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Original Article

Evaluation of the reproductive profile of *Subulina octona* (Gastropoda, Subulinidae) experimentally infected by *Paratanaisia bragai* (Digenea, Eucotylidae)

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(With 2 figures)

Abstract

Snails infected by trematodes may increase or decrease their reproductive activity in response to the presence of infection. Our aim was to verify the reproductive alterations in *Subulina octona* after infection by *Paratanaisia bragai*. The infected snails were individually exposed for 24 hours to 20 parasite eggs and four groups were formed (10, 20, 30 and 40 d.p.i.- days after infection), along with control groups. Every 10 days, the number of eggs in the reproductive tract, number of eggs hatched, galactogen content and histopathological changes were evaluated. The reproductive in the control and infected snails presented an alternating pattern, where periods of high production of eggs and newly hatched were followed by periods of low production. However, in relation to the amount of galactogen, both control and infected groups followed the same pattern of variation. In the histology, we observed the presence of male and female gametes with marked reduction in the number of oocytes. The results indicate that the intra-snail development of the parasite affects the reproductive biology of the host.

Keywords: digenetic trematode, host-parasite relationship; reproductive patterns.

Avaliação do perfil reprodutivo de *Subulina octona* (Gastropoda, Subulinidae) infectada experimentalmente por *Paratanaisia bragai* (Digenea, Eucotylidae)

Resumo

Moluscos infectados por trematódeos podem aumentar ou diminuir sua atividade reprodutiva em resposta à presença da infecção. Nosso objetivo foi verificar as alterações reprodutivas de *Subulina octona* após a infecção por *Paratanaisia bragai*. Os moluscos infectados foram individualmente expostos durante 24 horas a 20 ovos do parasito e quatro grupos foram formados (10, 20, 30 e 40 d.p.i. – dias pós infecção), com respectivos grupos controle. A cada 10 dias, o número de ovos no trato reprodutivo, o número de ovos eclodidos, o conteúdo de galactogênio e alterações histopatológicas eram avaliadas. O padrão reprodutivo nos moluscos controle e infectados evidenciou um comportamento alternante, nos quais períodos de alta produção de ovos e filhotes foram seguidos por períodos de baixa produção. No entanto, em relação ao conteúdo de galactogênio, moluscos controle e infectados seguiram o mesmo padrão de variação. Na histologia, observamos a presença de gametas masculinos e femininos com acentuada redução no número de óocitos. Os resultados indicam que o desenvolvimento intramolusco do parasito afeta a biologia reprodutiva do hospedeiro.

Palavras-chave: trematódeo digenético, relação parasito-hospedeiro, padrões reprodutivos.

1. Introduction

Paratanaisia bragai (Santos, 1934) Freitas, 1959 is a digenetic trematode able to parasite snails of the Family Subulinidae Fischer and Crosse, 1877, who acquire the infection by ingesting eggs present in the excretion products of infected definitive hosts. Several species of wild and domestic birds can be used as the definitive host by the parasite, among them there are species with economic importance as *Gallus gallus domesticus* (L.); *Meleagris*

gallopavo L. and *Phasianus colchicus* L. (Santos, 1934 *apud* Pinto et al., 2004; Gomes et al., 2005; Brener et al., 2006). The bird infection occurs by the ingestion of snails containing infective metacercariae, which develop into the adult stage in the host's renal collecting ducts (Santi et al., 2017).

The presence of this trematode has been recorded in South America, Central America, Oceania and Asia (Keller and Araujo, 1992; Kumar et al., 2009). In Brazil, we found records of the occurrence of *Paratanaisia* spp. in Paraná (Taroda et al., 2013), Goiás (Carneiro et al., 1975), Rio de Janeiro (Menezes et al., 2001; Santos, 1934 *apud* Pinto el at., 2004, Xavier et al., 2015), São Paulo (Silva et al., 2016; Santi et al., 2017; Santi et al., 2018) and Minas Gerais (Tavela et al., 2014; Teodoro et al., 2018). Most of these records involved birds care at veterinary hospitals, zoos or wildlife triage and conservation centers. These records show the great veterinary importance of trematodes from *Paratanaisia* genus, not only to the wildlife and its conservation but, also, by affecting domestic or domesticated birds, as parrots and cockatiel (Luppi et al., 2007; Santi et al., 2018). From the perspective of the *One Health* concept, where we must combine efforts from different areas to solve environmental, veterinary or human health problems, the study of *P. bragai* is even more important.

The biological cycle of this parasite was studied by Maldonado (1945), Keller and Araújo (1992) and Brandolini and Amato (2006), indicating the terrestrial snails *Subulina octona* (Brugüière, 1789) and *Leptinaria unilamellata* (d'Orbigny, 1835) as intermediate hosts in Brazil. These species are hermaphroditic animals considered to be agricultural pests and found in humid and shady places, such as flower gardens and vegetable gardens (Boffi, 1979; Araújo and Bessa, 1993). The participation of *S. octona* as intermediate host in the biological cycle of parasites of medical and veterinary importance increases the relevance of studies focusing on this species of snail (Durço et al., 2013).

Infection by larval trematodes can alter the reproductive activity of the snail host, making it important to observe parameters related to the ovipository activity and the eggs laid viability (Tunholi-Alves et al., 2011). Besides this, other indicators can also be evaluated to understand these changes, such as the galactogen content and the snail histopathology. Galactogen is a heteropolymer of galactose, synthesized in the albumen gland, as a constituent part of the perivitelline fluid, which is deposited inside the eggs to nutrition of embryos during their development and newly hatched snails (Gomot et al., 1989).

The reproductive behavior of snails during intramolluscan digenetic larval development has been widely studied, especially in aquatic snails belonging to the Family Planorbidae Rafinesque, 1815 (Crews and Yoshino, 1990; Tunholi et al., 2011; Faro et al., 2013). However, there is scarcity of data in the literature on the behavior of terrestrial snails in response to infection by trematodes (Pinheiro and Amato, 1995).

Snails may increase its reproductive efforts in an attempt to compensate future losses, a phenomenon characterized by Minchella (1985) and called as fecundity compensation. Tunholi et al. (2011) verified this phenomenon in *Biomphalaria glabrata* Say, 1818 experimentally infected with doses of 5 and 50 *Echinostoma paraensei* (Lie and Basch, 1967) miracidia. However, the infection by larval trematodes may lead to reduction of reproductive activity in snails, phenomenon called parasitic castration by Baudoin (1975), as observed by Pinheiro and Amato (1995) in the system *Eurytrema coelomaticum* (Giard et Billet, 1892) Looss, 1907/*Bradybaena similaris* (Férussac, 1821). The snail may present a total interruption or partial reduction of reproductive activity resulting from the direct mechanical action of the parasite on the reproductive structures or indirect action, as decrease of the snail's energy reserves or interference in the neuroendocrine system.

Considering the wide geographical distribution of this trematode, it is important to understand the dynamics between this parasite and their hosts, mainly the intermediate snail host, an important target to control programs. Thus, is important understand the reproductive profile of the intermediate host to clarifying the ecological and population dynamics of *P. bragai*. The objective of this study was to verify the reproductive changes caused by *P. bragai* parasitism in the terrestrial snail *S. octona* through evaluation of the reproductive parameters (number of eggs hatched/snail and number of eggs in the reproductive tract/snail), galactogen determination and histopathological analysis of the snails.

2. Methodology

2.1. Snails collection and maintenance

The snails were collected near of the Institute of Biological and Health Sciences (ICBS) of Federal Rural University of Rio de Janeiro (UFRRJ) (Latitude: -22,7634; Longitude: -43,6882). Subsequently, they were taken to the Biophysics Laboratory of the Physiological Sciences Department, UFRRJ, Brazil. These snails were maintained in terrariums (20 cm long x 12 cm wide x 6 cm high), with a layer of approximately 2 cm of sterilized earth at the bottom, which was moistened with distilled water in alternate days.

The snails were fed with fresh lettuce leaves (*Lactuca sativa* L.) and freshly peeled vegetables: carrot (*Daucus carota* L.), zucchini (*Cucurbita pepo* L.) and chayote (*Sechium edule* (Jacq.) Swartz, 1800). In addition, the diet was supplemented with ration for bird's growth enriched with calcium carbonate ($CaCO_3$), in proportion 3:1 (Bessa and Araújo, 1995).

The terrariums were cleaned twice a week, when the feed offered was renewed, the hatched snails were transferred to another similar terrarium, where they were maintained until reaching a shell length greater than 10mm and reaching sexual maturity (confirmed by the presence of eggs observed by shell transparency) for to be used in experimental procedures.

2.2. Experimental infection

The specimens of *P. bragai* used in this study were obtained from naturally infected pigeons (*Columba livia* Gmelin, 1789) collected in the Seropédica municipality, RJ, Brazil (Latitude: 22º44'38"S; Longitude: 43º42'27"W; Altitude: 26m) in 2018. The Ethics Committee on the Use of Animals (CEUA) of the Veterinary Institute of UFRRJ approved this stage of the experiment (Process Number 2225230617). The identification of the adult digenetic trematodes was made according to Yamaguti (1958) and Kanev et al. (2002) .

The pigeons infection was stated by excretions examination (De Carli, 1994), evidencing the presence of helminth eggs. Therefore, these naturally infected pigeons were necropsied and the adult trematodes were collected from kidney. After this, adult specimens were dissected to collect the eggs directly from the uterine cavity. The snails were previously subjected to food deprivation for 24 hours, followed by exposure to the trematode eggs.

The study used a total of 800 snails (n=800). Four hundred specimens of *S. octona* were exposed to the parasite in 24-well plates, each with one piece of chayote and 20 parasite eggs. The snails remained in these conditions for 24 hours to feed on all the chayote offered. The exposed snails were divided into 4 groups: 10, 20, 30 and 40 days after infection (d.p.i.) with 100 snails each group. In order to maintain population density along the experimental groups, another group of 100 snails were infected following the same procedures described before and maintained for replacement of dead mollusks throughout the observation period.

The control groups, consisting of 400 uninfected snails, divided in 4 groups of 100 specimens, were also placed in 24-well plates containing one piece of chayote and 20 µl of distilled water for 24 hours.

The snails were placed in terrariums containing 50 snails each and 2 cm of sterilized earth, moistened with distilled water.

2.3. Experimental analyzes

The snails were monitored for 40 days. Every 10 days, the numbers of hatched eggs and eggs in the adult reproductive tract were recorded. After this step, 97 snails from each group (control and infected) were dissected and the albumen gland was removed, weighed, identified and stored at -20 °C for further analysis of the galactogen content. All collection of biological material occurred in ice bath.

The galactogen content of the albumen gland was determined according Sumner (1924) and Pinheiro and Gomes (1994) and the results were expressed as mg galactose/g tissue, fresh weight.

In the same intervals, three snails from each group (infected and control) were dissected for shell removal and placed in the Duboscq-Brazil fixative (Fernandes, 1949) for 48 h for histological analysis. The tissues were processed according to routine histological techniques (Tolosa et al., 2003). Serial sections 5 µm thick were obtained and stained with hematoxylin-eosin (HE) and Gomori's trichrome. The slides were observed under an Olympus BX51 microscope and images were captured with the Olympus CellSens Standard software (CS-ST-V1, CellSens Standard, v.1.00).

2.4. Statistical analysis

The numerical results of the reproductive analysis were expressed as mean ± standard error of the mean and the control group was compared with the infected group through the t-test for unpaired data (α=5%). The relationship between infection time and reproductive parameters was determined by polynomial regression analysis (InStat, GraphPad, v.4.00, Prism, GraphPad, v.3.02, Prism Inc.).

3. Results

The experimental infection by *P. bragai* induced alterations in the reproductive biology, galactogen content and the tissue organization of the *S. octona* snails exposed to the parasite.

The total number of eggs observed inside the snails during the experimental period was, in average, 18.64 eggs/snail for the control group and 16.66 eggs/snail for the infected group, representing reduction of 10.62% in this parameter as consequence of the infection.

Reductions of 10.07%, 30.48% and 33.33% were observed in the number of hatched eggs per snail when analyzing the infected animals at 10 d.p.i. (2.50 ± 0.27), 20 d.p.i (1.3 ± 0.24) and 40 d.p.i. (0.80 ± 0.01), respectively, compared to the control groups analyzed after the same period (2.78 ± 0.24, 1.87 ± 0.34 and 1.2 ± 0.01, respectively). Only snails analyzed after 40 days of exposure presented a significant difference in relation to the corresponding control group. However, increase of 12.04% was observed in infected snails at 30 d.p.i. (3.07 ± 0.84) compared to the control snails (2.74 ± 1.35) (Table 1).

In the analysis of the relation between the number of hatched eggs and time of infection, the same oscillating pattern was observed in both groups (uninfected and infected), presenting a negative relation between these parameters, with lower values for infected snails than control snails. The relation between the parameters analyzed for infected snails (r^2=0.49) was not significant, but there was a strong relation for uninfected snails (r^2=0.91) (Figure 1A).

Ten days after infection, infected snails (3.11 ± 0.20) showed significant reduction (α = 0.001) in the number of eggs observed in the reproductive tract per snail, equivalent to 34.94% in relation to the control group (4.78 ± 0.12). At 20 d.p.i., the opposite was observed, with an increase of 5.6% in this parameter in the infected snails (4.34 ± 0.15) in relation to the control (4.11 ± 0.16). From this time on, the number of eggs declined again by 5.96% after 30 d.p.i. (4.26 ± 0.17) and 5.17% after 40 d.p.i. (4.95 ± 0.12), respectively, compared to the control snails evaluated in the same period (4.53 ± 0.16, 5.22 ± 0.07). The reduction observed in the number of eggs observed in the reproductive tract per snail of infected snails, after 40 days of infection was significant in relation to the control group (Table 1).

Table 1. Effects of the experimental infection by *Paratanaisia bragai* on the reproductive parameters, expressed as mean ± standard error of the mean, of the intermediate host *Subulina octona* during 40 days after infection (d.p.i.).

Days post infection (d.p.i.)	Eggs in the reproductive tract/snail		Hatched eggs /snail		Galactogen (mg of galactose/g of tissue, wet weight)	
	Control	Infected	Control	Infected	Control	Infected
10	4.78 ± 0.12[a]	3.11 ± 0.20[b]	2.78 ± 0.24[a]	2.50 ± 0.27[a]	65.34 ± 3.84[a]	63.65 ± 1.52[a]
20	4.11 ± 0.16[a]	4.34 ± 0.15[a]	1.87 ± 0.34[a]	1.3 ± 0.24[a]	67.59 ± 2.54[a]	79.60 ± 0.31[b]
30	4.53 ± 0.16[a]	4.26 ± 0.17[a]	2.74 ± 1.35[a]	3.07 ± 0.84[a]	79.23 ± 1.86[a]	93.55 ± 0.62[b]
40	5.22 ± 0.07[a]	4.95 ± 0.12[b]	1.2 ± 0.01[a]	0.80 ± 0.01[b]	79.78 ± 0.60[a]	74.12 ± 0.29[b]

[a,b] Means followed by different letters in the row differ from each other with significance of at least 5%.

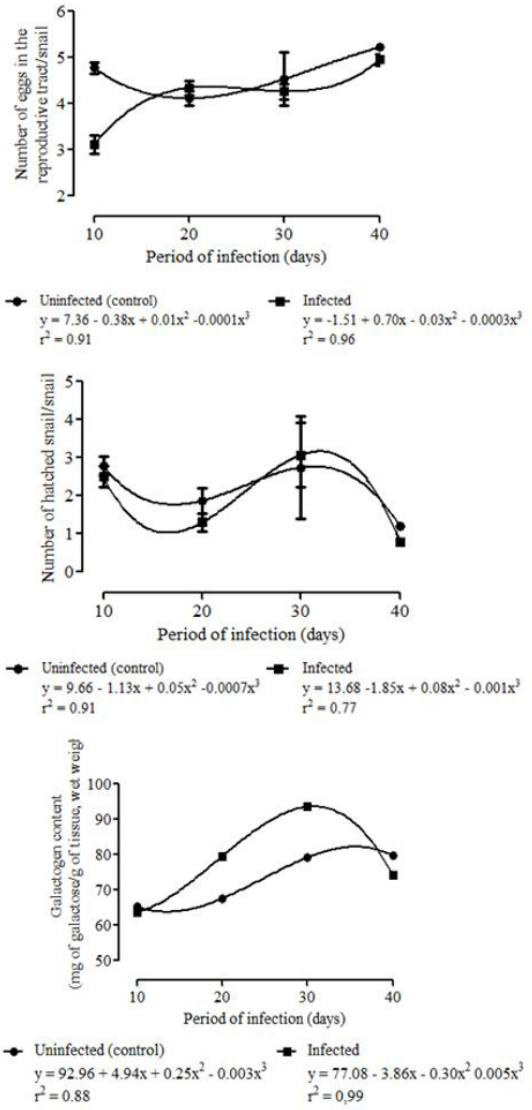

Figure 1. Relation between the period of infection and reproductive parameters analyzed in *Subulina octona* experimentally infected with *Paratanaisia bragai*. A) number of eggs observed in the reproductive tract, B) number eggs hatched per snail; and C) the galactogen content in *S. octona* experimentally infected by *P. bragai*, and the pre patent development of infection, 40 days after infection (d.p.i.).

Polynomial regression analysis showed alternating reproductive pattern in uninfected *S. octona*, in which there was periods of higher egg production followed by a subsequent period of lower egg production, with a strong positive relation between the analyzed parameters. This analysis allowed to verify that as result of *P. bragai* infection, there was inversion of the reproductive pattern of *S. octona*, resulting in a negative relation between the parameters analyzed, in which the snails began with lower egg production, until 20 d.p.i., after that the reproductive investment increased (Figure 1B).

A reduction of 2.59% in the galactogen content in the infected snails' albumen gland after 10 d.p.i. (63.65 ± 1.52 mg of galactose/g of tissue, wet weight) was observed in comparison with the control (65.34 ± 3.84 mg of galactose/g of tissue, wet weight). After 20 d.p.i. and 30 d.p.i, significant increases ($\alpha = 0.01$) were observed of 17.77% and 18.07% in the amount of galactogen measured in the infected snails (79.60 ± 0.31 and 93.55 ± 0.62 mg of galactose/g of tissue, wet weight, respectively) in relation to the control group (67.59 ± 2.54 and 79.23 ± 1.86 mg of galactose/g of tissue, wet weight, respectively). However, 40 d.p.i. the amount of galactogen in the infected snails (74.12 ± 0.29 mg of galactose/g of tissue, wet weight) again showed a significant decrease of 7.09% compared to the control (79.78 ± 0.60 mg of galactose/g of tissue, wet weight) (Table 1).

Regression analysis revealed a strong positive relation for both groups. At 20 d.p.i., the galactogen content was higher in the infected snails, but then declined at 30 d.p.i., but the two groups studied followed the same pattern of variation in the galactogen content (Figure 1C).

The histological analysis revealed that in the uninfected snails the reproductive function was preserved, with active gametogenesis, evidencing male and female gamete cells at different stages of development (Figure 2A). However, because of infection by *P. bragai* a significant reduction in the oocytes number in the ovotestes of infected *S. octona*, from 30 d.p.i. occurred (Figure 2B). In addition, the few oocytes observed in the infected snails presented irregular and granular cytoplasm and nucleus in apoptotic process.

4. Discussion

Maldonado (1945) first observed that *S. octona* was able to host *P. bragai* allowing its larval intramolluscan development. Keller and Araújo (1992) reported that this

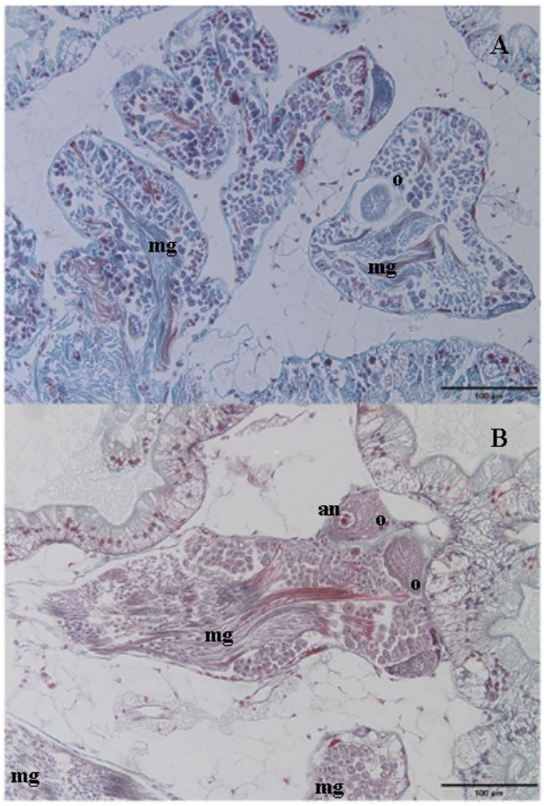

Figure 2. Histological analysis of gonadal tissue of the terrestrial snail *Subulina octona*. A) Uninfected snail, showing numerous male gametes (mg) in different stages of maturation and an oocyte (o). Scale bar = 100 µm. B) Snail infected by *Paratanaisia bragai* trematode showing numerous male gametes (mg) at different stages of maturation and two oocytes (o) with granular cytoplasm and nucleus in the process of apoptosis (an). Scale bar = 100 µm. Gomori's trichrome stain.

digenetic trematode did not develop in experimentally infected *S. octona*. Brandolini et al. (1997) showed that *S. octona* can act as an intermediate host of *P. bragai* under laboratory conditions and evaluated the effect of parasitism on some reproductive and biological aspects. However, in the present study, the results found by previous authors were complemented and the reproductive changes observed were associated with the metabolic alterations in host during the pre-patent period of parasite development.

The total number of eggs observed inside the parental snails was reduced because of the infection. It could also be evidenced an inversion of the oscillatory pattern in the egg production, no longer being a positive relation, whereby infected snails began their reproductive activity with a lower investment, followed by an increase in the egg production in relation to the uninfected snails. Sullivan et al. (1985) observed a similar phenomenon in other parasite-host systems, called fecundity compensation. In the present study, this compensation was only observed at 20 d.p.i., when then the number of eggs produced was slightly higher (5.6%) in the group of infected snails.

Notably, this reproductive effort increased at 20 d.p.i. in infected snails did not lead to an increase in reproductive success, since the number of eggs hatched was lower than that of control snails, even though there was a larger energy investment in the reproductive process, with an increase of almost 18% in the galactogen content in infected snails.

Despite the lower initial investment, evidenced by the reversal of the oscillating pattern in egg production, uninfected snails could not maintain the normal pattern of their reproductive biology, as observed in the uninfected animals. Hatchability maintained the same pattern in both groups, but infected snails, in average, had a lower hatching rate than the uninfected specimens (control).

Interestingly, the galactogen content was higher in the albumen gland of infected snails than in uninfected ones, evidencing that the parasite castration process observed in the *S. octona/P. bragai* system is not a nutritional type castration (Baudoin, 1975). In conclusion, even with an increased energy investment, there was failure of the compensatory processes, resulting in an effective castration process in parasitized snails.

The interference of larval digenetic trematode in the reproduction of the snail first intermediate host is a common phenomenon (Pinheiro and Amato, 1995). The term parasitic castration is used to refer to an interruption or a decrease in the reproductive activity of snails in response to parasitic infections. This can be caused by direct mechanisms, such as destruction of gonadal tissues of the host during intramolluscan larval development of the parasite, or indirectly, due interferences with neuroendocrine system (NES) or absorption, by the parasite, of circulating nutrients in the hemolymph required for the development and maintenance of the snail (Baudoin, 1975; Tunholi-Alves et al., 2011).

The results obtained in the present study showed that the presence of developing larvae of *P. bragai* caused a decrease in the reproductive activity of infected *S. octona*, mainly during the first 10 days of infection, when all the analyzed parameters presented a reduction in comparison with the control group. Brandolini and Amato (2006), monitoring the intramolluscan development of *P. bragai* in *S. octona*, observed that six, eight and ten days after infection, it was possible to observe first generation sporocysts near the wall of the intestine and spreading towards the digestive gland. The germinative capacity of the first generation sporocyst depends on the space availability in the host's body (Maldonado, 1945). Those authors also observed the presence of second generation sporocysts after 15 days of infection and after 32 days the presence of fully developed cercariae and metacercariae in the digestive gland.

The increase of the galactogen content during the 20 to 30 d.p.i. period indicates that the snails, to some extent, maintained the biosynthetic pathway of this polysaccharide near to normal value. In spite of the eggs production and high amount of galactogen, many embryos

were not viable, as evidenced by the reduced hatching rate of infected snails.

The histopathological analysis showed that the castration process observed in the system *P. bragai*/*S. octona*, is not nutritional, contrary to what Brandolini et al. (1997) reported. Brandolini and Amato (2006) reported that castration in this host-parasite system is mechanical, due to the presence of metacercariae in the ovotestis of snails. We did not identify the presence of larval trematodes in the ovotestis, and gonadal tissues did not show morphological or structural alterations due to mechanical pressure. Additionally, absence of development or reduced numbers of oocytes in infected hosts was observed.

So, the similar amount of galactogen and the absence of developing larvae in gonadal tissues indicate the existence of an endocrine process, leading to inhibition of oocyte maturation, probably due to the presence of excretory and secretory products (E/S) released by the larvae during their intramolluscan development. By the action of these E/S products, the parasite may modulate the NES, altering the development and functioning of the female reproductive system.

Generally, the literature about the digenetic trematode *P. bragai* is related to the occurrence of this parasite in different bird species that can act as definitive hosts. Few studies have sought to elucidate the relation of the parasite with its intermediate hosts *S. octona* and *L. unilamellata*. The obligatory character of the use of a mollusk as an intermediate host and the high specificity of the interaction between larval trematode and their mollusk host, makes this point of the parasite life cycle an excellent target for integrated disease control programs. Although to establish more effective control programs it is required a deep knowledge about the host-parasite interactions. By this reason, the present study, by the first time, bring some light to physiological aspects of the interaction *P. bragai*-*S. octona*, focusing the reproductive features of this system.

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