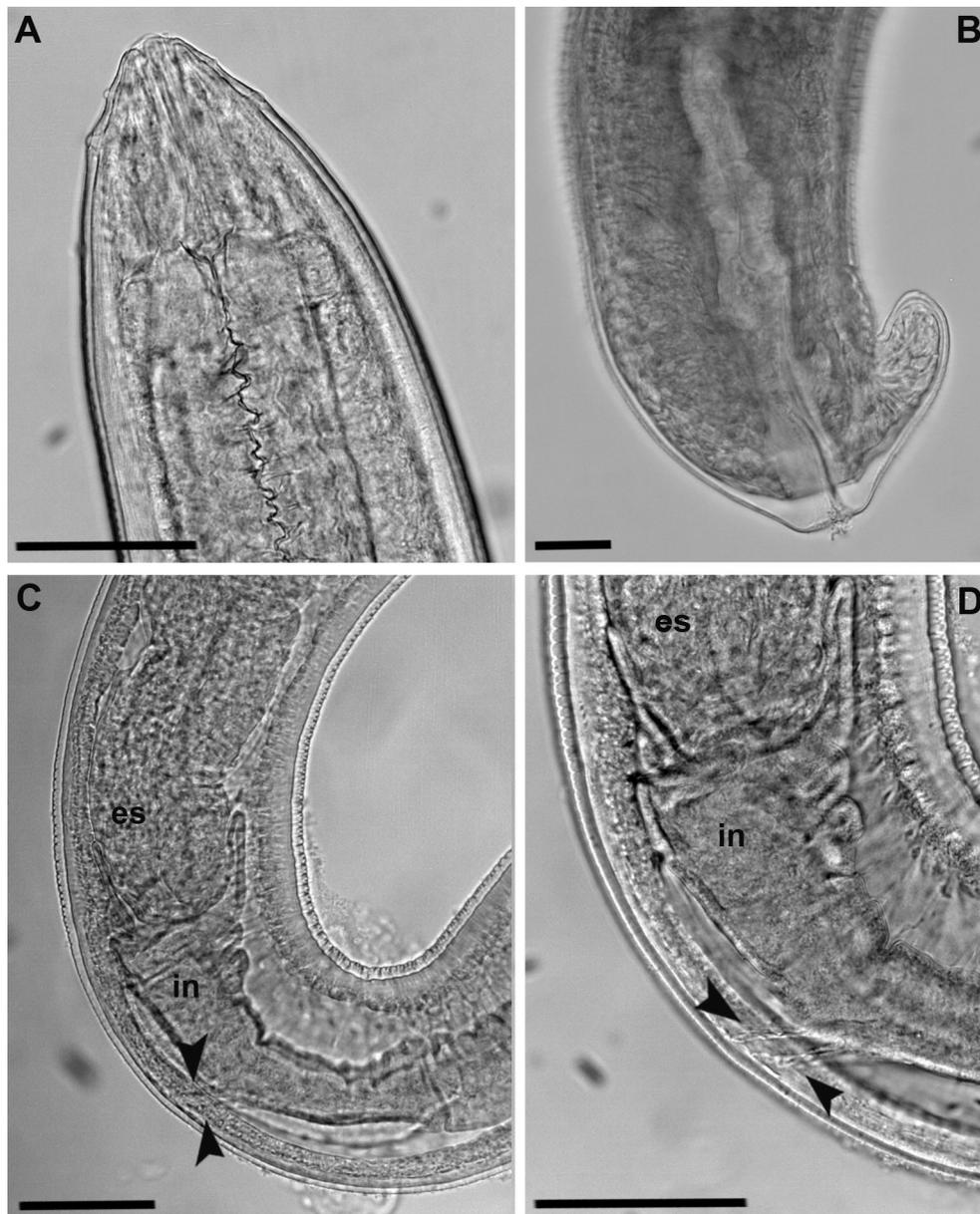


Fig. 2. Third-stage larva of *Dioctophyme renale* parasite *Hoplosternum littorale* (Hancock, 1828) (Siluriformes: Callichthyidae) in southern Brazil. **A** – Anterior extremity of female larva (Bar = 40 µm). **B** – Posterior extremity of male larva (Bar = 30 µm). **C** – Vulvar primordium (arrows) of female larva near to junction esophagus-intestine (es – esophagus, in – intestine) (Bar = 50 µm). **D** – Detail of vulvar primordium (arrow) of female larva (es – esophagus, in – intestine) (Bar = 50 µm).

worrying, since the dogs are definitive hosts and therefore egg disseminators. These eggs then reach the aquatic environment, where they infect intermediate hosts and subsequently paratenic hosts such as fish, and others vertebrates.

As to the presence of susceptible intermediate hosts, there is no knowledge about which host is responsible for the development of the third-stage larvae in South America. The freshwater oligochaete *Lumbriculus variegatus* (Müller, 1774) (Lumbriculidae: Clitellata) was identified as an intermediate host in North America (Mace and Anderson, 1975). This species was reported in Patagônia (Argentina) (Miserendino, 2007) and in Minas Gerais (Brazil) (Marchese et al., 2015), however there are no studies which demonstrate its participation in the nematode life cycle in the South American Continent. It is important to emphasize the possibility of other oligochaete species acting as intermediate hosts in the region.

Hoplosternum littorale in its turn shows an aerial accessory

respiration which allows its survival in temporary environments (Hostache and Mol, 1998; Brauner et al., 1999) where a large concentration of sediments in small puddles occur and consequently of the fauna in draught periods, what may potentialize the ingestion of oligochaetes infected by *D. renale*. This concentration effect may also facilitate the infection in dogs, especially in the urban environment as in the case of the area this study.

Epidemiological studies made in high prevalence areas of canine diotophymatosis in Santa Catarina (Brazil) (Pedrassani et al., 2017), and in Buenos Aires province (Argentina) (Radman et al., 2017) pointed out as risk factors, the habit of the dogs of drinking water from canals or the ingestion of fish or anurans. Though, there is no studies that indicate the occurrence of *D. renale* larvae in fish in these areas. In relation to the anurans, *R. icterica* (*C. icterica*) was reported as host of larvae in Santa Catarina with a prevalence and mean intensity of infection of 5.17% (3/58) and 5.33 larvae/host (1–10 larvae), respectively

Table 1

Mean measurements (mm) of third-stage larvae of *Diocotophyme renale* recovered from naturally infected *Hoplosternum littorale* (Hancock, 1828) (Siluriformes: Callichthyidae) in southern Brazil. n – number of specimens examined; SD – standard deviation.

	Male (n = 5)	Female (n = 5)
	mean ± SD	mean ± SD
Body length	3.41 ± 0.962	4.848 ± 1.265
Pharynx length	0.041 ± 0.008	0.039 ± 0.005
Distance of first papillae row to anterior extremity	0.009 ± 0.002	0.008 ± 0.004
Distance of second papillae row to anterior extremity	0.021 ± 0.006	0.019 ± 0.005
Distance of nerve ring to anterior extremity	0.075 ± 0.002	0.077 ± 0.011
Esophagus length	1.11 ± 0.119	1.543 ± 0.332
% esophagus of body length	33.94 ± 6.831	32.21 ± 2.886
Width at level of esophageal-intestinal junction	0.131 ± 0.017	0.126 ± 0.029
Distance of vulvar primordium to anterior extremity	–	1.761 ± 0.433
Rectum length	0.134 ± 0.013	0.114 ± 0.019

(Pedrassani et al., 2009). In the area of the present study, a high prevalence of larvae in freshwater turtles, *T. dorbigni*, was verified, 87.5% (28/32) and mean intensity of infection of 13.9 larvae/host, pointing out the presence of the parasitosis in the region (Mascarenhas and Müller, 2015). In the fishes of this area the indices of infection were low, as well as in other studies (Measures and Anderson, 1985; Abdallah et al., 2012), which suggests that these hosts do not represent the main source of infection for domestic dogs. According to Mascarenhas et al. (2018) the participation of fish, turtles and snakes as a source of infection for dogs in urban areas should be seen with caution, since these hosts must be ingested by dogs for that occurs infection

with the third-stage larvae. In this case, the difficulties that dogs can have in the capture and predation of these vertebrates in urban areas to the point of effectively feeding back the cycle must be considered. However, the authors emphasized that the records of *D. renale* larvae in fish, turtles and snakes serve as an alert, and these hosts considered as the sentinels of the parasitosis (Mascarenhas et al., 2018). Due to the low parasitological indices found in fish and the difficulties in these of being predated by dogs, it is suggested that possibly the main source of infection for domestic dogs are oligochaetes, ingested together with water from canals, ditches and swamps. The urban area sampled, in the present study, is characterized for an intense urbanization adjacent to the wetlands near the São Gonçalo Canal, where domestic dogs in a vulnerable condition may be observed, which have access to the swamp areas as well as the pluvial canals.

It stands out that the Siluriformes *H. littorale* shows itself to be a good reservoir of *D. renale* larvae in the region and may act as a source of infection for wild carnivores, such as *Galictis cuja* (Molina, 1782), *Lontra longicaudis* (Olfers, 1818) (Mustellidae) and *Leopardus geoffroyi* (Felidae), which have already been reported as hosts in the region (Pesenti et al., 2012; Echenique et al., 2018; Trindade et al., 2018) (Fig. 3). Wild Mustelidae are the natural hosts of *D. renale*, where domestic dogs are considered accidental definitive hosts due to the diverse ectopic locations in which the nematode was reported (Radman et al., 2017).

Fig. 3 illustrates the diversity of domestic and wild hosts reported with larvae and adults of *D. renale* in the Southern Region of Brazil by several authors (Pesenti et al., 2012; Mascarenhas and Müller, 2015; Mascarenhas et al., 2017; Rappeti et al., 2017; Mascarenhas et al., 2018; Echenique et al., 2018; Trindade et al., 2018), as well as the present study. In this context, it is concluded that complementary studies are necessary in order to generate knowledge, which may help in the development of control and prophylaxis programs of diocotphyomatosis in the region. Such studies should consider the infection indices

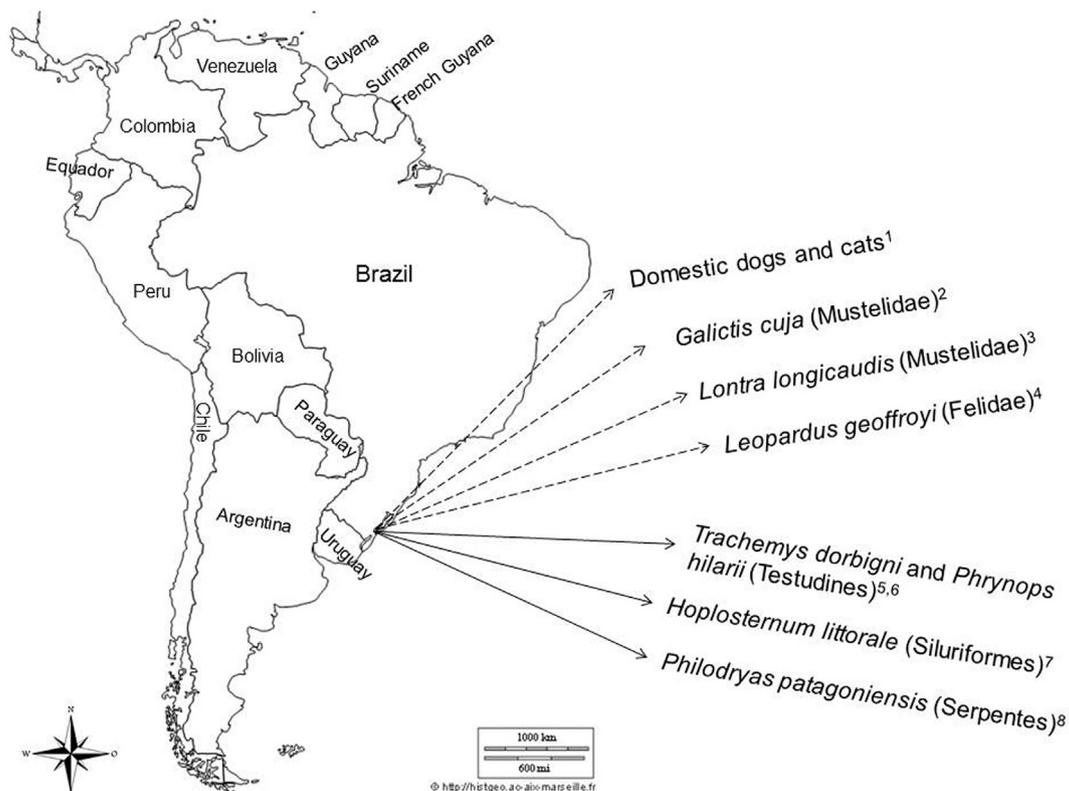


Fig. 3. Diversity of hosts registered with adults (dashed arrows) and larvae (continuous arrows) of *Diocotophyme renale* (Goeze, 1782) (Enoplida: Diocotphymatidae) in southern Brazil. References: 1 – Rappeti et al. (2017); 2 – Pesenti et al. (2012); 3 – Echenique et al. (2018); 4 – Trindade et al. (2018); 5 – Mascarenhas and Müller (2015); 6 – Mascarenhas et al. (2017); 7 – Mascarenhas et al. (2016) and present study; 8 – Mascarenhas et al. (2018).

in possible paratenic hosts as well as research related to the intermediate hosts which are unknown in the South American Continent.

In relation to the presence of larvae in fish, it is important to investigate larvae of *D. renale* in different species of fish, especially in endemic areas for the parasitosis and that have fishing activity, to verify the occurrence of larvae and the indices of infection in fish of economic interest, since this parasite has zoonotic potential. Cases of human infection of *D. renale* have been reported mainly in asiatic countries (Hanjani et al., 1968; Urano et al., 2001; Ignjatovic et al., 2003; Sardjono et al., 2008; Li et al., 2010; Gu et al., 2012; Katafigiotis et al., 2013; Venkatrajaiah et al., 2014; Tokiwa et al., 2014; Chauhan et al., 2016; Yang et al., 2016; Norouzi et al., 2017). In Brazil there is only one reported case, in 1945 in Maranhão State (Lisboa, 1945). *Hoplosternum littorale* has no economic importance in the studied region (Southern of Brazil) however, in the Northeast of Brazil it is often used as a source of human food (Albuquerque and Barthem, 2008). It should be noted, that in *H. littorale* the larvae were found encysted in the celoma cavity and stomach serous, whilst in other hosts, such as freshwater turtles the larvae were also found in the muscles in addition to the celoma cavity (Mascarenhas and Müller, 2015; Mascarenhas et al., 2017). Therefore, to investigate the larvae a complete host exam must be considered.

5. Conclusion

The occurrence of *D. renale* larvae in *H. littorale* is an alert for the presence of the parasite in the region; however, the role of fish as a source of infection for dogs in the region should be considered with caution given the difficulties that dogs may face in capturing and preying fish to the point of effectively maintaining the urban cycle of the parasitosis. Also, the low larvae numbers reported in the total sample of fish indicate that these hosts do not exert an important role in the transmission of *D. renale* among domestic animals in the studied region. Thus, it is suggested that the main source of infection for domestic dogs are oligochaetes ingested together with water from canals, ditches and swamps.

Conflicts of interest

The authors declare that there is no conflict of interest.

Ethical statement

The study was licensed by Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio/n° 47397) and approved by the Ethical Commission of Animal Experimentation of UFPel (CEEA – 1859).

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