Reproductive Response Of Bulimulus Tenuissimus (*Mollusca*) As An Intermediate Host Of Angiostrongylus Cantonensis (*Nematoda*)

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ABSTRACT

The snail Bulimulus tenuissimus is a terrestrial gastropod widely distributed in the Americas, Asia, Africa and some countries of other continents. It typically occurs in the same areas as Angiostrongylus cantonensis, which needs a mollusk as intermediate host. In spite of this, the literature is scant about the relationship of these organisms. Specimens of B. tenuissimus were experimentally infected with L1 larvae of A. cantonensis. Weekly, until the third week post exposure, the reproductive parameters were analyzed and snails were dissected for collection of hemolymph, along with the albumen gland and tissues for histological analysis. The total number of eggs laid, total number of newly hatched snails and number of newly hatched snails/eggs laid were higher in the infected snails, but the differences observed were not significant. The galactogen content in the albumen gland was significantly reduced from the second week post exposure onward. Also, in spite of presence of larvae in the gonadal region of the snail with granuloma-like structures and some histological changes, gametogenesis occurred regularly in the infected hosts. The results are worrisome, since they showed that B. tenuissimus is an intermediate host to A. cantonensis which supports infection without changes in its reproductive biology and activity. The present study sheds more light on a snail that is a newly recorded experimental and natural intermediate host of A. cantonensis, which is widely distributed and occurs in areas were neural angiostrongyliasis has been recorded. Therefore, this snail can act in the nature as an important agent for dispersion of neural angiostrongyliasis, posing a serious problem to human and animal public health.

Keywords: comparative physiology, host-parasite relationship, reproductive biology, Metastrongylidae, Bulimulidae.

1 INTRODUCTION

The molluscs are important elements in the life cycle of many parasites, thus attracting strong interest as targets control programs. The snail *Bulimulus tenuissimus* (d'Orbigny, 1835) is a terrestrial gastropod, from family Bulimulidae (Salgado and Coelho, 2003), widely distributed in North America – specifically Mexico (Fahy 2003) and North Carolina (USA) (Robinson and Slapcinsky 2005) - and South America (Agudo-Padrón 2014, Agudo-Padrón and Lenhard 2011, Breure and Avila 2016). Despite its widespread distribution in the Americas, where it serves as an intermediate host for several parasitic trematodes and nematodes, as well as attacking crops (mainly those for subsistence, such as vegetable gardens), there are few studies of the physiology, biology and behavior of this snail species (Silva et al. 2008, 2011).

The nematode Metastrogylidae *Angiostrongylus cantonensis* (Chen, 1935) is a parasite naturally found in rodents, but occasionally parasitizes humans causing neural angiostrongyliasis or eosinophilic meningoencephalitis (Hsu et al. 1990). The first case of neural angiostrongyliasis was reported in 1945 (Australia) (Prociv and Carlisle 2001). Since then, the presence of human infections by *A. cantonensis* has been recorded in Southeast Asia and the Pacific Islands (Kim et al. 2014) and in different Brazilian regions (Thiengo et al. 1995, 2010, Caldeira et al. 2007, Morassuti et al. 2014). The larval development of *A. cantonensis* occurs in a snail from L1 to L3, after which the larvae are shed to infect a definitive host. In the literature several species of molluscs have been identified as *A. cantonensis* intermediate host, but almost all studies have been conducted for epidemiological purposes. Recently, B. *tenuissimus* was reported as a natural and experimental host of this nematode (Ramos-de-Souza et al. 2018; Martins et al. 2018).

Brockelman performed the first studies of the host-parasite relationship focusing on physiological changes in the terrestrial snil *Achatina fulica* induced by *A. cantonensis* infection in the 1970s, (Brockelman et al. 1976, Brockelman 1978, Brockelman and Sithitavorn 1980). Only in the present decade, have new studies been conducted investigating different snail as potencial host and their physiological response to parasitism by *A. cantonensis* (Tunholi-Alves et al. 2011, 2014, 2015). Tunholi-Alves and co-workers were the first to report the reproductive changes caused in the freshwater snail *Biomphalaria glabrata* infected with *A. cantonesis*, describing the occurrence of partial parasitic castration of *B. glabrata* infected by *A. cantonensis* larvae, probably in response to the depletion of energy reserves, since the authors did not observe injuries to the gonadal tissues (Tunholi-Alves et al. 2011). Lima et al. (2017) also reported reproductive changes in *Biomphalaria tenagophila* and *Biomphalaria straminea* infected with *A. cantonesis*. But, there is still doubt about terrestrial snails. Since the natural definitive hosts are rodents, why are there so few studies about host-parasite relationship including *A. cantonensis*-terrestrial snails?

The present study aimed, for the first time, to find evidence of the changes in the reproductive biology of *B. tenuissimus*, through biological, biochemical and histological analyses, as a consequence of infection by *A. cantonensis*.

2 MATERIALS AND METHODS

2.1 MAINTENANCE OF THE SNAILS AND FORMATION OF GROUPS

Samples of the mollusc *B. tenuissimus* were manually collected, in the early morning, from vegetable gardens located in Seropédica, RJ, Brazil (Latitude 22º 44' 38" S; Longitude 43º 42' 27" W; Altitude 26m). The collected molluscs were maintained in plastic recipients under laboratory conditions, with the bottom covered by vegetable soil (2cm), bedewed on alternate days by using tap water.

The molluscs were fed with fresh lettuce leaves and slices of chayote, cucumber and carrot ad libitum, with the food being replaced on alternate days.

The eggs were collected and transferred to new recipients prepared as described above. The newly hatched snails that reached 90-100 days-old and had, at least, 10 mm in shell length, were used to form six groups: three control (uninfected) groups (n=90), and three infected groups (n=90), each group with 30 specimens. All the groups were formed using triplicates (n_{total}=180). The experimental observations lasted for three weeks, which corresponds to the pre-patent period of infection in the intermediate host (Lima et al. 2017).

2.2 PARASITES

Molluscs *A. fulica*, naturally infected, were collected in the municipality of São Gonçalo, RJ, Brazil in 2015, in the area surrounding the home of a patient diagnosed with eosinophilic meningoencephalitis Third-stage larvae (L3) of *A. cantonensis*, obtained from naturally infected *A. fulica* were inoculated in *R. norvegicus* in the Laboratory for Biology and Parasitology of Reservoir Mammals (LBPMR) of Oswaldo Cruz Institute (Fiocruz), where the cycle is maintained under laboratory conditions. All the first-stage larvae (L1) utilized in this study came from this experimentally maintained life cycle of *A. cantonensis*.. This study was approved by the Oswaldo Cruz Foundation Ethics Committee on Animal Use (CEUA/IOC-025/2018).

2.3 EXPERIMENTAL INFECTION OF THE SNAILS

Rodents *R. norvegicus* experimentally infected and maintained as previously described were used to feces obtaining, from which the first-larvae were collected (Baermann technique - Willcox and Coura 1989). Then, L1 larvae of *A. cantonensis* were spread on thin slices of fresh cucumber placed in 24-well plates molluscs *B. tenuissimus* were individually placed on top of the cucumber slices, being exposed to 1,200 L1 larvae. The plates were closed, allowing the contact among molluscs and larvae for 24 h. The uninfected snails were submitted to the same procedure with distilled water instead of the L1 larvae. The onset of infection was monitored under a stereomicroscope and some molluscs were observed ingesting the larvae. After 24h the content of each well was observed under a stereomicroscope to detect larvae (L1 stage) (Tunholi-Alves et al. 2011). After that, the molluscs exposed/infected were transferred to the terrariums to form the experimental groups.

2.4 REPRODUCTIVE ACTIVITY ANALYSIS

The molluscs placed in each terrarium were observed daily for three weeks. The number of live snails was recorded and the earth of each terrarium was gently examined to look for the eggs. Next, the eggs found were counted and transferred to new terrariums (nursery) and daily observed until their hatching.

The reproductive parameters observed were the following:: total number of eggs laid, total number of newly hatched snails, and number of newly hatched snails/eggs laid.

2.5 DETERMINATION OF THE GALACTOGEN CONCENTRATION

To galactogen determination, albumen gland was obtained from dissection of 25 molluscs (each group, n_{total}=25) under stereomicroscope, without anesthesia, with tissues maintained in ice bath during dissection and stored at -20 ºC. Galactogen was extracted according to Pinheiro and Gomes (1994), and its content was determined by the 3,5 DNS technique (Sumner 1924) and expressed as mg galactose/g tissue, wet weight.

2.6 HISTOLOGICAL AND HISTOCHEMICAL ANALYSES

Five molluscs foram each group (n_{total}=15) had their shells of removed and the tissues were fixed in Duboscq-Brasil fixative (Fernandes, 1941), for 24 hours at 4 ºC, and were processed according routine histological techniques (Humason 1979). Sections (5 µm) were obtained. The slices were stained using hematoxylin and eosin or Gomori’s trichrome techniques to observe the tissue morphology. The galactogen deposits were evidenced by Periodic Acid Schiff (PAS) reaction (Humason, 1979).

2.7 STATISTICAL ANALYSES

The total number of eggs laid, total number of newly hatched snails, and number of newly hatched snails/eggs laid results were expressed as mean ± standard error of mean (X ± SEM). The relation between the parameters analyzed and the time of infection was determined by polynomial regression test (Graph Pad Prism, Prism Inc.). The means observed in the different periods were analyzed by Tukey-Kramer test and ANOVA (InStat, v. 4.00, Prism Inc.).

3 RESULTS

The total number of eggs laid by *B. tenuissimus* did not vary significantly between uninfected and *A. cantonensis*-infected snails or during the different infection periods (Tab. 1). The mean number of eggs laid was higher in infected snails throughout the three weeks after exposure, where the highest value was observed in the first week post exposure (236.43 ± 45.94), 7.0% higher than that observed among uninfected snails. One-way ANOVA indicated no significant difference among the values observed in the different weeks ($P<0.05$). Polynomial regression revealed a weak positive relation between the period of infection and the total number of eggs laid by infected *B. tenuissimus* (Fig. 1A), showing a tendency to reach the normal oviposition value at the end of pre patent period.

The viability of the eggs laid by *B. tenuissimus* infected with *A. cantonensis* was evaluated by analyzing the total newly hatched snails from eggs laid by infected snails in comparison with uninfected ones. In spite of the absence of significant difference among the values obtained ($P<0.05$), the same variation pattern observed for the number of eggs laid was observed here. The greatest hatchability was observed in the third week post exposure (241.75 ± 34.69), 23.30% higher than that observed in the control group (Fig. 1B).

The ratio increased continuously with the progress of infection reaching the maximum value in the third week after exposure (1.07 ± 0.15), an increase of 20.49% in relation to the control group (Table 1). Also, this was the only period of infection in which the ratio between the newly hatched molluscs and the laid eggs was significantly higher than that observed in the control group ($P<0.05$). In addition, there was a weakly significant relationship between the ratio (number of newly hatched snails/eggs laid) and the period of infection (Fig. 1C).

The galactogen content showed a weak positive relation with infection period. However, despite the reduction (Fig. 1D) in the third week after exposure, one-way ANOVA ($P<0.05$) did not revealed significant differences between the values observed for the control group and the three periods of infection analyzed.

Histological analyzes of uninfected snails showed ovotestis with normal characteristics, presenting oocytes and spermatozoa in different developmental stages (Fig. 2A), and the albumen gland with regular tubular structure in the uninfected snails (Fig. 2B). The presence of oocytes and spermatozoa in the ovotestis of *B. tenuissimus* experimentally infected with *A. cantonensis* was confirmed throughout the prepatent period of infection (Figs. 2C-D), indicating the normal functioning of the gonads. Only in the first week post exposure, were larvae observed in the ovotestis tissues. Fig. 2C shows a granuloma-like structure with two larval profiles, where the tissues as a whole are structurally changed in comparison with the uninfected snails. Nevertheless, the observation of histological sections of other infected snails in the same period after exposure (Fig. 2D) indicated a gonad with normal appearance, evidencing differences in the individual response to parasitism in this host-parasite system. Similar results were observed in the snails in the third week after exposure, where some had morphological alterations (fig. 2E) and others had no alterations in the structure of the ovotestis (Fig. 2F). Despite the changes observed, all the sections analyzed contained oocytes and spermatozoa with morphology preserved.

4 DISCUSSION

Changes in reproductive biology have been recorded in many relationships between different organisms for more than 100 years (McCrady 1874, Malm 1881). However, Giard, in a sequence of studies published from 1886 to 1913 (Giard 1886, 1887, 1889, 1911-1913, Giard and Bonnier 1887) was the first to propose the use of the term “parasitic castration” to refers to reproductive changes observed in host-parasite relationships. Research in this field has been focused on the effects of trematode larval development and the consequences on the reproduction of the snails’ first intermediate host during their life cycle (McClelland and Bourns 1969, Becker 1980, Tunholi et al. 2011).

In many experimental models, total or partial inhibition of the reproductive activity in the relationships between helminths and molluscs has been recorded (Baudoin 1975). In spite of the great emphasis on the issue in the international scientific literature, the effects of the parasitic infection by larval nematodes on the reproduction of intermediate snail hosts have largely been neglected. This lack of information is particularly glaring regarding the relationship of *A. cantonensis* with its intermediate snail host. Although the species was first described in 1935, the first studies about physiological changes in *A. fulica* infected with *A. cantonensis* were only published in the 1970s (Brockelman 1978, Brockelman et al. 1976).

This study presents the first report of the effects of experimental infection with *A. cantonensis* L1 larvae on the reproductive biology of *B. tenuissimus* and our data indicate the infection did not significantly affect the reproductive parameters analyzed. Lima et al. (2017) compared the effects of the infection by this species of nematode on the reproduction of *B. straminea* and *B. tenagophila* and observed marked parasitic castration in both parasitized molluscs. Tunholi-Alves et al. (2011) observed similar results for *B. glabrata* infected by *A. cantonensis* larvae. Interestingly, even though this parasite uses a terrestrial rodent as definitive host, most studies focus on interactions with the intermediate hosts freshwater snails.

The total number of eggs laid by infected *B. tenuissimus* varied more in the snails in the first week after exposure compared to the uninfected ones, but from this point onward only small differences were observed with the values of the control group. Based on the number of newly hatched snails, the infection seemed not affect the viability, since mean values were always higher than those observed among uninfected snails, resulting in a rising mean ratio between these parameters throughout the development of the infection. These results differ from those recorded by the authors in both studies mentioned above. The increasing ratio of newly hatched snails/eggs laid showed that the infection leads to higher energy demand and burden on reproductive biology of *B. tenuissimus* in the first week after exposure. However, with the progress of the infection, a reduction of the reproductive efforts by the infected snails clearly occurred, increasing the number of newly hatched snails/eggs laid, which can be interpreted as rising reproductive success of the parasitized hosts. At the end of the prepatent period, the infected snails had a reproductive success rate 20.49% higher than the uninfected ones, showing the occurrence of a fecundity compensation process in response to the parasitism by *A. cantonensis* larvae. Minchella called fecundity compensation the sharp increase of snail's egg laying following parasite exposure (Minchella and LoVerde 1981; Minchella 1985) describing it as an adaptive physiological response to trematode parasitism. Davis et al. (2022) described this phenomenon as "life-history response, occurring when hosts increase their current reproductive output to make up for expected losses in future reproduction due to parasitic infection". The term is currently used more broadly to indicate patterns of increased reproductive effort in host-parasite relation, as a way for compensate future or past losses. In addition, Tunholi et al.(2011) showed that the infection of the freshwater snail *B. glabrata* triggered the same kind of response to *Echinostoma paraensei* larval infection. Duffield et al. (2017) stated that reproductive investment made in fecundity compensation "can be allocated towards an increase in the quantity and/or quality of offspring", and our results showed that *B. tenuissimus* infected with *A. cantonensis*, not only increase the eggs production (number of eggs/snails), but also produces embryos with high viability and hatchability.

Galactogen is a galactose polymer restricted to the albumen gland. It is produced as an important source of energy for the embryo during its development in the egg (Goudsmit and Ashwell 1965, Nieland and Goudsmit 1969). Therefore, this polysaccharide is an essential component to assure the normal development of the embryo and newly hatched snails. Therefore, the progressive reduction observed in our study seems to be more related to the use of this substance by the snail to the increase egg production, since galactogen reduction is inversely related to the reproductive success, expressed as number of newly hatched snails/number of eggs laid by *A. cantonensis*-infected *B. tenuissimus*. A similar pattern was observed in *B. straminea* infection (Lima et al. 2017). Also, Tunholi-Alves et al. (2011) observed that in *B. glabrata* infected with *A. cantonensis*, all the reproductive parameters analyzed were significantly reduced. In turn, Pinheiro and Amato (1995) observed parasitic castration in *Bradybaena similaris* infected with larvae of the digenetic trematode *Eurytrema coelomaticum*, but fecundity compensation did not happen in this host-parasite relationship. This diversity of reproductive responses observed in the different host-parasite associations, indicates that each relationship involves a particular response, reflecting the coevolution of the organisms and their adaptations to parasitic coexistence. For this reason, further studies about these new interrelationships are needed, to obtain information that in the future, can be used in programs to control parasitic diseases caused by these helminths.

The histological analysis showed that changes in the gonad tissues may occur, but it was not a general response of all the infected snails, reflecting an intraspecific variation pattern in the tissue response. Even when histological changes were observed, it was possible to observe gametes in different developmental stages, indicating that gametogenesis was occurring normally. These morphological findings reinforce the oviposition activity and egg viability found in our study. Thompson and Kavaliers (1994) stated that in many host-parasite systems, parasitism does not result in physical damage or destruction of the host's reproductive organs, but even so, parasitic castration occurs. In *B. tenuissimus*-*A. cantonensis* system it seems that the opposite occurred: in spite of the presence of larvae and the disorganization of the structural features of the ovotestis and albumen gland tissues, the reproductive activity proceeded normally.

In conclusion, the results presented in our study are very worrisome and denotes are important to veterinary medicine and public health. Pien and Pien (1999) stated that *A. cantonensis* has spread from Southeast Asia to the South Pacific, Africa, India, the Caribbean, Australia and North America, but this distribution certainly became wider in the ensuing decade (Foronda et al. 2010). In the present study, *B. tenuissimus* was able to withstand exposure to 1,200 L1 larvae and the prepatent intramolluscan larval development did not significantly affect the reproductive activity, according to the parameters analyzed, or the internal morphology.

Three factors should be considered: 1 - the presence of snails of the *B.* genus in many countries of North and South America, in many of which the occurrence of *A. cantonensis* has also been recorded; 2 – the snail *B. tenuissimus* is frequently found in gardens and other planted areas, especially subsistence crops; and, 3 - the habits of smallholders of not using personal protective equipment. These characteristics cause a high risk of exposure to infection by *A. cantonensis* larvae.

Neural angiostrongyliasis is a neglected disease that is now disseminated in many countries. Our results bring new information and call attention to a largely overlooked snail host, but one whose infection can became a serious problem to public and wildlife health.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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FIGURES

Figure 1. Reproductive changes in *Bulimulus tenuissimus* experimentally infected with 1,200 L1 of *Angiostrongylus cantonensis* for three weeks. Week 0 represents the average of the control groups during the three weeks of analysis, since there was no significant difference during the three weeks.

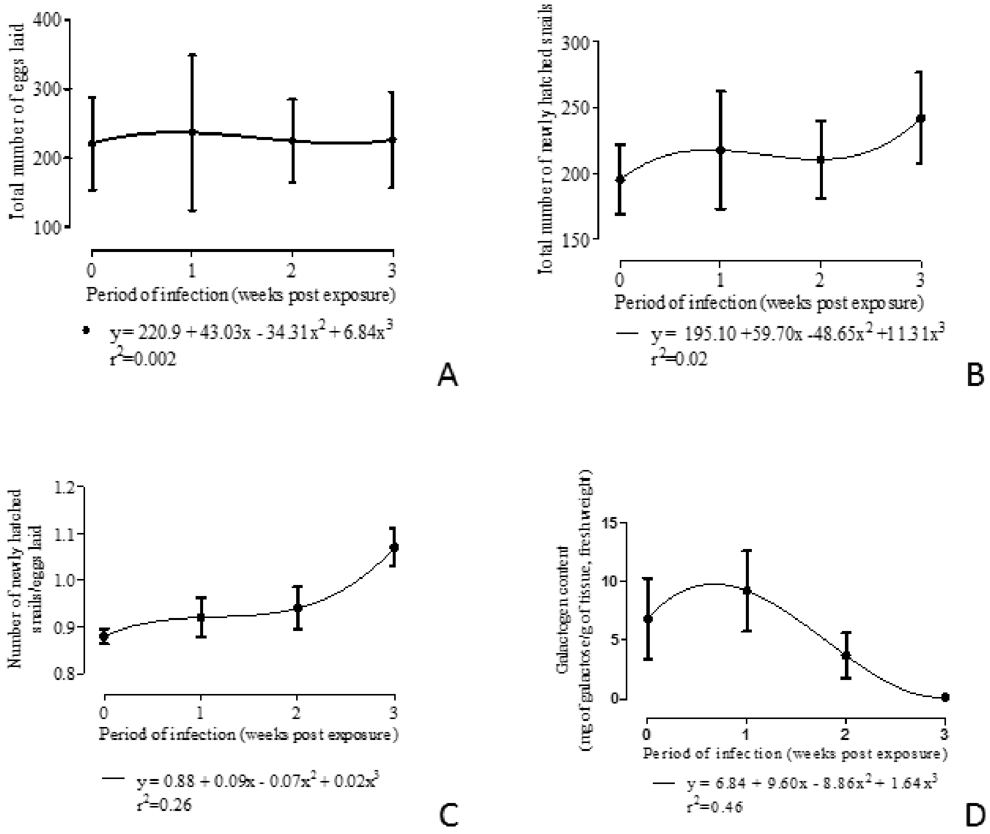

Figure 2. Histological sections of the ovotestis region of *Bulimulus tenuissimus* experimentally infected with 1,200 L1 larvae of *Angiostrongylus cantonensis*, for three weeks. A. Uninfected snail showing normal aspect of the gonad, containing sperm (s) at different stages of development and oocytes (ov). B. Albumen gland (a) of uninfected snail. C-F. Ovotestis region of infected snail, with larvae developing in the tissues of the organ (white arrow) along with hemocyte infiltrates (black arrow), albumen gland (a), but still with the presence of sperm (s) and oocytes (ov) in different stages of development. Granuloma (g) formed around the developing larva.A -B, stained by hematoxylin and eosin; C- F, stained by Gomori's trichromic.

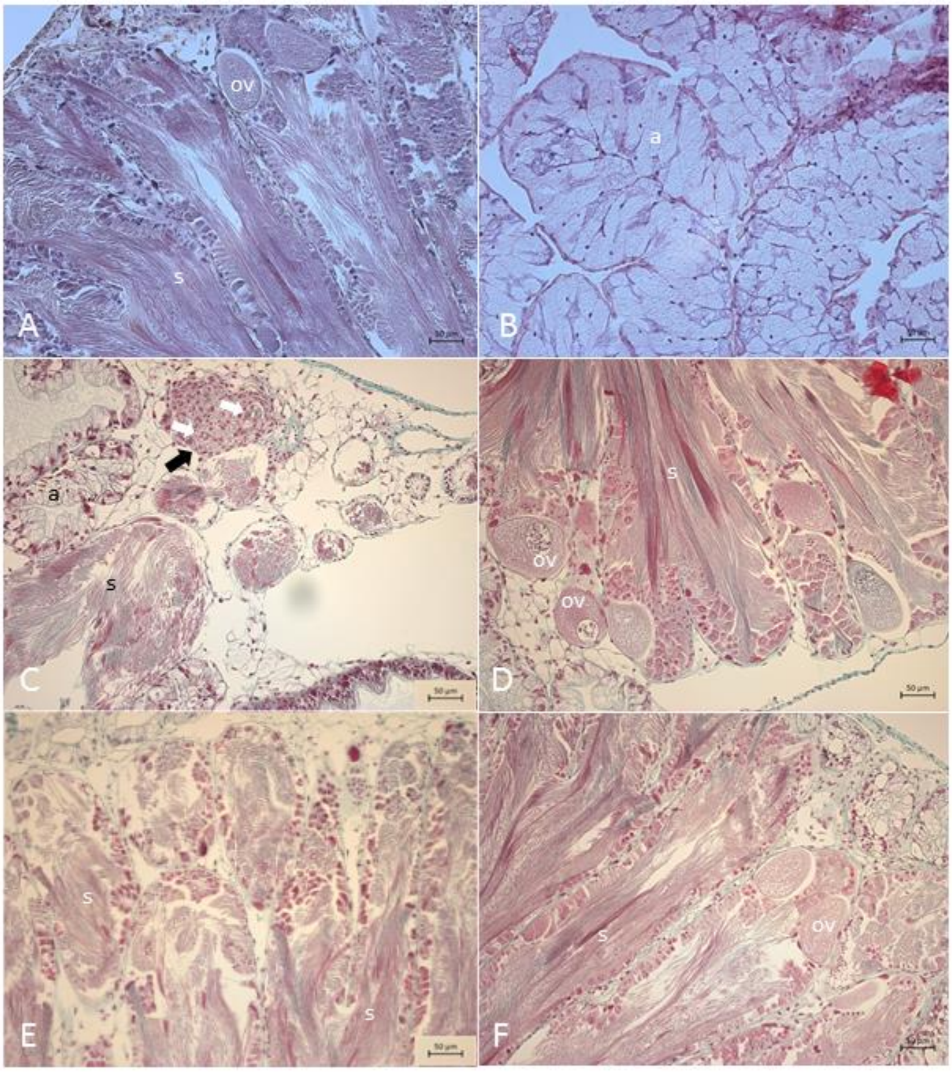

Table 1 Changes in the total number of eggs laid and number of newly hatched snails/eggs laid by *Bulimulus tenuissimus* during three weeks after exposure to *Angiostrongylus cantonensis* L1 larvae. Week 0 represents the average of the control groups during the three weeks of analysis, since there was no significant difference among the values observed in the three weeks analyzed.

Period of infection (Weeks)	Total number of eggs laid	Number of hatched snails/eggs laid
	X±SD	(%)
0	237.00 ± 33.55	0.89
1	236.43 ± 45.94	0.92
2	209.00 ± 25.47	0.93
3	226.00 ± 30.09	1.07