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Renicolidae infection in Manx shearwater (*Puffinus puffinus*): is parasitism implicated on renal lesions? --Manuscript Draft--

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Abstract:	<p>Renicolid digeneans are frequently observed in the renal tubules and ureters of seabirds, such <i>Puffinus puffinus</i> , a migratory species distributed along the Brazilian coast. However, few studies have focused on the relationship between renicolid infection and health status in seabirds. Thus, the aim of this study was to describe i. renal and systemic alterations, ii. the renicolids, and iii. the biological aspects associated with the parasitism in <i>P. puffinus</i> . Gross and histological assays were performed in 93 <i>P. puffinus</i> stranded on the Paraná coast, southern Brazil. Renicolids were submitted to morphological and molecular assays. Females, juveniles, and poor body condition were the most frequent characteristics observed. A high prevalence of renicolids was found in <i>P. puffinus</i> (71/93). In the kidney, the main microscopic finding was mild nephritis. The renal lesions were significantly associated with the parasite infection. The morphological and molecular analyzes confirmed the species as <i>Renicola sloanei</i> (100% and 95.9% of nucleotide identity with <i>R. sloanei</i> strains from <i>P. puffinus</i> and from <i>Spheniscus demersus</i> , respectively). Cardiac and skeletal muscle degeneration and necrosis were the most frequent systemic changes in both parasitized and non-parasitized animals, which are probably associated with the migratory period. Therefore, the results suggest that the presence of renicolids might lead to important renal alterations, possibly contributing to a decrease in the health status of <i>P. puffinus</i> along the migratory route.</p>
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Author Comments:	<p>We refer to the Parasitology Research the manuscript article entitled “Renicolidae infections in Manx shearwater (<i>Puffinus puffinus</i>): is the parasitism implicated on renal lesions?” for consideration and possible publication.</p> <p>The aim of the manuscript is to present the description of renicolid trematodes in renal tubules and their implications for renal tissue in <i>Puffinus puffinus</i> stranded on Paraná state, southern of Brazil. This seabird species has migratory behavior and is often found stranded on the Brazilian coast and other countries. However, few studies have presented relevant information concerning the health status in this species. In this manuscript we confirmed that the renal trematode is <i>Renicola sloanei</i>. In addition, we showed that the presence of the parasite induces significant renal alterations that might contribute to animal debility due to the migration process.</p> <p>We declare the interest and approval of the manuscript by all authors. We would like to inform the author’s contribution: Andressa M. Rorato N. de Matos (delineating of the study, molecular and morphological analysis, histopathology, and writing), Mário R. C. Meira Filho (parasitological analysis and writing), Elis Lorenzetti and Fernanda L. L. Pereira (molecular analysis and writing), Eloiza T. Caldart (statistical evaluations and writing), Thiago Bizari and Ricardo L. N. de Matos (histological analysis), Camila Domit (critical revision), and Ana Paula F. R. L. Bracarense (delineating of the study, writing, and critical revision).</p> <p>Finally, we declare that this manuscript, an original work, was not submitted to another journal and that none of the authors have a conflict of interest.</p>
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Renicolidae infection in Manx shearwater (*Puffinus puffinus*): is parasitism implicated on renal lesions?

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Abstract

Renicolid digeneans are frequently observed in the renal tubules and ureters of seabirds, such *Puffinus puffinus*, a migratory species distributed along the Brazilian coast. However, few studies have focused on the relationship between renicolid infection and health status in seabirds. Thus, the aim of this study was to describe *i.* renal and systemic alterations, *ii.* the renicolids, and *iii.* the biological aspects associated with the parasitism in *P. puffinus*. Gross and histological assays were performed in 93 *P. puffinus* stranded on the Paraná coast, southern Brazil. Renicolids were submitted to morphological and molecular assays. Females, juveniles, and poor body condition were the most frequent characteristics observed. A high prevalence of renicolids was found in *P. puffinus* (71/93). In the kidney, the main microscopic finding was mild nephritis. The renal lesions were significantly associated with the parasite infection. The morphological and molecular analyzes confirmed the species as *Renicola sloanei* (100% and 95.9% of nucleotide identity with *R. sloanei* strains from *P. puffinus* and from *Spheniscus demersus*, respectively). Cardiac and skeletal muscle degeneration and necrosis were the most frequent systemic changes in both parasitized and non-parasitized animals, which are probably associated with the migratory period. Therefore, the results suggest that the presence of renicolids might lead to important renal alterations, possibly contributing to a decrease in the health status of *P. puffinus* along the migratory route.

KEY WORDS: Seabirds, renal histopathology, health status, *Renicola sloanei*, trematodes, migratory seabirds.

Introduction

Puffinus puffinus (Brünnich, 1764), a procellariiform known as the Manx shearwater, migrate to southern Atlantic - Argentinian, Uruguayan, and Brazilian - seawaters mainly in spring and summer, between September and November (Guilford et al. 2009; Freeman et al. 2013). The Manx shearwater have been considered marine environment sentinels and their health condition and mortality rates can contribute to assess the environmental conditions (Cardoso et al. 2014). Mortality of Manx shearwater in Brazilian seawaters was associated with the migration process (Vooren and Brusque 1999). In addition, multiple sources of impacts might affect seabird species along their life cycle, including anthropogenic (Wilcox et al. 2015) and natural issues (Siebert et al. 2012). Several studies worldwide have reported an association between lesions and the presence of parasites in different seabird species (Matos et al. 2020). In general, the studies focus on ecological and health assessments; however, data regarding the parasitism and lesions in *P. puffinus* on the coast of Brazil are scarce (Melo et al. 2012; Mariani et al. 2019).

Among the endoparasites found in *P. puffinus*, the Renicolidae Dollfus, 1939, a group of small digeneans that frequently inhabit the renal tubules and ureters of seabirds, has been described (Campbell and Sloan 1943; Wright 1954a, 1956; Gibson 2008). In Brazil, renicolids have been reported in several seabirds such as *Sterna* spp., *Larus dominicanus* Lichtenstein, 1823, *Sterna hirundinacea* Lesson, 1831 (Wright 1954b), *Sula leucogaster* (Boddaert, 1783) (Wright 1954b; Thatcher 1993), *Thalasseus maximus* (Boddaert, 1783) (Thatcher 1993), *Spheniscus magellanicus* (Forster, 1781) (Jerdy et al. 2016), and *P. puffinus* (Mariani 2016). A recent research conducted in southern Brazil has reported the morphological and molecular features of *Renicola sloanei* Wright, 1954 in *P. puffinus* (Matos et al. 2019).

Different intrinsic and extrinsic factors might influence the effects of the parasite infection. There is a general recognition that host features such as age, sex (Gutiérrez et al. 2017) and sexual maturity (Thieltges et al. 2006) influence the community structures of parasites and/or the susceptibility to parasitism; however, studies describing these associations are scarce (Gutiérrez et al. 2017), especially in seabirds. In addition, migratory periods, natural diseases, immunosuppression, and environmental changes might also influence the abundance, diversity, and distribution of parasites (Penn 2001; Lafferty 2013; Falkenberg et al. 2019). This way, parasitism might affect specific organs and modify the host physiology and ecological behavior. Particularly, data concerning renal parasitism in *P. puffinus* by renicolid are scarce (Wright 1956). Studies in other seabird species report both significant renal lesions (Campbell and Sloan 1943; Hill 1952; Riley and Owen 1972; Mahdy and Shaheed 2001; Jerdy et al. 2016 as well as no changes (Munyer and Holloway 1990; Horne et al. 2011; Rubio-Godoy et al. 2011).

The Manx shearwater was one of the main stranded seabird species found on Brazilian southeast and south coast (Paraná, São Paulo, and Santa Catarina states) (Petrobras 2019). However, results concerning ecological aspects and health status in seabirds occurring in the Paraná state are limited (Santos-Moraes and Krul 1995; Krul 2004; Carniel and Krul 2012; Daudt et al. 2017). Therefore, considering the relevance of this species on the Brazilian coast, the aim of this study was to evaluate whether the occurrence of renal parasitism is associated with renal lesions or with other variables such as sex, sexual maturity, body condition in *P. puffinus* stranded on the coast of Paraná state, southern Brazil. It also aimed to describe the main findings in other systems and the morphological and molecular aspects of renicolids.

Materials and methods

Area of the study and animals

The sampling area was the coast of Paraná state (25°44'S and 48°29'W), southern Brazil. The area is characterized by a coastline of 105 km, including Baía de Guaratuba in the south and the Paranaguá Estuarine Complex (PEC) in the north. The PEC area includes the islands of Mel, Superagui and Peças.

The animals analyzed were obtained between September 2015 and October 2018 through the Santos Basin Beach Monitoring Project (Projeto de Monitoramento de Praias da Bacia de Santos - PMP-BS), one of the monitoring programs required by Brazilian Institute of Environment and Renewable Natural Resources for the environmental licensing process of oil production and transport by Petrobras in the presalt province. The study was performed only with stranded animals. No animal was intentionally caught or killed. Consequently, submission to a Brazilian institutional ethics committee on animal usage was not required. Nevertheless, a field permit was granted by the Ministry of Environment-MMA (SISBIO 640/2015).

The carcasses were submitted to standard gross examination, and only carcasses with fresh or moderate condition of decomposition were used (Pugliares et al. 2007). The birds were identified by species-specific morphological characteristics (Onley and Scofield 2007). Sex and sexual maturity (immature and mature) were determined by feathering and gonadal evaluations (gross and microscopic). In addition, the nutritional state (body condition) was scored according to pectoral muscle mass amount (Romagnano 1999): normal – well muscled; poor – keel bone very palpable; cachectic – keel bone almost without muscle.

Tissue samples (brain, cerebellum, lungs, heart, liver, kidneys, skeletal muscle, stomach, intestines, thyroid/parathyroid glands, thymus, bursa of Fabricius, and spleen) were collected. Specifically, the cranial lobe of kidney (approximately 20 to 40mm) was sampled.

Histological analysis

Tissues were fixed in 10% buffered formalin solution, sectioned at 4 μ m and stained with hematoxylin and eosin (HE) for histological analysis. To reach consensus on the alterations observed, all assessments (macro and microscopic) were evaluated by three pathologists. Muscle necrosis (skeletal and cardiac) was evaluated using Mallory phosphotungstic acid hematoxylin staining (PTAH) (Behmer et al. 1976) ($n = 28$ animals). In addition, a quantitative analysis was performed in kidney sections by counting the number of renicolids per tissue section (mean 60 mm^2).

Analysis of renal digeneans

The digenean trematodes isolated from the kidneys were fixed in AFA solution (ethanol, formaldehyde, and acetic acid); no worms were compressed during fixation. The digeneans were stained with Mayer's carmalum and Delafield's hematoxylin and mounted on histological slides with a synthetic medium (Entellan[®], Merck, Germany) (Amato and Amato 2010).

Measurements of renicolids were taken using an image analysis software (Opticam Microscopy Technology 0500R, Doral, FL, USA). The parameters were measured according to previous studies (Wright 1954b, 1956). In addition, histological slides of the kidneys were used to measure the lengths and widths of the eggs. The criteria to include the eggs in this assessment were the presence of a brown-shell, a miracidium, and a longitudinal section arrangement.

Nucleic acid extraction, PCR, and DNA sequencing

The extraction of genetic material from the digeneans was carried out using a commercial kit (DNeasy Blood and Tissue™ kit, QIAGEN®, Valencia, CA). The amplification of the ITS2 region was performed using the primer pair SPIR1 (5'-GAGGGTCGGCTTATTATCTATCA-3') and SPIR2 (5'-TCACATCTGATCCGAGGTCA-3') (Stacy et al. 2010). The polymerase chain reaction (PCR) conditions, analysis of the amplicons, and sequencing were performed according to Matos et al. (2019). The nucleotide (nt) sequences were compared to the sequences deposited in GenBank using BLAST software (GenBank, RRID:SCR_002760) (NCBI 2018).

Statistical analysis

A descriptive statistical analysis was conducted to characterize the occurrence of parasitism, the number of trematodes, the systemic lesions, and the morphological parameters of digeneans. The association measures of the variables evaluated by odds ratio (OR) were obtained by the multiple logistic regression using R 3.3.2 software (R Project for Statistical Computing, RRID:SCR_001905) (R Core Team. 2016) considering a significance level of 5% and 95% confidence interval (CI).

Results

Occurrence of parasitism in *Puffinus puffinus*

Parasitological analysis was performed in the kidney of 93 *P. puffinus* presenting a fresh condition of the carcasses. Renal parasitism occurred in 71 (76.3%) animals, while 22 (23.7%) were uninfected. For both conditions, females, juveniles, showing a poor body condition were more frequent (Table 1). No significant association was observed between the presence or absence of renicolids and sex, sexual maturity, and body condition.

The number of digeneans per renal section was counted in 65 animals; in six animals only eggs were observed in the lumen of the renal tubules. The mean number was 0.22 ± 0.25 parasites/mm² (min-max 0.01-1.09). There

is no significant difference between the number of parasites in males (0.023 parasites/mm²) and females (0.019 parasites/mm²).

Gross and histological assays of *Puffinus puffinus*

Macroscopically, the trematodes appeared as small black and white multifocal areas inside the renal parenchyma (Fig. 1a). Microscopically, 70.97% (571/806) of the worms were found in pairs inside a cyst-like structure in the lumen of renal tubules. From the 93 *P. puffinus*, 60 of the infected (84.5%, 60/71) and 14 of the non-infected (63.6%, 14/22) showed some degree of histological changes (Table 1).

The main histological finding in both parasitized and no parasitized was mild to moderate lymphocytic infiltrate (Fig. 1b and 1c). In the infected animals were also observed ductal ectasia with occlusion of neighboring tubules ($n = 71$), focal to multifocal tubular necrosis ($n = 17$), epithelial tubular hyperplasia ($n = 14$) (Fig. 1d), and epithelial cell tubular cytoplasmic vacuolization ($n = 10$) (Fig. 1c). The lymphocytic inflammatory infiltrate circumscribed the parasitized collecting ducts and/or the interstitial medullar tissue (Fig. 1b and 1c). In 13 animals, concomitant inflammatory infiltrate and necrosis were observed. Discrete tubular hyperplasia, with two or three cell layers, was mainly observed in the collecting ducts (Fig. 1d). Multiple histological changes were frequently observed. In two animals, all the described changes were observed, characterized by moderate cell tubular cytoplasmic vacuolization, discrete to moderate lymphocytic infiltrate, multifocal tubular necrosis, and moderate epithelial tubular hyperplasia. In addition, the number of parasites in those animals was 0.005 and 0.022 parasites/mm².

Using a multiple logistic regression, a significant association was observed between the presence of renicolids and the occurrence of renal lesions ($p = 0.021$ / CI OR 1.3-18.3) (Table 1). Moreover, parasitized animals showed 4.8 more ratio to present renal lesions than no parasitized. In addition, renal lesions were observed in 97.30% (36/37) of the males (75.68% parasitized) and in 68% (34/50) of the females (58% parasitized). Thus, an association between sex and renal lesion ($p = 0.013$ / CI OR 1.8-126.6) was verified with males showing 15 more ratio to had renal lesions than the females (Table 1).

Frequently, parasitized (58/71) and non-parasitized (20/22) seabirds presented lesions in other systems. The main lesions detected in both the parasitized and non-parasitized animals were cardiac and skeletal muscle degeneration and necrosis (Fig. 2a), pulmonary congestion and hemorrhage, and lymphocytic hepatitis (Table 1). Skeletal and cardiac muscle degeneration and necrosis were confirmed using PTAH staining in all animals analyzed ($n = 28$) (17 parasitized and 11 non-parasitized animals) (Fig. 2b).

Digenean morphological and molecular assays

Measurements were taken only in mature digeneans ($n = 84$). In general, most of the parasites presented a large uterus containing numerous eggs. The means of the parameters used for the statistical analysis are detailed in Electronic Supplementary Material 1. Morphologically, the digeneans showed a rough oval body with a blunt anterior extremity that gradually tapered toward the opposite end (Electronic Supplementary Material 2). A large variation in body length was observed (1051-2338 μm). The cuticle of some individuals presented small spines. The oral sucker was subterminal or terminal, and the pharynx was barrel-shaped. The excretory vesicle was extended in the posterior end. The vitelline glands were arranged in extracecal follicles and were distributed in four-fifths of the body, from the distal pharynx region to the proximal ventral sucker area (Electronic Supplementary Material 2). The lobed ovary was located anterolateral to the ventral sucker, and the two lobed testes were located on both sides of the ventral sucker. The genital pore was located on the median line. The uterus was long and coiled, with a large uterine sac (Electronic Supplementary Material 2).

The measures of the eggs ($n = 317$) on kidney slides (Fig. 2c) were obtained from 60 renicolids present in 30 *P. puffinus* and were similar to the eggs observed on the parasite slides (Fig. 2d) (width 30 ± 1 ; min-max 27-34 μm ; length 16 ± 1 ; min-max 13-20 μm). The number of eggs measured per renicolid in renal section varied between two and 13.

Renicolid genetic materials from 14 *P. puffinus* were amplified, and due to the adequate quality and quantity of the nt, in two samples the ITS2 region were sequenced. The results of the molecular analysis showed that the renicolids from *P. puffinus* presented a high nt identity with *R. sloanei* from *S. demersus* (95.9%) (GenBank accession number KU563710) (Heneberg et al. 2016), *P. puffinus* (100%) (GenBank accession numbers MH021181 and MH021182) (Matos et al. 2019), and with *Cercaria pythionike* Rothschild, 1938 (96.6%) (GenBank accession number DQ489707). Moreover, the sequences exhibited a relatively low nt identity with other renicolids, including *R. sterna* (Heneberg et al., 2016) (68.8%; GenBank accession number KU563707), *R. lari* Timon-David, 1933 (68.3%; GenBank accession numbers KU563706 and KU563709), and *R. pinguis* (Mehlis in Creplin, 1846) (74.2%; GenBank accession numbers KU563705) (Heneberg et al. 2016). In addition, in this study, the phylogenetic analysis of *R. sloanei* strains from *P. puffinus* clustered together with previously described *R. sloanei* strains (Heneberg et al. 2016; Matos et al. 2019) (Electronic Supplementary Material 3).

Discussion

Information concerning the abundance, prevalence, distribution, and impacts of seabird parasites on their hosts is limited (Hoberg 1996), and beach systematic monitoring programs may provide a unique opportunity for assessing ecological aspects and disease surveillance among multiple seabird species. Helminths are considered common parasitic fauna in seabirds; however, they can cause negative effects in their hosts (Galaktionov 1996). In the present study, it was observed a high prevalence of parasitism and a significant association between the presence of renicolids and renal alterations in stranded Manx shearwaters.

A high prevalence of renal digeneans was observed in this study (76.34%, 71/93), similarly to a previous report in *P. puffinus* (80%, 8/10) (Wright 1956) and in other seabird species such as *Sula nebouxii* Milne-Edwards, 1882 (80%, 16/20) (Rubio-Godoy et al. 2011). In *S. demersus* from the east coast of South Africa, the prevalence of kidney trematodes ranged from 21 (3/14) to 74% (17/23) during four years of study (2006-2009) (Horne et al. 2011). Conversely, the prevalence of *Renicola* spp. in Brazilian stranded *S. magellanicus* was 5.5% (3/54) (Jerdy et al. 2016). The differences among the infection rates in the species are probably related to the habitat and diet; moreover, migratory birds may be exposed to increased diversity of parasites (Gutiérrez et al. 2017). An increase in parasitism levels in definitive and intermediate hosts was also associated with poor environmental quality (e.g., degradation and environmental contamination) (Lafferty and Kuris 1999). It is important to emphasize that in the present study the number of specimens evaluated was higher than in previous studies as a result of the beach monitoring program.

We were interested in evaluating the influence of host sex and maturity in parasite prevalence. Several parasitic species depend on the food chain to complete their biological cycle, resulting in different interactions with their hosts (Lafferty 2013), and might differ among species, sexual maturity, and sex. In *Somateria mollissima* (Linnaeus, 1758) from Germany the prevalence of renicolids was higher in immature and adult females (14.3%; 14/102) than in adult males (8.3%; 8/102) (Thieltges et al. 2006). Immature animals were more frequent in *P. puffinus* from the northeastern coast of Brazil (Mariani 2016) and in *S. nebouxii* parasitized by renicolids (Rubio-Godoy et al. 2011). A negative correlation between the corporal condition and number of renal trematodes was reported in *S. nebouxii* males parasitized with *Renicola thapari* Caballero, 1953 (Rubio-Godoy et al. 2011). In the present study, females, immature animals showing a poor body condition were more frequent; however, there is no association among the presence of renal parasites and sex, maturity or body condition.

Data associating renal lesions in *P. puffinus* and *Renicola* spp. infection were restricted to a single previous report (Wright 1956). The lesions were characterized as mild luminal dilatation of the parasitized tubules and occlusion of neighboring tubules, and no clinical effect (Wright 1956). In other species, inflammatory infiltrate (lymphocytes, histiocytes, and eosinophils) has been observed (Campbell and Sloan 1943; Horne et al. 2011; Jerdy et al. 2016). In the present study, the main histological changes were like those previously described, including the mild intensity (Campbell

and Sloan 1943; Wright 1956; Horne et al. 2011; Jerdy et al. 2016). Interestingly, despite the mild intensity of the alterations, a significant association between renal lesions and the presence of trematodes in *P. puffinus* was observed. Previous studies are limited to histological descriptions, and no statistical analysis was performed to evaluate this relationship.

Another interesting result was the greater number of parasites in males, and the greater ratio to present renal lesions than females. Similar results were observed in *S. nebouxii* males parasitized with *R. thapari* (Rubio-Godoy et al., 2011); however, in *Podiceps cristatus* (Linnaeus, 1758), males and females showed equal *R. pinguis* parasitism (Sitko and Heneberg 2015). The difference founded in this study might be associated to the longer period of migration observed in *P. puffinus* males (Guilford et al. 2009), and/or different forage habitats used by males and females during the nestling period (Gray and Hamer 2001; Guilford et al. 2009). Also, males and females might be exposed to different intermediate hosts and/or parasites burden. However, considering the high CI OR (1.8-126.6), more studies must be conducted to a better understanding of these differences.

Lesions in other systems were evaluated, and the most frequent, in both parasitized and non-parasitized birds, were muscle degeneration and necrosis. Causes of muscular necrosis might include nutritional deficiencies (mostly vitamin E and/or selenium deficiencies in piscivorous birds), excessive muscular effort, ischemia, metabolic disorders, and physical injuries (Vleet and Ferrans 2007; Schmidt et al. 2015). Considering the rate of muscle cell necrosis and the frequent poor corporal condition observed in *P. puffinus* immatures in the present study, the main cause of fragility and death is probably related to exercise-induced damage, such as previously described by Vooren and Brusque (1999). It is also important to consider that mass mortality is frequently observed in Procellariiformes, occurring predominantly during the migration process. Mortality is related to weather and/or inadequate diet, and the affected birds show emaciation, pectoral muscle and multiorgan atrophy and complete fat exhaustion (Sick 1997; Stidworthy and Denk 2018), reinforcing exercise-induced damage as the main contributing factor. Therefore, an increase or decrease in parasitism might occur in response to stressful environmental conditions and/or host factors, even in specific host-parasite interactions (Eiras 1994; Lafferty and Kuris 1999). In addition, an interesting finding was that the two animals showing all the renal histological changes displayed a small number of parasites/mm². This result suggests that other comorbidities, such as viral or toxic diseases might be associated in the lesions occurrence.

Other aim of this study was to describe the morphological and molecular aspects of the renicolids. Most of the worms were in pairs inside a cyst-like structure. This characteristic is observed in several trematodes' families and probably is a strategy to increase the genetic diversity (Heneberg et al. 2014). The morphological characteristics of parasite species have been widely used for diagnosis and species identification. In this study, the distribution of vitelline glands (organized in extracecal follicles from the distal pharynx region to the proximal central sucker area) and size of

the eggs coincided with *R. sloanei* from penguins (Wright 1954a) and *R. glacialis* Riley & Owen, 1972 from *Fulmarus glacialis* (Linnaeus, 1761), Northern Fulmar (Riley and Owen 1972). An important parameter that can be used to distinguish *R. sloanei* from other species of renicolids is the connection between the arms of the excretory vesicle (Wright, 1954a). This parameter was observed in *Renicola* spp. from *P. puffinus* and *Fratercula arctica* (Linnaeus, 1758) (Wright 1956), in *R. glacialis* from *F. glacialis* (Riley and Owen 1972), and in *R. sloanei* from *P. puffinus* (Matos et al. 2019). However, in the present study, due to prominent uterine sacs, the excretory vesicles in the renicolids from *P. puffinus* were not observed.

Other measurements (e.g., body, pharynx, and sucker sizes), except for the size of the eggs, are highly variable among *Renicola* species (Wright 1954a, 1956; Riley and Owen 1972; Jerdy et al. 2016). Thus, the size of the eggs can be considered one of the main parameters for species identification. In the present study, the egg size presented high similarity with the egg size of *R. sloanei* from *P. puffinus* (30x16 µm) (Matos et al. 2019), *R. glacialis* from *F. glacialis* (30x17 µm) (Riley and Owen 1972), *Renicola* spp. from *S. magellanicus* (28x15 µm) (Jerdy et al. 2016) and *R. sloanei* from *Pygoscelis antarcticus* (Forster, 1781), *Eudyptes chrysolophus* (Brandt, 1837), and *Uria aalge* (Pontoppidan, 1763) (28-34x16-18 µm) (Wright 1954a). It is also possible to measure the egg size in the renal parenchyma of infected animals. It is important to note that the measurements must be performed in the same type of egg (mature eggs with dark brown shells and miracidia) on both tissue and parasite slides. In addition, eggs of *Renicola* spp. from *S. magellanicus* (Jerdy et al. 2016) also showed similar measurements in both the specimen and the host tissue.

Morphological characterization is a useful method for species identification. However, identification can be limited, requiring the use of molecular techniques (Routtu et al. 2014). The high nt identity (ribosomal DNA gene) with *R. sloanei* from *S. demersus* (Heneberg et al. 2016) and *P. puffinus* (Matos et al. 2019) and the low nt identity with other renicolids reinforce the identification of *R. sloanei*.

In conclusion, the systematic monitoring of the Paraná coast, southern Brazil, allowed the analysis of a significant number of stranded seabirds. Continuous monitoring programs are important strategies that contribute to the knowledge of the abundance, diversity, and ecological aspects of marine animals. Taken together, the results of this study indicated the prevalence of renicolid parasitism in a Manx shearwater and that this interaction led to a negative effect or an unbalanced host-parasite relationship, since they induced renal lesions. The parasite was identified as *Renicola sloanei* through morphological and molecular analyses. In addition, it was shown that the egg size is a significant measure for species identification. Parasites occur in nearly every population, interacting with the host in complex ways. Further studies evaluating seabird health and environmental degradation can contribute to decrease the gaps in the knowledge concerning the interaction among parasites, their hosts, and the environmental health.

Declarations

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Figure Legends

Fig. 1 Kidney gross and microscopical findings in *P. puffinus* stranded on the coast of Paraná state. (a) Gross findings showing renicolids (arrow) in the kidney. (b) Lymphocytic infiltrate (arrow) surrounding a parasitized collecting duct. (c) Inflammatory infiltrate (arrowhead) and tubular cytoplasmic degeneration (arrows). The insert shows an area of cytoplasmic degeneration in epithelial tubular cells (Bar 10 µm). (d) Epithelial ductal hyperplasia (arrow) in a parasitized duct. b, c, and d - HE, Bar 50 µm

Fig. 2 (a and b) Microscopical aspects of muscle cells from *P. puffinus* stranded on the coast of Paraná. (a) Segmental skeletal muscle degeneration (arrows) (HE, Bar 50 µm). (b) Skeletal muscle degeneration showing loss of striations (arrow) (PTAH, Bar 50 µm). (c and d) Characterization of mature eggs used for measurements (length and width) of *R. sloanei* from *P. puffinus*. (c) Eggs in a kidney histological section (arrows) (HE, Bar 20 µm). (d) Microscopical aspect of eggs of isolated *R. sloanei* (arrows) (Mayer's carmalum stain, Bar 20 µm)

Table 1 Number (n) and prevalence (%) of sex, sexual maturity, body condition, and lesions found in parasitized (Yes) and nonparasitized (No) *P. puffinus* stranding on coast of Paraná state, southern Brazil and the significant results of multiple regression logistic

Variables	Total	Presence of <i>Renicola</i>		
		Yes n (%)	No n (%)	
Sex ^a				
Female	50	38 (76.00)	12 (24.00)	
Male	37	29 (78.38)	8 (21.62)	
Sexual maturity ^b				
Juvenile	70	53 (75.71)	17 (24.29)	
Adult	17	13 (76.47)	4 (23.53)	
Body condition ^c				
Poor	66	47 (71.21)	19 (28.79)	
Cachectic	21	19 (90.48)	2 (9.52)	
Normal	2	2 (100)	0 (0)	
Main renal lesions				
Inflammatory infiltrate	63	50 (79.37)	13 (20.63)	
Tubular cell necrosis	20	17 (85.00)	3 (15.00)	
Tubular epithelial hyperplasia	16	14 (87.50)	2 (12.50)	
Tubular epithelium degeneration	11	10 (90.91)	1 (9.09)	
Without lesions	19	11 (57.89)	8 (42.11)	
Main lesions in other systems				
Pulmonary congestion	53	42 (79.25)	11 (20.75)	
Myocardium necrosis	48	37 (77.08)	11 (22.92)	
Muscle necrosis	30	20 (66.67)	10 (33.33)	
Pulmonary haemorrhage	17	9 (52.94)	8 (47.06)	
Lymphocytic hepatitis	16	13 (81.25)	3 (18.75)	
Significant associations with occurrence of renal lesions ^d				
	Occurrence of <i>Renicola</i>		Sex ^a	
	Yes	No	Female	Male
n/Total	60/71	14/22	34/50	36/37
%	84.51	63.64	68%	97.30%
p-Wald	0.021			0.013
Adjusted OR	4.8	1.0	1.0	14.9
CI 95% OR	1.3 - 18.3			1.8 - 126.6

^a six animals with sex indeterminate; ^b six animals with sexual maturity indeterminate; ^c four animals with body condition indeterminate; ^d multiple regression logistic

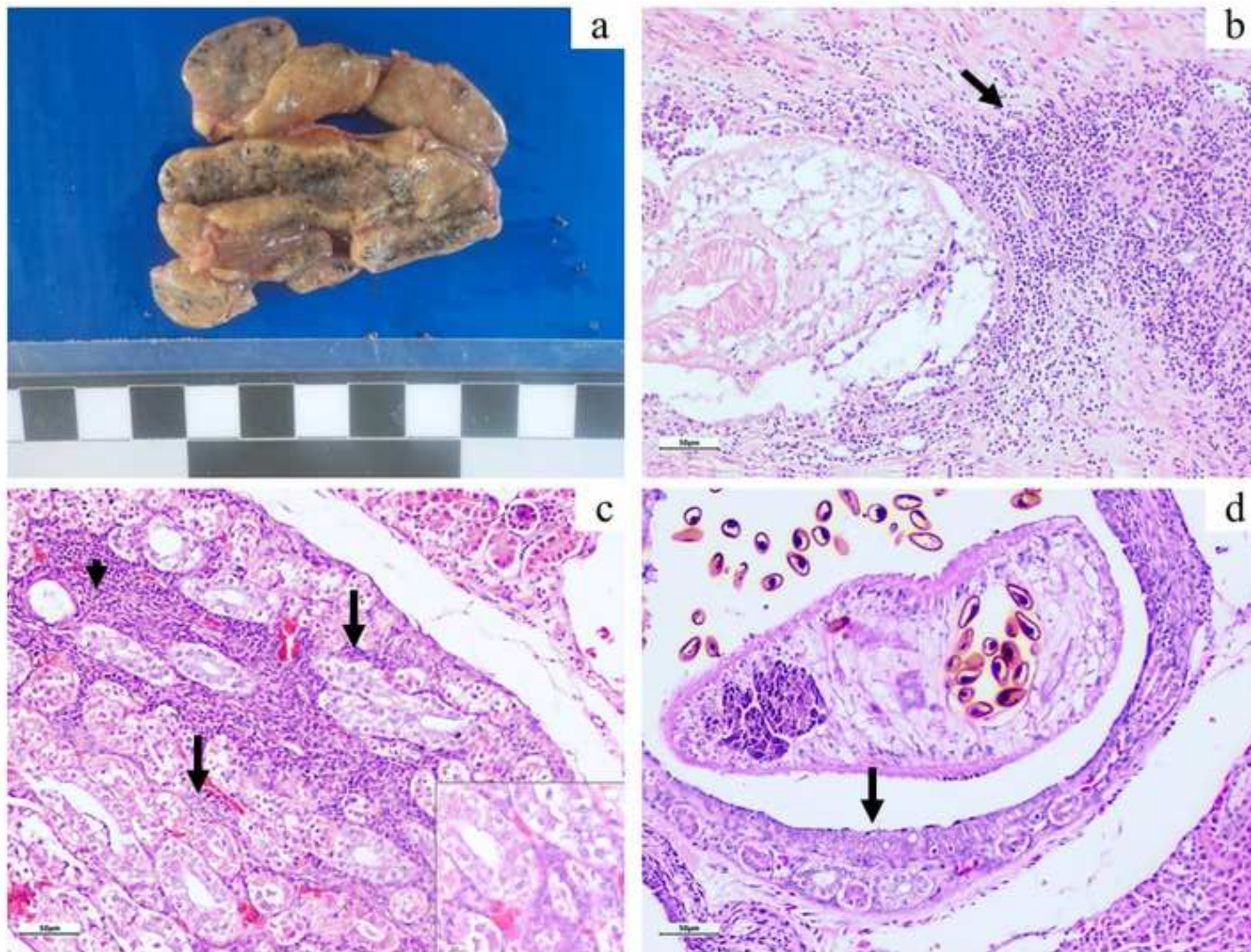
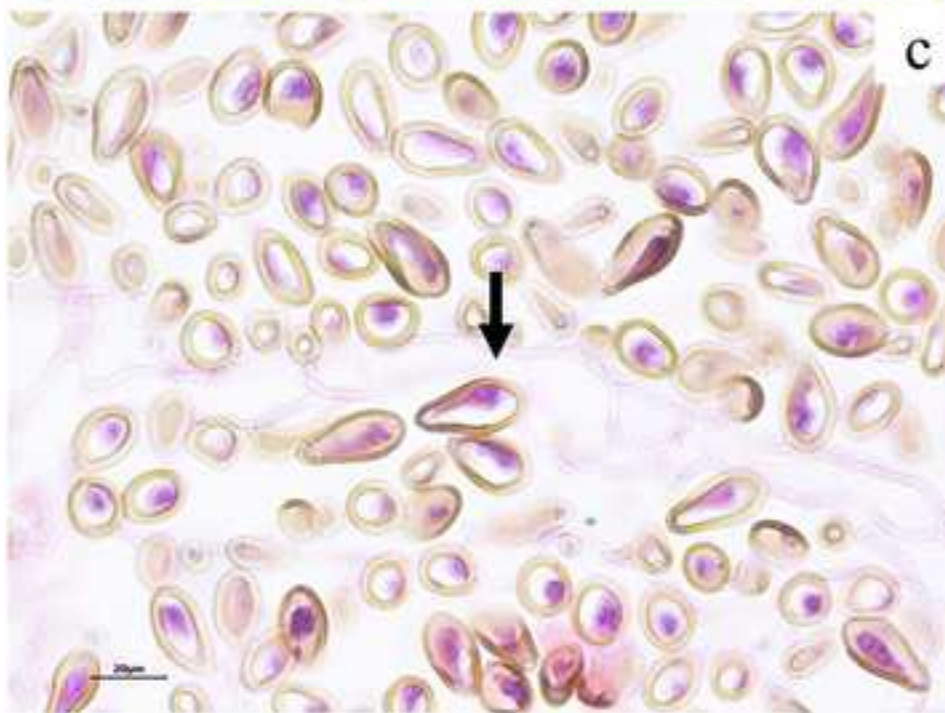
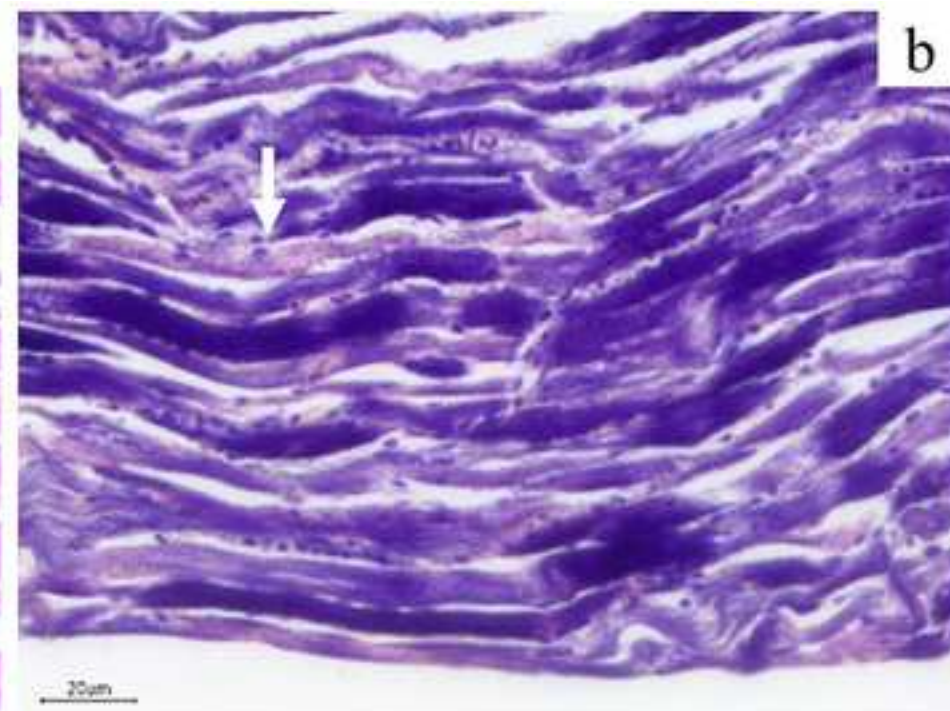
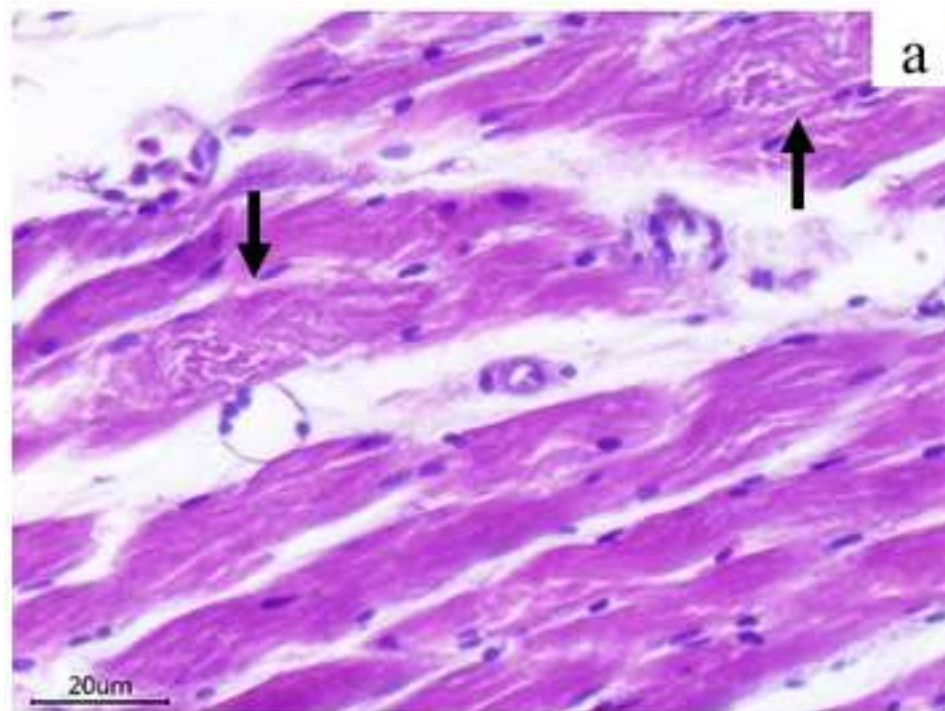


Fig. 2

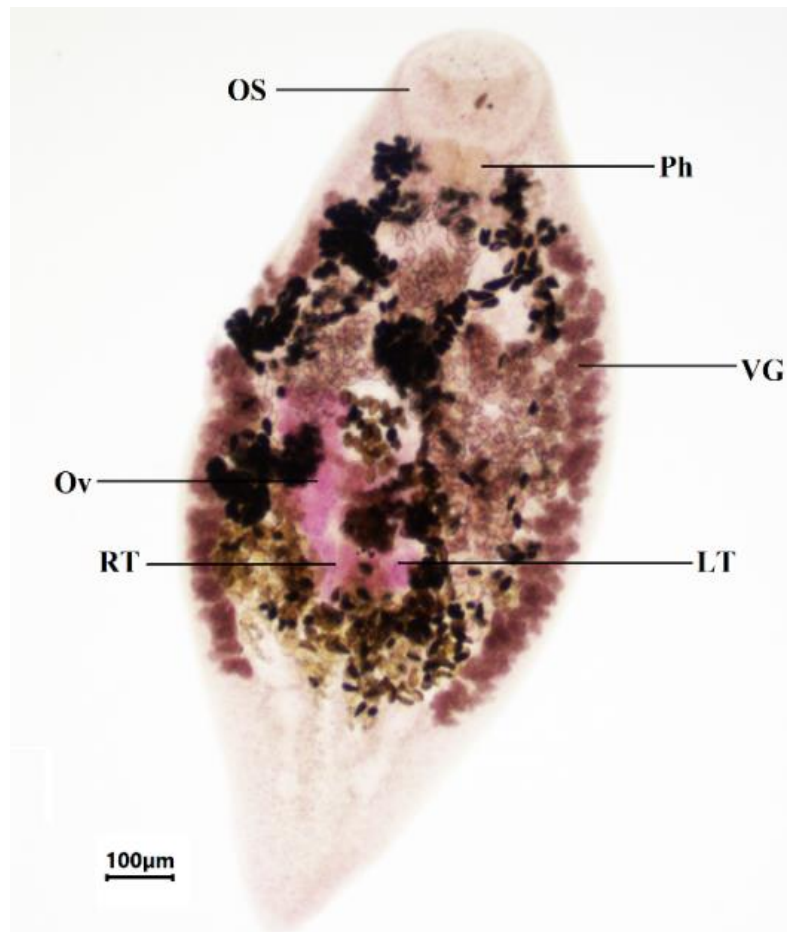


Electronic Supplementary Material 1 Means, standard deviations (SDs) and number measurements (N) of digeneans *Renicola sloanei* infecting *Puffinus puffinus* stranded on the coast of Paraná state, Southern Brazil

Measure	N	Mean \pm SD (Range)
Body lenght	20	1805 \pm 344 (1051-2338)
Body width ^a	17	851 \pm 166 (518-1130)
Body width ^b	4	711 \pm 41 (668-776)
Oral sucker lenght	53	212 \pm 53 (93-366)
Oral sucker width	54	243 \pm 62 (100-442)
Pharynx lenght	33	90 \pm 19 (31-130)
Pharynx width	35	97 \pm 16 (69-133)
Ventral sucker lenght	25	115 \pm 29 (96-165)
Ventral sucker width	24	117 \pm 21 (91-165)
Eggs lenght	653	30 \pm 2 (27-36)
Eggs width	653	16 \pm 1 (14-19)
Genital pore at ventral sucker ^c	3	78 \pm 33 (38-130)
Lenght:width body ratio	10	2.2 \pm 0.3 (1:1.7-2.7)
Oral:ventral sucker lenght ratio	16	1.8 \pm 0.3 (1:1.2-2.4)
Oral:ventral sucker width ratio	15	2.1 \pm 0.4 (1:1.1-2.6)

^a The maximum body width. ^b The width at the level of the ventral sucker. ^c Measured from the posterior end of the genital pore to the anterior end of the ventral sucker

Electronic Supplementary Material 3 Adult *Renicola sloanei* from *Puffinus puffinus* stranded on the coast of Paraná state. (Mayer's carmalum stain, Bar 100 μm)



OS - Oral sucker; Ph - Pharynx; VG - Vitelline glands; LT - Left testes; RT - Right testes; Ov - Ovary.

Electronic Supplementary Material 4 Phylogenetic analysis of Brazilian *Renicola sloanei* field strains.

The tree based on the ITS2 region was constructed using the maximum likelihood method with a Kimura-two parameter model + gama distribution (K2+G). The sequences from this study are marked with solid circles. The bootstrap values are shown at the branch nodes (values < 80% are not shown). The scale bar at the bottom of the tree represent the number of nt substitutions per site. The GenBank accession numbers of the other strains are provided. The *Prosthogonimus cuneatus* strain was used as an outgroup

