Distribution of cysts of *Strongyluris* sp. (Nematoda) in the pallial system of *Achatina fulica* Bowdich, 1822 from Vila Dois Rios and Vila do Abraão, Ilha Grande, Angra dos Reis, Rio de Janeiro

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(With 6 figures)

Abstract

This work aimed to assessing *Strongyluris* sp. cysts distribution pattern in the several inner organs from pallial system of *Achatina fulica* Bowdich, 1822. Also we verified if there is a relationship between the mollusk size and the number of specimens from parasites collected from two touristic villages in Ilha Grande (Angra dos Reis, Rio de Janeiro state): Vila Dois Rios (VDR) and Vila do Abraão (ABR). The samples were obtained through a field work conducted bimonthly during 2007, 2008, 2010, and 2011, at both locations. Height and width were measured from shells collected, and the all specimens were classified in different classes: class 1 - <4.0 cm, class 2 - 4.1-9.0 cm and class 3 - > 9.0 cm. After the specimens were dissected in order to find and count the number cysts in the pallial system. In specimens from both locations, the pulmonary and secondary veins showed a high number of cysts. No significance difference was found both in the abundance of cysts among the specimens in ABR ($p=0.138$) and VDR ($p=0.181$). *Achatina fulica* showed different intensities of cyst infection based on the size classes: the class-3 specimens, at both locations, showed the greatest cyst average (ABR Anova $F=3.8$; $p=0.02$); (VDR T of Student $T=-2.04$; $p=0.04$). The results suggested that the highest number of cysts in the vascularized area in pallial system of *A. fulica* was a consequence of a greater hemolymph circulation in that area, delivering more nutrients for larvae development. We think that bigger individuals host a higher number of cysts, as they usually present a larger biomass and a larger area of the pallial system, allowing an efficient parasite colonization. Other possible explanation could be the long exposure of the molluscs of class 3 to the parasites, which allowed a longer time to the larvae to allocate themselves.

Keywords: Atlantic Forest, invasive specie, land snail, parasitism, Nematode.

Distribuição de cistos de *Strongyluris* sp. (Nematoda) no complexo pallial de *Achatina fulica* Bowdich, 1822 da Vila Dois Rios e da Vila do Abraão, Ilha Grande, Angra dos Reis, Rio de Janeiro

Resumo

O objetivo do estudo foi analisar o padrão de distribuição dos cistos de *Strongyluris* sp. nos diversos órgãos do complexo pallial de *Achatina fulica* Bowdich, 1822 e verificar se existe relação entre o tamanho do molusco e o número de parasitos em espécimes procedentes de dois vilarejos da Ilha Grande, Vila Dois Rios (VDR) e Vila do Abraão (ABR), município de Angra dos Reis, Rio de Janeiro. As coletas foram realizadas bimestralmente nos anos de 2007, 2008, 2010 e 2011 nas duas localidades. A altura e largura das conchas foram medidas, os espécimes classificados em classe 1 - <4,0 cm; classe 2 - 4,1-9,0 cm e classe 3 - > 9,0 cm e posteriormente dissecados sob estereomicroscópio para a busca e contagem dos cistos no complexo pallial. Em ambas localidades, VDR e ABR, as veias pulmonares e secundárias apresentaram predominância de alocação dos cistos. Não foi encontrada diferença na abundância de cistos entre os espécimes de ABR ($p=0,138$) e VDR ($p=0,181$). *Achatina fulica* apresentou intensidades diferentes de infecção de acordo com as classes de tamanho: os espécimes pertencentes a classe 3, nas duas localidades, apresentaram a maior média de cistos visíveis (ABR Anova $F=3,8$; $p=0,02$); (VDR T de Student $T=-2,04$; $p=0,04$). Os resultados sugerem que o maior número de cistos na região vascularizada de *A. fulica* foi decorrente do maior aporte de hemolinfa nesta área, proporcionando mais nutrientes para o desenvolvimento das larvas. Acreditamos que indivíduos maiores albergam um elevado número de cistos, pois, geralmente, apresentam maior biomassa e maior área da cavidade pallial, possibilitando uma eficiente colonização dos parasitos, outra causa pode ser explicada pelo maior tempo de exposição dos moluscos da classe 3 aos parasitas, que possibilitou um período maior para as larvas se alocarem.

Palavras-chave: Mata Atlântica, espécie invasora, molusco terrestre, parasitismo, Nematoda.

1. Introduction

The gastropod *Achatina fulica* Bowdich, 1822, native from Eastern Africa (Mead, 1961), is considered one of the hundred worst invasive species in the world (IUCN, 2013). One of the hypothesis on the invasion of this mollusk in Brazil establish that *A. fulica* was introduced in the 1980's, in a farming fair promoted by the Brazilian government, as a commercial purpose of replacing the *escargot* (usually *Cornu aspersum* Müller, 1774). Then, the country became one of the main producers for the European market (Colley and Fischer, 2009). Currently, the mollusk is present in 24 of the 26 Brazilian states, including islands and environments of protected areas (Thiengo et al., 2008; Faraco, 2011).

Achatina fulica is considered a harmful species to the environment, as it can possibly change the structure of communities and the composition of native populations (CBD, 2011), caused by the direct competition for resources against native mollusk species (Simberloff, 2002), such as *Megalobulimus* spp. and *Thaumastus* spp. (Carvalho et al., 2003; Fischer and Colley, 2004; Thiengo et al., 2008; Zenni and Ziller, 2010; Faraco, 2011).

In terms of public health, *A. fulica* is a potential intermediate host of nematodes of medical importance, such as *Angiostrongylus costaricensis* Morera and Céspedes, 1971 and *Angiostrongylus cantonensis* (Chen, 1935), related to the abdominal and meningocerebral angiostrongyliasis (Thiengo et al., 2007; Maldonado Júnior et al., 2010). It can be also a potential both an intermediate and paratenic host of some parasites of wild animals species of *Aelurostrongylus* Cameron, 1927 (Thiengo et al., 2008; Oliveira et al., 2010) could parasite lung both domestic and wild cats (Ribeiro and Lima, 2001), *Rhabditis* Dujardin,

1845, that lodge in the external auditory canal of cattle, causing otitis (Martins Júnior, 1985; Campos et al., 2002; Thiengo, 2009; Barbosa et al., 2016) and *Strongyluris* Mueller, 1894, which are intestinal parasites mainly found in lizards (Kohn et al., 1973; Vicente et al., 1993; Rocha and Vrcibradic, 2003).

In 2008, *Strongyluris* larvae were found encysted inside the pallial cavity of specimens of *A. fulica* from Ilha Grande (Thiengo et al., 2008). According to Seehabutr (2005) and Franco-Acuña et al. (2009) several nematodes are parasites of *A. fulica*, but the understanding of their relationship has not yet been clarified. Moreover, as it is a possible paratenic host, facilitated by its soft body, habitat, and generalist habits (Fischer et al., 2010), *A. fulica* can harbor countless nematodes, which could parasitized the vertebrate fauna when ingested. When spread to new areas, the mollusk also would introduce new parasite species, contributing to the parasitism of the local fauna (Simberloff, 2002; Thiengo et al., 2008).

In order to better understand and clarify the role of *A. fulica* as an intermediate host for *Strongyluris* larvae, this work have as goal aimed to assessing the *Strongyluris* spp. cysts distribution pattern inside the organs of the pallial system and evaluating if exist a relationship between the mollusk size and the number of parasited specimens in Ilha Grande.

2. Material and Methods

The specimens were obtained from Vila Dois Rios (23°10'55.73"S; 44°11'30.81"W) and Vila do Abraão (23°08'20.30"S; 44°10'07.19"W), located at Ilha Grande, a township of Angra dos Reis, in the south of the Rio de Janeiro state (Figure 1).

Figure 1. Location map of Ilha Grande in Rio de Janeiro State showing the collecting sites, Vila do Abraão and Vila Dois Rios, pointed out by the red squares. Scale in kilometers. Map: L. E. M. Lacerda.

The samples were collected bimonthly in 2007, 2008, 2010, and 2011, at both communities. According to the suggestion made by Fischer and Colley (2004) and Albuquerque et al. (2008), the sampling localities were selected based on the mollusk preference in terms of rest and foraging places (hedgerows, vegetable farming, gardens, garbage, rubble, empty lots, and woods edges).

The specimens collected in 2007 and 2008 were sacrificed by drowning, fixed, and preserved with 70%GL alcohol, following the technique described in Thomé (1975). The animals collected in 2010 and 2011 were immediately dissected, after sacrifice (Salgado, 2010).

The height and width of the shells were measured with a 0.05 mm-accuracy Mitutoyo caliper rule, according to Salgado (2010); the specimens were classified as <4.0 cm - class 1, 4.1-9.0 cm - class 2, and >9.0 cm - class 3 (Tomiyama and Nakane, 1993; Fischer et al., 2008). In order to retrieve the animal, the shells were cracked with a hammer, wrapped in a newspaper to reduce the impact caused by the hits and to avoid damaging the pallial system. The dissection was conducted by inserting fine point scissors, from the mantle edge, at the pneumostome level, initially cutting near the anus and then along the rectum, exposing the pallial cavity roof, folded to the left. This animal was thoroughly examined with naked eye and a Leica MZ6 stereomicroscope in order to find and count the cysts in different organs.

Nematode identification was based on morphology characters according described in Bain (1970), Anderson (2000), Franco-Acuña et al. (2009) and Oliveira et al. (2010).

Non-parametric Kruskal-Wallis test (H test) was used to analyze the cyst distribution pattern in the soft parts of *A. fulica*. The comparison between the size classes and the number of cysts was evaluated by parametric Student t test and ANOVA. After that, we conducted the

a posteriori Tukey test to compare the average pairs after the variance analysis.

3. Results

We analyzed 851 specimens, from them 544 (64%) were from Vila do Abraão (ABR) and 307 (36%) from Vila Dois Rios (VDR), with heights ranging from 1.8 to 12.7 cm in ABR, and 1.4 to 11.7 cm in VDR. We found 141 (16.6%) specimens parasitized with *Strongyluris* spp. cysts, totaling 1,898 cysts. In VDR, 45 (32%) specimens were parasitized, totaling 629 (33%) cysts. In ABR, we found 96 (68%) parasitized specimens, in a total of 1,269 (67%) cysts.

We observed a predominance of the presence of cysts in the pulmonary veins and in the secondary pulmonary veins (SPV) or in both structures (Figures 2 and 3). In the samples from ABR, 1,070 cysts were found in SPV (84.3%), 164 cysts in the kidney (13%), 20 cysts in the pericardial

Figure 2. *Achatina fulica* Bowdich, 1822 pallial cavity of a specimen from Vila Dois Rios, Ilha Grande, Angra dos Reis, RJ, Brazil, with *Strongyluris* sp. cysts adhering to the secondary veins. The black arrow indicates a cyst 0.8 mm in diameter. Photo: J. L. Oliveira.

Figure 3. *Achatina fulica* Bowdich, 1822 from Vila Dois Rios, Ilha Grande, Angra dos Reis, RJ, showing cysts of *Strongyluris* sp. in the roof of pallial cavity. (A) cyst within the pulmonary vein indicated by the black square; the arrow indicates the large pulmonary vein; (B) cysts inside the secondary veins indicated by the white square. Scale bar = 1mm. Photos: S. B. Santos.

sac (1.5%), and 15 cysts in the rectum (1.20%). In the samples from VDR, 592 cysts were found in SPV (95%), 14 cysts in the kidney (2%), 7 cysts in the pericardial sac (1%), and 16 cysts in the rectum (2%) (Table 1).

We did not find significant differences in the abundance of cysts among the organs of specimens in ABR (Kruskal-Wallis, $n=96$, $g.l.=3$; $H=5.515$, $p=0.138$) and VDR (Kruskal-Wallis, $n=45$, $g.l.=3$; $H=4.878$; $p=0.181$) (Figure 4 and 5).

In order to compare the size classes, 95 infected specimens from ABR were analyzed (one of them presented a broken shell apex and was removed from the analysis, because it was not possible to measure its height), and found that 10% (10) belonged to class 1, 62% (59) to class 2, and 28% (26) to class 3. In VDR, 44 specimens were assessed, and 59% of them (26) belonged to class 2,

and 41% (18) to class 3. There was not observed parasitized specimens in class 1. *Achatina fulica* presented different intensities of infection based on the size classes. The results showed that the specimens of class 3, in both locations, presented a higher average of cysts. The variance analysis performed for the three size classes in ABR revealed that difference: class 1: 2.1 ± 1.5 ; class 2: 8.7 ± 24.8 ; class 3: 28 ± 49.5 ; Anova $F=3.8$; $p=0.02$. Tukey post-test revealed a remarkable difference ($p=0.03$) only between class 1 and the others. Student's t-test for the VDR data also indicated an significant difference in the average of cysts between the size classes (class 2: 8.15 ± 16.1 and class 3: 22.3 ± 29.7); (Student's t-test= -2.04 ; $p=0.04$).

According morphological data Franco-Acuña et al. (2009) e Oliveira et al. (2010), the larvae found were identified as L3 larvae of *Strongyluris* sp. (Figure 6).

Table 1. Organs of *Achatina fulica* Bowdich, 1822 from Vila do Abraão and Vila Dois Rios, Ilha Grande, Angra dos Reis, Rio de Janeiro, parasitized by *Strongyluris* sp. by locality, total number of cysts and average of cysts per organ.

	Secondary pulmonary veins (SPV)		Kidney		Pericardial sac		Rectum	
	ABR	VDR	ABR	VDR	ABR	VDR	ABR	VDR
Total of parasitized organs per locality	90	45	19	7	8	3	7	4
Total cysts per organ	1,070	592	164	14	20	7	15	16
Average $\pm$ SD	11.8 $\pm$ 30.1	13.1 $\pm$ 22.1	8.6 $\pm$ 27.3	2 $\pm$ 1.9	2.5 $\pm$ 1.6	2.3 $\pm$ 1.5	2.1 $\pm$ 2	4 $\pm$ 2.9

ABR: Vila do Abraão; VDR: Vila Dois Rios.

Figure 4. Number of cysts of *Strongyluris* sp., expressed as mean $\pm$ standard deviation, in the analyzed organs from *Achatina fulica* Bowdich, 1822 collected in Vila do Abraão, Ilha Grande, Angra dos Reis, Rio de Janeiro, from 2007 to 2011. SPV - Secondary pulmonary veins.

Figure 5. Number of cysts of *Strongyluris* sp., expressed as mean $\pm$ standard deviation, in the analyzed organs from *Achatina fulica* Bowdich, 1822 collected in Vila Dois Rios, Ilha Grande, Angra dos Reis, Rio de Janeiro, from 2007 to 2011. SPV - Secondary pulmonary veins.

Figure 6. *Strongyluris* sp. from *Achatina fulica* Bowdich, 1822 collected at Vila Dois Rios, Ilha Grande, Angra dos Reis, Rio de Janeiro. (A) anterior end demonstrating the bulbiform esophagus (BE); (B) buccal capsule (BC); (C) posterior end with the anus (AN) and dorsoventrally curved tail. Photo: A.P.M. Oliveira.

4. Discussion

Strongyluris Mueller, 1894 is a intestinal parasite belongs to the family Heterakidae, mostly found in lizards (Kohn et al., 1973; Vicente et al., 1993; Anderson, 2000) and rarely in anuran amphibians (Bursey et al., 2003). *Strongyluris* spp larvae were found in terrestrial molluscs in Brasil, like *Bradybaena similaris* (Férussac, 1821), *Bulimulus tenuissimus* (d'Orbigny, 1835), *Belocaulis angustipes* (Heyneman, 1885), *Megalobulimus* sp., *Phyllocaulis variegatus* (Semper, 1885), *Sarasinula marginata* (Semper, 1885) and *Subulina octona* (Bruguière, 1792). for the first time by Thiengo (1995).

Anderson (2000) described the life cycle of the species of *Strongyluris* as monoxenic; we consider therefore the eggs with infective larvae are being offered on the soil and leaf litter by the definitive host for *Achatina fulica*, which ingests them while searching for food. However, Barreto-Lima and Anjos (2014) indicated *S. oscari* as a heteroxenic parasite, using arthropods as intermediate hosts. Then, we can consider that the infection of *Achatina fulica* by *Strongyluris* spp. larvae could be explained by the role of the snail as an intermediate host, as observed by Valente et al. (2016). This hypothesis can be accepted, as Fischer et al. (2010) mentions that the habitat and habits of *A. fulica* facilitate the parasitism in this species.

It is suggested that the greatest number of cysts in the vascularized area of *A. fulica* was a consequence of a great hemolymph circulation and, consequently, nutrients availability in that area, which delivered more requirements for larval development, as proposed by Bain (1970), based on experiments. The author infected cockroaches *Blattella germanica* species (Linnaeus, 1775) and mosquitoes *Culex* sp. with larvae of *S. brevicaudata* Mueller, 1849. As a result he noted that third-stage larvae were found in the general body cavity, region rich in hemolymph in the two species. In the mosquitoes, besides the presence of larvae in the body cavity, some were located in the chest within thin-walled cysts filled with fat globules. The search for nutrients for larva development by parasites in the intra-mollusk phase can be observed in an essay with *Bradybaena similaris* (Férussac, 1821) infected by a larval trematode, conducted by Pinheiro and Amato

(1994). The authors noticed an outstanding decrease in the hemolymph glucose content of the parasitized mollusk after a few days of infection.

Given that the mantle region is one of the main storage sites of glycogen in terrestrial gastropods, the hemolymph that supplies this area is rich in glucose (Pinheiro, 2007). The mantle region is a strategic site to warrant larval development by providing the types and amount of nutrients required.

Hemolymph in *A. fulica* also features high cholesterol levels, a result of the metabolism of lipids in these animals (Lustrino et al., 2010). This factor can also explain the preference of larvae allocation in the mantle organs, as for filling the cysts and folding the larvae, are necessary amounts of fat that possibly were easily acquired from hemolymph flowing in this region.

We believe that larger individuals house a higher number of cysts, as they usually show a larger area in the pallial cavity roof which in turn allows an efficient parasite colonization, or because the longer exposure of the molluscs from class 3 to the parasites, which allowed a longer time to the larvae to allocate themselves (Franco-Acuña et al., 2009). The non-registration of cysts on individuals of class 1, may have been due to not verifying the tissues through histological sections, to the search for microscopic sizes cysts.

Another hypothesis can also be explained by the probable relation between the food preference and the ontogenetic development of the species (Fischer et al., 2008). Fischer et al. (2008) observed in a study of the African snail diet that individuals who reach larger sizes (adults) consumed specially plant litter. With this behavior in mind, we can perceive a possible infection route of that species by the *Strongyluris* spp., as the nematode eggs are certainly being offered on the soil and the plant litter by the definitive host, in order to be freely ingested by *A. fulica* adult specimens. Other possible infection routes could take place through geophagia, coprophagia, and necrophagy, considering that these sources are contaminated. These eating habits are also common in larger specimens (Mead, 1961; Albuquerque et al., 2008).

The immunological stress caused by the senescence can also increase animals vulnerability to parasites (Mead, 1961). The gradual reduction in the organism physiological functions of old mollusks (class 3) probably facilitated the access and the establishment of parasites in the studied specimens.

This work stresses the need for more studies about the relation of the helminth fauna with the invasive mollusks and the potential role of the African snail as a intermediate host for parasites that can reach the wild fauna in Brazil (Thiengo and Fernandez, 2010). These data contribute to the increase of information on the role of *A. fulica* in life cycle of parasites of medical and veterinary significance (Faraco, 2011). Besides increase of knowledge the present study show and reinforce others as Franco-Acuña et al. (2009) and Maldonado Júnior et al. (2010), that, in spite of the great importance of *A. fulica* acts as intermediate host to *A. cantonensis*, as proven by other authors, the risk that this snails bring to public health and environment is greater than usually we can imagine. There are some kind of parasites that are found in *A. fulica* that are not known and we do not even know what kind of damages they can cause to human or domestic and wild animals. By this reason, the present study, supported by previous ones, focused the importance to analyze the parasitic fauna in *A. fulica*, as well as the possible relationship between the parasite and the snail and vertebrate host.

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