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Original Article

Reproductive alterations of *Biomphalaria glabrata* (Say, 1818) infected with *Angiostrongylus cantonensis* (Chen, 1935) and exposed to *Euphorbia milii* var. *hislopii* latex

Alterações na atividade reprodutiva de *Biomphalaria glabrata* (Say, 1818) infectada com *Angiostrongylus cantonensis* (Chen, 1935) e exposta ao látex de *Euphorbia milii* var. *hislopii*

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Abstract

The natural phytochemical latex of *Euphorbia milii* var. *hislopii* is one of the most promising natural molluscicides for the control of *Biomphalaria glabrata*, and has been widely studied under laboratory conditions for selective control of schistosomiasis transmission. However, the effect of this product on *B. glabrata* infected by other helminths had not yet been investigated. The present study reports evaluation of the effect of *E. milii* var. *hislopii* latex on the survival and reproductive activity of *B. glabrata* infected by *Angiostrongylus cantonensis*. For this purpose, the following groups were formed: control (C), exposed (E), infected (I) and infected and exposed for different time intervals (1 day - I+E-1D, 7 days - I+E-7D, 14 days - I+E-14D, 21 days - I+E-21D and 28 days - I+E-28D). The experimental infection was performed with L1 larvae of *A. cantonensis* and exposure to 0.08 mg/L (LC_{50}) of *E. milii* latex for a period of 24 hours. We analyzed the effects of *E. milii* latex on the survival of snails during four weeks, reproductive parameters and possible histophysiopathological changes in the gonad and albumen gland of the snails. Regarding survival, there was reduction of more than 50% in the groups exposed to latex (E and I + E) compared to the control group. As for the number of ovigerous masses, eggs, and average number of hatched snails, significant increases were observed in the I+E-1D group at the 4th week compared to the control group and the other weeks in the same group. *Angiostrongylus cantonensis* larvae were observed in the gonad and albumen gland from day 21 and 28 of infection in groups I and I+E, respectively, with granuloma-like formation. At these observation periods and in these groups, an increase in galactogen was observed in the albumen gland, which influenced egg laying, suggesting the existence of a fecundity compensation mechanism phenomenon. It was possible to conclude that both stressors – *A. cantonensis* infection and exposure to *E. milii* latex – directly influenced the survival and reproductive parameters of *B. glabrata*.

Keywords: mollusca, nematode, reproduction, granuloma-like, histopathophysiological.

Resumo

O fitoquímico natural látex de *Euphorbia milii* var. *hislopii* é um dos mais promissores moluscicidas naturais para o controle de *Biomphalaria glabrata*, tendo sido amplamente estudado em condições laboratoriais como controle seletivo da transmissão da esquistossomose. No entanto, o efeito deste produto em *B. glabrata* infectada por outro helminto, ainda não tinha sido investigado. O presente trabalho tem por objetivo avaliar o efeito do látex de *E. milii* var. *hislopii* na sobrevivência e atividade reprodutiva de *B. glabrata* infectada por *Angiostrongylus cantonensis*. Para isso, foram formados os seguintes grupos: controle (C), exposto (E), infectado (I) e infectado e exposto em diferentes intervalos de tempo de infecção (1 dia – I+E-1D, 7 dias – I+E-7D, 14 dias – I+E-14D, 21 dias – I+E-21D e 28 dias – I+E-28D). A infecção experimental foi realizada com larvas L1 de *A. cantonensis* e a exposição com 0,08 mg/L (LC_{50}) do látex de *E. milii* por um período de 24 horas. Foram analisados os efeitos do látex de *E. milii* quanto à sobrevivência dos moluscos ao longo de quatro semanas, aos parâmetros reprodutivos e as possíveis alterações histofisiopatológicas na gônada e na glândula de albúmen dos moluscos analisados. Em relação à sobrevivência

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foi possível observar redução de mais de 50% nos grupos expostos ao látex (E e I + E) em comparação ao grupo controle. Quanto ao número de massas ovígeras, ovos e número médio de moluscos eclodidos pode-se observar um aumento significativo no grupo I+E-1D na 4ª semana em comparação ao grupo controle e as demais semanas no mesmo grupo. Larvas de *A. cantonensis* foram observadas na glândula e na glândula de albúmen, a partir dos 21º dias e 28º dia de infecção nos grupos I e I+E, respectivamente, com a formação de granuloma-*like*. Nesses períodos de observação e nesses grupos pode-se observar na glândula de albúmen, um aumento do conteúdo de galactogênio, o que influenciou a postura dos ovos, sugerindo um fenômeno de compensação da fecundidade. Foi possível concluir que ambos agentes estressores, infecção por *A. cantonensis* e exposição ao látex de *E. milii* influenciou diretamente a sobrevivência e os parâmetros reprodutivos de *B. glabrata*.

Palavras-chave: mollusca, nematoda, reprodução, granuloma-*like*, histofisiopatológicas.

1. Introduction

Helminth infection and exposure to phytochemicals are known to modulate the survival and reproductive parameters of intermediate host snails (Moore, 2002; Oliveira-Filho et al., 2014; Alberto-Silva et al., 2015, 2020). Infection can cause partial or total disruption of oviposition and egg hatching, in addition to biochemical and histopathological changes, leading to a reduction in the population of infected snails (Faro et al., 2013; Guerino et al., 2017). Regarding infection, these effects have been studied in models such as *Biomphalaria glabrata* (Say, 1818) infected by *Schistosoma mansoni* (Sambon, 1907) (Minchella and Loverde, 1981; Vasconcellos and Schall, 1986; Lima, 2010; Faro et al., 2013; Alberto-Silva et al., 2015); *B. glabrata* infected by *Echinostoma paraensei* Lie and Basch, 1967 (Tunholi et al., 2011), *B. glabrata* co-infected by *E. paraensei* and *Angiostrongylus cantonensis* (Chen, 1935) (Bonfim et al., 2014; Garcia, 2014); *B. glabrata* infected by *A. cantonensis* (Tunholi-Alves et al., 2011); *Biomphalaria straminea* (Dunker, 1848) and *Biomphalaria tenagophila* (D'Orbigny, 1835) infected by *A. cantonensis* (Lima et al., 2017) and *Bulimulus tenuissimus* (D'Orbigny, 1935) infected by *Angiostrongylus cantonensis* (Martins et al., 2019).

Regarding exposure to phytochemicals, the World Health Organization (WHO, 1983), recommends control of the host snail population, through integrated management as one of the strategies to control transmission of parasites of medical and veterinary importance. However, the use of molluscicides to control populations of intermediate hosts is still used in some countries, such as those in Africa. The phytochemical latex of *Euphorbia milii* var. *hislopii* has been studied for its schistosomostatic (Augusto et al., 2017) and molluscicidal properties related to survival and reproductive parameters of snails such as *Achatina fulica* (Ferussac, 1821) (Crignis et al., 2012), *Leptinaria unilamellata* D'Orbigny, 1835 (Afonso-Neto et al., 2010), *Bulimulus tenuissimus* (D'Orbigny, 1935) (Patrício et al., 2019), *Lymnaea columella* (Say, 1817) (Vasconcellos and Amorim, 2003) and *B. glabrata* (Mello-Silva et al., 2007; Lima, 2010; Alberto-Silva et al., 2020). This phytochemical presents several advantages, such as biodegradability, low cost, efficiency in sublethal doses (less than 2.0 mg/L) (Vasconcellos and Schall, 1986) and selective effect on infected snails. This natural product had not been tested on freshwater or terrestrial snails infected by *A. cantonensis*.

The nematode *A. cantonensis* is the etiological agent of human neural angiostrongyliasis or eosinophilic meningitis. It causes inflammation of the meninges by larvae of this parasite (Ibrahim, 2007), and is endemic to Asia (Alicata, 1988) and emerging in Brazil, with its first record in 2006 in the state of Espírito Santo (Caldeira et al., 2007). Humans are considered accidental hosts, since the natural cycle of this parasite is maintained in rodents, such as *Rattus norvegicus* (Berkenhout, 1769) and terrestrial and/or freshwater snails. The snail *B. glabrata* is very important in maintaining the experimental cycle of several parasites (Tunholi-Alves et al., 2014) such as *S. mansoni* and *A. cantonensis*, besides having been used as a model in several studies of the phytochemical latex of *E. milii* var. *hislopii* (Vasconcellos and Schall, 1986).

Helminth infection and/or exposure to phytochemicals can alter the survival and reproductive parameters of *B. glabrata* in nurseries, allowing or not its repopulation. Thus, this study aimed to evaluate the effects of *A. cantonensis* infection and/or exposure to *E. milii* var. *hislopii* latex on the survival, reproductive parameters, and possible histophysiopathological changes in the gonad and albumen gland of *B. glabrata* under laboratory conditions.

2. Material and Methods

2.1. Ethical considerations

This study was approved by the Animal Use Ethics Committee of Oswaldo Cruz Foundation (CEUA-IOC - 025/2018) and is registered Brazil's National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen no. A9666E5).

2.2. Experimental infection and group formation

The biological cycle of the parasite *A. cantonensis* is maintained in the bioterium of the Lauro Travassos Pavilion at Fiocruz according to Garcia et al. (2014). First-stage larvae (L1) were obtained by collecting and processing feces from *Rattus norvegicus* (Willcox and Coura, 1989). The study was carried out using 1,320 snails of the species *B. glabrata*, Sumidouro strain, kept during the experiment under laboratory conditions, with controlled temperature (25 °C ± 2 °C), 12/12 hour photoperiod. The snails measured 8-12 mm in diameter and had approximate age of 90 days. They were into the following groups: control (C), exposed (E), infected (I) and infected and exposed (I+E) at 1 (1D), 7 (7D), 14 (14D), 21 (21D) and 28 (28D) days. The experimental infection was performed individually in 24-well plates with 0.50 ml of dechlorinated water, where approximately 1,000 first stage larvae (L1) of *A. cantonensis* were deposited. To perform the exposure, the snails were divided into groups of ten specimens and exposed to an

aqueous suspension of 0.08 mg/L of lyophilized *E. milii* var. *hislopii* latex for a period of 24 hours, using 1 liter glass beakers. All groups were observed for up to 28 days after infection.

2.3. Collection and solution preparation

Samples with volume of 5 mL of *E. milii* var. *hislopii* latex were collected from the Fiocruz garden of the Manguinhos campus (22°52'29.64"S; 43°14'43.39" W), Rio de Janeiro, Brazil. Latex of *E. milii* var. *hislopii* were collected and freeze-dried, according to Augusto et al. (2016).

Both the collection procedures and the experiment to determine the lethal (LC_{90}) and sublethal (LC_{50}) concentrations of *E. milii* var. *hislopii* latex followed the protocols described by Vasconcellos and Amorim (2003).

2.4. Analysis of survival and reproductive parameters

After 24 hours of exposure, the snails were removed from the suspension and placed in dechlorinated water. For survival analysis, the number of live snails, from all groups analyzed, was recorded weekly during four weeks. The following reproductive parameters were analyzed: (E) mean number of eggs; (Em) mean number of egg masses; (Hs) mean number of snails hatched; (E/S) eggs per snail; (Em/S) egg masses per snail; (Hs/S) snails hatched per snail; Hs/E: snails hatched per egg; (E/Em) eggs per egg mass; and (Hs/Em) snails hatched per egg mass.

The ten snails from each group were kept in plastic aquaria (22 cm length, 14.5 cm width, and 12.5 cm height) with 3 L capacity, filled with 2 L of dechlorinated water, replaced once a week together with addition of 0.5 g of $CaCO_3$ for snail calcium replacement, and containing a 3 cm^2 Styrofoam plate as substrate for oviposition. Survival and reproductive parameters were analyzed with the aid of a stereoscopic microscope after counting the ovigerous masses and eggs. They were identified and transferred to Petri dishes containing 10 ml of dechlorinated water, kept at a controlled temperature of 25° C ± 2 °C, with 12/12 photoperiod. The number of hatched snails was counted 15 days later, as described by Alberto-Silva et al. (2020). The snails were fed *ad libitum* with fresh lettuce leaves (*Lactuca sativa* L.) three times a week, and all experiments carried out in triplicate.

2.5. Biochemical analysis

Galactogen extraction from the albumen gland was performed according to the method of Pinheiro and Gomes (1994) and quantified by the 3.5 DNS method (Sumner, 1924), and expressed as mg galactose/g tissue fresh weight.

2.6. Histological analysis

A total of 100 snails were removed from the shells and the soft tissues were kept in Carson's Formalin Millonig fixative (Carson et al., 1973) for 24 hours at a temperature of 25 °C ± 2 °C. The fixed tissues were processed according to routine histological techniques (Tolosa et al., 2003) and inclusion was performed using a Shandon HistoCentre self-inclusion apparatus. Semifine sections (5 µm thick) were obtained using a Leica RM 2135 rotary microtome and were stained with hematoxylin and eosin (Silva, 2016) or Masson's trichrome (Silva, 2014).

2.7. Statistical analysis

The results obtained were expressed as mean ± standard deviation of the mean and subjected to one-way ANOVA and Tukey's multiple comparison test for comparison of the means at 5% significance ($p < 0.05$) (GraphPad Prism 5.0).

3. Results

3.1. Survival

The survival results of the different groups (C, E, I, I+E 1D, I+E 7D, I+E 14D, I+E 21D, I+E 28D), as a function of time were analyzed during 4 weeks (Figure 1). The control group

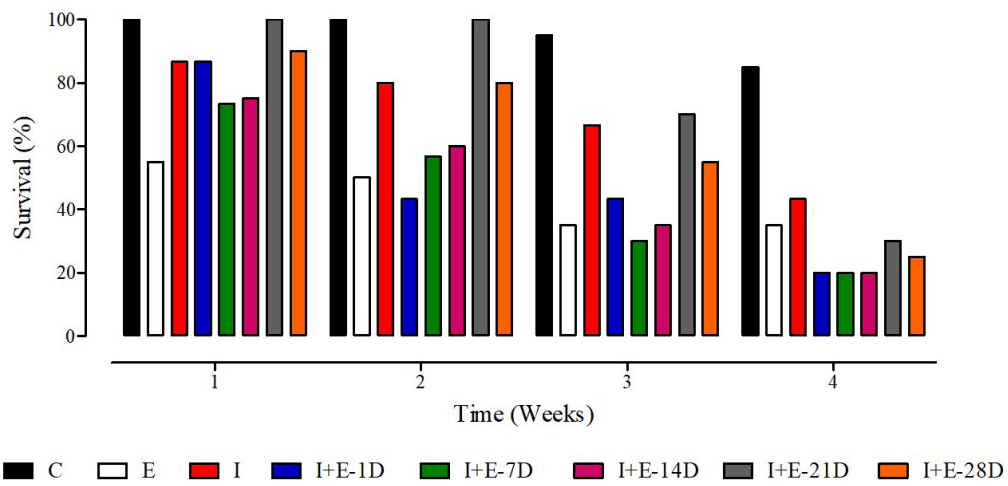

Figure 1. Effects of *Angiostrongylus cantonensis* infection and/or exposure to *Euphorbia milii* var. *hislopii* latex on the survival (%) of *Biomphalaria glabrata* in groups C, E, I, I+E-1D, I+E-7D, I+E-14D, I+E-21D and I+E-28D after 1, 2, 3, and 4 weeks.

had survival ranging from 85 to 100%, inversely related to observation time, with the lowest rate observed at the end of four weeks. Exposure of *B. glabrata* to *E. milii* var. *hislopii* latex (E) caused a reduction in the survival rate of the snails throughout all periods analyzed compared to the control. The lowest survival rates were observed in the third and fourth weeks of observation, 35% in both intervals. Figure 1, presenting the lowest average percentage viability (43.75%).

Snails infected with *A. cantonensis* (I) maintained high viability in the first two weeks of infection (86.6% and 80%, respectively), with more significant reductions after 3 (71.6%) and 4 (58.3%) weeks of infection.

Exposure of *B. glabrata* infected with *A. cantonensis* to *E. milii* var. *hislopii* latex at one (I+E-1D: 48.3%), seven (I+E-7D: 44.97%) and 14 (I+E-14D: 47.5%) days of infection resulted in a mean viability similar to that observed for the snails exposed (E) to the same product. The snails from the exposed groups 21 (I+E-21D: 75%) and 28 (I+E-28D: 62.5%) days after infection with *A. cantonensis* had the highest mean viability within this infected and exposed subgroup.

3.2. Number of eggs laid per snail (E/S)

Exposure to *E. milii* var. *hislopii* latex (E) in the first week of infection fully inhibited oviposition of uninfected *B. glabrata*. There was an evident recovery of this parameter in the ensuing weeks of observation, suggesting a compensatory process of oviposition. The number of eggs/snail was always higher than that observed for the control snails (C). In the last two weeks of observation, the number of eggs laid per snail was 175.36% higher for snails in group E.

The effects of infection with *A. cantonensis* (I) on the number of eggs laid per snail were also negative, but there was no compensatory process regarding this parameter, with its maintenance at values always lower than those observed for the control snails, where the greatest reduction was observed in the second week of infection. There was a recovery tendency, with increasing values throughout the development of the pre-patent period, but without reaching the same value as the uninfected snails.

When we evaluated the effects of exposure to *E. milii* var. *hislopii* latex on snails infected with *A. cantonensis*, the number of eggs laid per snail showed a compensatory trend in the groups exposed 1 day after infection, in the fourth week of exposure only. In the group exposed seven days after infection, this compensation of fecundity was observed from the second week of observation, reaching an average percentage variation of +146.20% compared to the control group.

3.3. Number of egg masses laid per snail (Em/S)

Exposure of the snails to *E. milii* var. *hislopii* latex interrupted the laying of egg masses in the first week, with a tendency to recover during the second and third weeks of observation. However, by the fourth week after exposure, an increase of 550% was observed, confirming the reproductive compensation phenomenon. The same pattern was observed for the snails infected (I) with *A. cantonensis*, but the inhibitory effect was milder, with no complete interruption of egg masses in any of the periods observed.

The analysis of the snails infected and subsequently exposed to *E. milii* var. *hislopii* latex followed the pattern observed when analyzing the number of eggs laid per snail, with a reduction or interruption of egg laying in the first week in all infected and exposed groups. In the following observation periods, the groups exposed one day (I+E-1D) and seven days (I+E-7D) after infection always maintained higher values than those observed for the control group, being 219.8% and 111.9%, respectively, higher on average than the control snails.

3.4. Number of eggs per egg mass (E/Em)

In the snails exposed to latex (E) of *E. milii* var. *hislopii* laying of egg masses was interrupted, so the ratio between the number of eggs per egg mass in the first week of exposure was zero. In the following weeks, this ratio was always significantly higher than that observed in the unexposed snails (C), with 52.97% higher average egg production per egg mass of the latex-exposed snails.

Infection of *B. glabrata* by *A. cantonensis* resulted in a lower ratio of number of eggs per egg mass than that observed for the uninfected snails (C) throughout the entire pre-patent period, which is in line with what was observed above regarding the number of eggs laid per snail, which was also always lower in the infected snails. This ratio for the infected snails was on average 74.14% lower than that observed in the uninfected and unexposed snails (C) (Table 1).

Snails exposed to *E. milii* var. *hislopii* latex after infection with *A. cantonensis* from all groups always ha lower ratios of number of eggs per egg mass than observed in the control groups, indicating production of a greater number of egg masses by these snails, but without these having an equivalent number of eggs.

3.5. Viability of offspring of A. cantonensis infected snails exposed to E. milii var. hislopii latex

The relationships between the number of snails hatched per egg laid, the number of snails hatched per snails used, and the number of snails hatched per egg mass in each group indicated the effect of infection, exposure, and the association of these two factors on the viability of offspring produced.

The number of hatched snails per egg (Hs/E) showed large variation among the different groups. However, in the group exposed to *E. milii* var. *hislopii* latex (E), only in the first week of observation was this value zero, with no significant variation, being higher than the values observed for the control group (C) in all other periods. Snails infected with *A. cantonensis* (I) only had lower ratio between hatched snails and laid eggs compared to control snails (C) after three weeks (-76.19%), resulting in an average of 5.16% compared to the control group.

With respect to the ratio between the number of hatched snails per egg mass (Hs/Em), both exposure to *E. milii* var. *hislopii* latex (E) and infection with *A. cantonensis* (I) promoted the absence (E) or reduction in the number of hatched snails (I). However, from the second week

Table 1. Effects of *Angiostrongylus cantonensis* infection and exposure to *Euphorbia milii* var. *hislopii* latex on the reproductive parameters of *Biomphalaria glabrata*: E/Em: eggs per egg mass and Hs/Em: hatched snails per egg mass.

GROUPS	1st Week		2nd Week		3RD WEEK		4TH Week	
	X ± SD		X ± SD		X ± SD		X ± SD	
	E/Em	Hs/Em	E/Em	Hs/Em	E/Em	Hs/Em	E/Em	Hs/Em
C	10.80±0.85^{a,A}	3.40±0.85^{a,A}	12.53±1.10^{a,A}	8.25±2.48^{a,A}	7.82±1.39^{a,A}	1.65±0.2^{a,A}	9.00±2.83^{a,A}	1.75±0.35^{a,A}
E	0.00±0.00^{a,A}	0.00±0.00^{a,A}	16.25±1.77^{a,A}	11.25±3.89^{a,A}	19.35±3.75^{b,A}	11.33±7.0^{a,A}	12.13±8.31^{a,A}	2.75±3.89^{a,A}
I	4.33±3.79^{a,A}	1.44±1.26^{a,A}	1.50±2.60^{a,A}	1.17±2.02^{a,A}	3.00±3.00^{a,B}	0.11±0.19^{a,B}	1.17±1.26^{a,A}	0.25±0.43^{a,A}
I+E – 1D	10.17±9.57^{a,A}	8.50±7.70^{a,A}	4.53±4.32^{a,A}	0.67±1.16^{a,A}	3.90±3.76^{a,A}	1.42±2.45^{a,A}	9.92±2.13^{a,A}	3.25±1.75^{a,A}
I+E – 7D	5.40±6.56^{a,A}	4.39±4.90^{a,A}	5.43±4.84^{a,A}	1.50±2.18^{a,A}	8.67±4.31^{a,A}	3.57±3.30^{a,A}	8.53±7.43^{a,A}	2.14±3.14^{a,A}
I+E – 14D	0.00±0.00^{a,A}	0.00±0.00^{a,A}	7.85±2.61^{a,A}	0.00±0.00^{a,A}	3.75±5.30^{a,A}	2.00±2.83^{a,A}	4.00±5.66^{a,A}	2.50±3.54^{a,A}
I+E – 21D	2.50±0.71^{a,A}	0.00±0.00 ^{a,A}	5.75±0.35^{a,A}	0.00±0.00^{a,A}	2.00±2.83^{a,B}	2.00±2.83^{a,A}	0.00±0.00^{a,A}	0.00±0.00^{a,A}
I+E – 28D	1.50±0.00^{a,A}	0.25±0.35^{a,A}	3.00±4.24^{a,A}	2.00±2.83^{a,A}	1.30±1.84^{a,B}	0.84±1.18^{a,A}	0.00±0.00^{a,A}	0.00±0.00^{a,A}

a,b = Means and standard deviations followed by different letters in rows differ at 5% significance; A,B = Means and standard deviations followed by different letters in columns differ at 5% significance.

of analyses on, the snails infected with *A. cantonensis* maintained significantly lower values of this ratio in comparison with the control group (C), while the exposed snails (E) produced a greater number of offspring per egg mass than the control snails (C) (Table 1).

The ratio between the number of snails hatched per egg mass laid by the snails of the different groups (Hs/Em) was either totally prevented (E) or reduced (I) in the groups exposed to *E. milii* var. *hislopii* latex or infected with *A. cantonensis* in the first week of analysis (Table 1). From the second week of observation, all values obtained for the exposed snails were higher than those observed for the control snails, representing a mean difference of 182.86% between them. In the infected snails, there was no such recovery during the pre-patent period, with an average reduction of 80.63% compared to the control group.

In the groups exposed to *E. milii* var. *hislopii* latex after infection with *A. cantonensis*, there was only an increase in the number of snails hatched per egg mass in the first week for the groups exposed one day (I+E-1D) and seven days (I+E-7D) after infection with the nematode larvae. Thereafter, only the second group (I+E-7D) maintained this ratio higher than that observed for the control snails (C), resulting in an increase of 19.87%. The exposed group after 28 days of infection (I+E-28D) had the lowest absolute value of all groups, 54.36% lower than the control group.

Exposure to *E. milii* var. *hislopii* latex totally inhibited snail reproduction in the first week of analysis. However, in the following three weeks, the hatchability of the snails in relation to the number of parent snails was significantly higher in the exposed group (E) than in the control snails (C), reaching a mean value greater than 424.85%. The infection altered the percentage of eggs laid. The eggs laid by snails infected (I) with *A. cantonensis* had a significantly lower hatching percentage (-69.31%) than the eggs laid by the control snails (C).

Exposure of *A. cantonensis* infected snails to *E. milii* var. *hislopii* latex (E) caused a reduction in almost all groups analyzed, but even in group I+E-7D the observed difference was not significant in relation to the number of hatched snails per parent snail in the first week of observation. However, in the second week, snails from all infected and exposed groups produced eggs with lower hatchability than those from the control group (C), resulting in a lower ratio between the number of hatched snails and number of parent snails. In the last two weeks, snails from the groups exposed to *E. milii* var. *hislopii* latex after one (I+E-1D: +457%), seven (I+E-7D: +328.5%) and 14 (I+E-14D: 78.15%) days of infection with *A. cantonensis*, showed increased hatchability in comparison with the control group.

3.6. Galactogen content in albumen gland

Regarding the galactogen content (mg galactose/g tissue, fresh weight) in the albumen gland of *B. glabrata* infected with *A. cantonensis* and/or exposed to *E. milii* var. *hislopii* latex, no significant difference was observed in the control groups (C), the exposed groups (E) and the I+E group over the 28 days of the experiment. No significant difference was observed between groups C and E. Significant reductions were observed between the infected-1 day, infected-7 day, infected-14 day, and all infected+exposed groups on the one hand and groups C and E on the other. There was a significant increase in the infected-21 day group compared to group C, but the same was not observed in group E, while in the infected-28 day group there was no difference. There was a significant increase in galactose content in the I-21D and I-28D groups compared to the infected-1 day, infected-7 day, and infected-14 day groups (Table 2).

3.7. Histology

Analyzing the gonad, in all groups it was possible to observe preserved acinar organization with active gametogenesis (spermatogenesis and oogenesis) and unchanged morphology (Figure 2). Sperm and oocyte formation at different stages of development was also observed in groups E, I and I+E (A to F) at all intervals analyzed.

Table 2. Galactogen content (mg galactose/g tissue fresh weight) in the albumen gland of *Biomphalaria glabrata* infected with *Angiostrongylus cantonensis* and/or exposed to *Euphorbia milii* var. *hislopii* latex. There was no significant difference within the control groups (C) and within the exposed groups (E).

GROUPS	X ± SD
C	0.88±0.13[a]
E	1,32±0.33[a]
I	0.27±0.85[b]
I+E–1D	0.23±0.05[b]
I+E–7D	0.06±0.04[b]
I+E–14D	0.10±0.03[b]
I+E–21D	0.24±0.06[b]
I+E–28D	0.19±0.13[b]

a,b = Means and standard deviation followed by different letters differ at 5% significance level ($p < 0.05$).

From group I-21D onwards (C) the gonad appeared normal, with sperm formation, evidence of a larval profile, with granuloma-like formation, in group I+E-21D (D). Granuloma-like formation around the larva can be observed in horizontal view, but there is no structural change in the gonad. In group I-28D (E), the gland was also observed with normal appearance and evidence of larval profile, where it is C-shaped, and it is possible to see only the far end of the head and tail on the cut surface, with vertical view. In group I+E-28D (F), the gland has normal appearance and granuloma-like formation due to cellular infiltration (Figure 3).

The histological analysis of the whole snail showed the presence of larvae in the connective tissue from one week of infection (Figure 4), without deformation and surrounded by granuloma-like formation. In the control (A), exposed (B), I-1D (C) and I+E-1D (D) groups, the albumen gland is not deformed, with normal structure in the I-7D group (E). It is possible to observe infiltration of cells, leading to

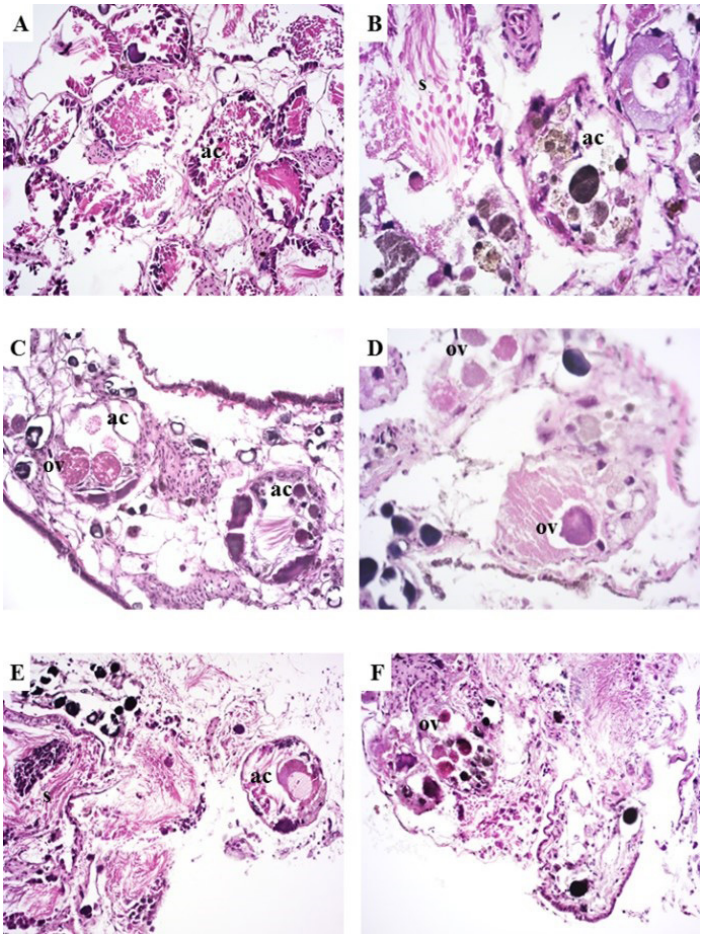

Figure 2. Histological sections of the gonadal region of *Biomphalaria glabrata* infected with *Angiostrongylus cantonensis* and/or exposed to *Euphorbia milii* var. *hislopii* latex: A. Control group-20X - Showing normal aspect of the gonad, with acini (ac) and occurrence of gametogenesis; B. Exposed-40X - Normal gland, with presence of spermatogenesis (s) and oogenesis, in the acini (ac); C. Infected-1 day-20X - Normal looking gland, with acini (ac), sperm formation and oocytes at different stages; D. Infected+Exposed-1 day-40X - Normal looking gland, with acini with oogenesis (ov); E. Infected-7 days-20X – Normal gland, with acini (ac) and gametogenesis; and F. Infected+Exposed-7 days-20X – Normal gland, with acini (ac) and gametogenesis at different stages. A-F stained with hematoxylin and eosin.

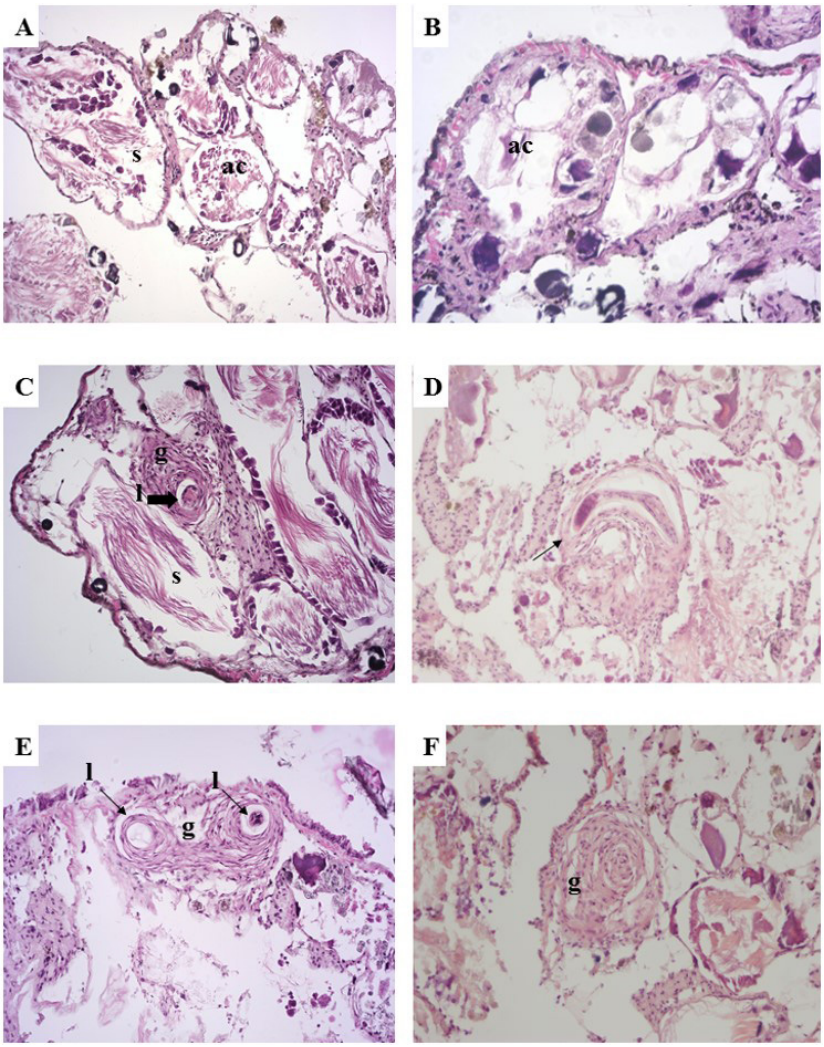

Figure 3. Histological sections of the gonadal region of *Biomphalaria glabrata* infected with *Angiostrongylus cantonensis* and/or exposed to *Euphorbia milii* var. *hislopii* latex: A. Infected-14 days-20X - Normal looking gland with acini (ac) and formation of spermatozoa (s) and oocytes in different stages; B. Infected+Exposed-14 days-40X - Normal looking gland, acini (ac) with oocyte formation in different stages; C. Infected-21 days-20X - Normal gland, with spermatozoa (s) and granuloma-like structure formation (g) around larval profile (l); D. Infected+Exposed-21 days-20X - Normal gland, granuloma-like formation around larva (arrow); E. Infected-28 days-20X - Normal gland, granuloma-like formation (g) around larval profile (l); and F- Infected+Exposed-28 days-20X - Normal gland, granuloma-like formation (g). A-F stained with hematoxylin and eosin.

granuloma-like formation, and evidence of larval profile in the central part, without deposition of collagen. In group I+E-7D (F), there is granuloma-like formation due to the occurrence of cellular infiltration.

Larvae were observed in the interacinar connective tissue of the albumen gland, cephalopod mass, kidney, nidamental gland, prostate gland and near the heart (Figure 5). In group I-14D (A), there is evidence of larval profile, where two regions with cellular infiltrate can be observed, probably because the larva is C-shaped, so it is possible to see the end of the head and tail, seen vertically. In group I+E-14D (B), it is possible to observe the C-shaped larva in horizontal view, besides the larval trace with vertical view, where the end of the head and tail region can be seen, with the presence of cellular infiltrate and granuloma-like formation. In group I-21D (C), there is granuloma-like formation, without collagen deposition, where larval remnant can be seen in vertical view. In groups I+E-21D (D) and I-28D (E), it is possible to observe larval fragment, in horizontal view in group I-28, and in group I+E-28D (F) between the acini of the albumen gland it is possible to observe larval vestige, with granuloma-like formation.

4. Discussion

The effect of *E. milii* var. *hislopii* latex on *B. glabrata* infected by *A. cantonensis* was different from the effect

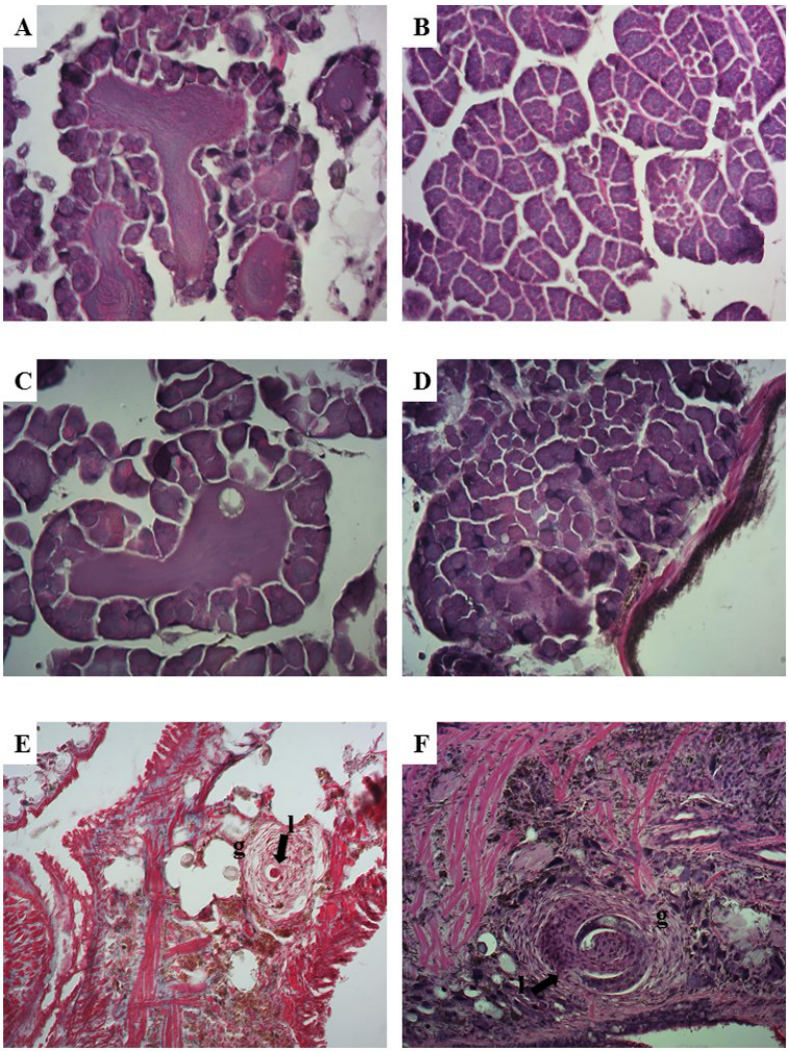

Figure 4. Histological sections of *Biomphalaria glabrata* infected with *Angiostrongylus cantonensis* and/or exposed to *Euphorbia milii* var. *hislopii* latex: A. Control-40X - albumen gland without deformation; B. Exposed-20X - albumen gland without deformation; C. Infected-1 day-40X - albumen gland without deformation; D. Infected+Exposed-1 day-40X - albumen gland without deformation; E. Infected-7 days-20X - Cellular infiltrate with granuloma-like formation (g), with evidence of larval profiles (l), no collagen present; and F. Infected+Exposed-7 days-20X - Cellular infiltrate with granuloma-like formation (g), with evidence of larval profile (l). E stained with Masson's trichrome, the others with hematoxylin and eosin.

of the same product on *B. glabrata* infected by another helminth, *Schistosoma mansoni*. This work demonstrated that the effect of the natural product varies depending on the type of parasite, cycle and parasitic strategy in the same intermediate host. When exposed to *E. milii* var. *hislopii* latex, specimens infected with *S. mansoni* showed even greater depletion of carbohydrate contents in the hemolymph and tissues, causing host death. Infection of *Biomphalaria glabrata* specimens altered the contents of carbohydrates, lipids and proteins in the hemolymph, possibly causing their death (Mello-Silva et al., 2010).

Reproductive parameters were different in *B. glabrata* exposed to the same phytochemical but infected with different parasites. Exposure to latex of *E. milii* var. *hislopii* inhibited oviposition of uninfected *B. glabrata* in the first week of observation. The same result was observed by Mello-Silva et al. (2007) and Alberto-Silva et al. (2015). However, from the second week on, the exposed mollusks produced an increasing number of eggs compared to the control snails. Thus, the latex exposure causes initial disruption of the snail's oviposition activity, but the loss occurring in the first week of exposure is offset by an increased number of eggs laid per snail in the later periods than observed in the unexposed snails.

We also observed an increase in the reproductive effort of these snails, which from the second week on produced a greater number of eggs per egg mass. These eggs were fertile, resulting in a greater number of snails hatched per egg laid, per parent snail, and per egg mass. This was the only group that showed a higher concentration

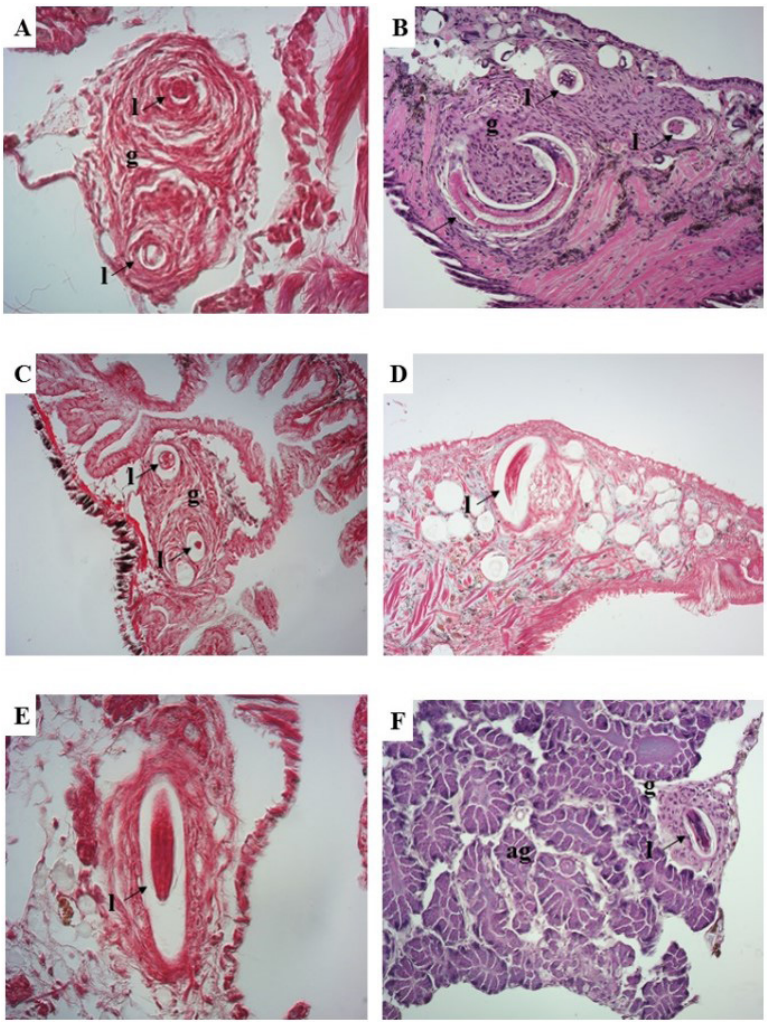

Figure 5. Histological sections of *Biomphalaria glabrata* infected with *Angiostrongylus cantonensis* and/or exposed to *Euphorbia milii* var. *hislopii* latex: A-B. With evidence of larval profile (l). A. Infected-14 days-40X - Cellular infiltrate with the granuloma-like formation (g), without the presence of collagen; B. Infected+Exposed-14 days-20X - With evidence of larval profile: C-shaped (arrow), with horizontal view and another with vertical view, presence of cellular infiltrate, with granuloma-like formation (g); C. Infected-21 days-20X - Cellular infiltrate with the granuloma-like formation (g), without the presence of collagen; D. Infected+Exposed-21 days-20X - Evidence of larval profile (l); E. Infected-28 days-40X - Cellular infiltrate with granuloma-like formation, no collagen present; and F. Infected+Exposed-28 days-20X - Evidence of larval profile (l) in the connective tissue between albumen gland acini (ag), with surrounding granuloma-like formation (g). B and F stained with hematoxylin and eosin, the others with Masson's trichrome.

of galactogen in the albumen gland than in the control snails, corroborating the previous results, providing the nutritional support to ensure an increased reproduction rate and post-exposure reproductive success. The gonadal tissues had well-preserved structures, with well-formed spermatozoa and acini containing granules of excretory material, indicating the metabolic activity of these cells. These results reinforce the idea expressed above that exposure of *B. glabrata* snails to *E. milii* var. *hislopii* latex can control neural angiostrongyliasis in a selective manner, without long-term effects on the snail population, which clearly showed the ability to recover reproductive activity by compensating for the losses suffered in the first week of infection.

The phenomenon of reproductive compensation has been observed by several authors as a strategy employed by snails after the action of some stress agent, as verified by Mello-Silva and collaborators (2007) in *B. glabrata* infected and not infected by *S. mansoni*, where three weeks after exposure to the LC_{50} (1.0 mg/L) of *E. milii* var. *hislopii* latex, there was an increase in egg production with a smaller number of egg masses, while in the second week there was a 25.14% reduction in the number of eggs.

According to Minchella and LoVerde (1981), infected hosts may respond to parasitic castration and threat to survival by increasing reproductive investment immediately after infection to compensate for future reproductive losses. These authors called this phenomenon "fecundity

compensation" or "terminal investment" (Duffield et al., 2017), in which reproductive investment aims to increase the quantity and/or quality. However, Duffield et al. (2017) pointed out that fecundity compensation occurs not only in response to parasite infection, but also occurs in response to other factors such as age, nutritional condition, and predation that can negatively impact future reproduction. Thus, the reproductive response of *B. glabrata* to exposure to *E. milii* var. *hislopii* latex also is a fecundity compensation process, by which the loss in the first week of exposure, with total interruption of oviposition activity, is offset not only with a greater number of eggs laid, but also with higher hatching and survival rates, as proposed by the terminal investment hypothesis (Williams, 1966).

In snails infected with *A. cantonensis*, the number of eggs laid per snail was lower in all weeks of observation. However, it was possible to observe an effort by the snail to sustain its reproductive output, with the production of a greater number of egg masses per snail at the end of the observation period (28 days). Despite this reproductive effort observed at the end of the pre-patent period, the eggs produced by *B. glabrata* infected with *A. cantonensis* showed low viability, with the number of snails hatched per egg laid, number of snails hatched per egg mass, and number of snails hatched per parent snail were always lower than that observed in the control group. These data are consistent with the significant reduction in the amount of galactogen in the albumen gland of infected snails, hampering egg production and viability. The low availability of nutrients in the perivitelline fluid of these eggs is one of the factors responsible for their failure to develop and hatch. The histopathological examination revealed the presence of granuloma-like structures in the gonadal region, along with vacuolization and reduction in the acinar area of this organ, demonstrating the disorganization of these tissues. Although there were gametes formed and in formation in the gonad, the tissue disorganization observed likely compromised the hemolymph flow to this region and the supply of nutrients necessary for the successful development of these cells. This together with the low concentration of galactogen resulted in low viability of the hatched snails.

Among the many forms of modulation exerted by stressors in snails, the most common are total and partial castration, reproductive compensation and increase in mortality (Minchella and Loverde, 1981; Mello-Silva et al., 2007; Faro et al., 2013; Alberto-Silva et al., 2015, 2020). All of these events were observed in our study to a greater or lesser degree. Evaluating only the infection process, Faro et al. (2013), working with the *B. glabrata/S. mansoni* model, comprehensively studied the phenomenon of parasitic castration. They analyzed the pre-patent and patent periods of infection (total of 62 days), focusing on the interference in reproductive parameters and survival. The authors observed a reduction in fecundity and fertility of the positive snails (with elimination of cercariae), where these only began oviposition after the 50th day of infection. During the experiment, there was 100% survival in all groups. Friani et al. (2022), using the same model plus exposure to 1.0 mg/L of *E. milii* latex, observed 100% survival in infected snails, 88.5% in exposed snails, and 66.6% survival in snails infected and exposed to latex.

Tunholi-Alves et al. (2011), in a study of *B. glabrata* infected by *A. cantonensis*, observed partial castration, with a reduction in the number of egg masses/infected snail compared to the control group, in addition to a reduction in the average number of eggs/egg mass. These reductions were significant in the second and third weeks after infection. In relation to hatchability there was also a reduction in the percentage of viable eggs of infected snails (85.53%) compared to the control group (97.98%).

In the present study, no morphological changes in the albumen gland region, were observed in any group, which points to an influence of the neuroendocrine/nutritional nature on the observed changes in galactogen contents. Jong-Brink (1995) reviewed the mechanisms involved in the modulation caused by schistosomatids in their snail hosts to obtain resources to ensure their larval development, focusing on the effects of *Trichobilharzia ocellata* infection in *Lymnaea stagnalis*, through interference in the functioning of caudo-dorsal cells (CDCs), which synthesize calfluxin (CaF), in turn modulating calcium influx into the digestive gland cells and thus influencing the entire oxidative energy metabolism of these cells, as well as light green cells (LGC) and pave cells (CC).These cells secrete insulin-like peptides, which modulate carbohydrate metabolism in the mollusk.

In the present study, we observed the occurrence of an intense parasitic castration process in *B. glabrata* infected with *A. cantonensis*, with no compensatory process for this loss of fecundity durin the analyzed period.

The results of first part of this study serve as a basis for analyzing the effect of the conjugation of infection of *B. glabrata* with *A. cantonensis* and exposure to *E. milii* var. *hislopii* latex on the reproductive parameters considered here.

Exposure to *E. milii* var. *hislopii* latex of *B. glabrata* infected with *A. cantonensis* reduced or interrupted oviposition in the snails of all groups. However, the dynamics observed for snails exposed one and seven days after infection were different, with no influence on the viability of eggs laid and hatchability. Even though there was a lower number of eggs laid per snail, the number of snails hatched per egg laid was equal to the values obtained for the control snails (uninfected and unexposed) in these two groups. Infection and exposure to *E. milii* var. *hislopii* latex together triggered in these two groups an effective reproductive strategy, with production of fewer egg masses containing more eggs per mass. The process reduced the energy expenditure for the production of ovigerous masses, whereby the balance was directed to gonadal nutrition and production of gametes and embryos to assure high viability.

This physiological/nutritional bias is reinforced by the absence of significant histopathological changes in the gonad of snails infected with *A. cantonensis* and exposed to *E. milii* var. *hislopii* latex, although there was encapsulation of larvae with granulomatous structure formation in this region.

The development from L1 to L3 of *A. cantonensis* led to the occurrence of reproductive changes such as tissue damage and altered glucose content. We observed

evidence of larval profiles, surrounded by granuloma-like formations, through hemocyte infiltrate in the gonad of groups I and I+E from 21 days of infection. However, these factors did not influence the occurrence of gametogenesis. Changes in the snail tissues were also not observed in the albumen gland in these groups. Additionally, the reduced galactogen content in the albumen gland of the snails infected with *A. cantonensis* and exposed to *E. milii* var. *hislopii* latex corroborates the strategy of optimizing existing resources, by producing fewer ovigerous masses and thus reducing reproductive effort, directing these resources to the production of embryos. These were fewer in the first two weeks of analysis and then reached higher numbers than in control snails in the final two weeks of analysis.

We also observe that exposure to *E. milii* var. *hislopii* latex reduced the survival of *B. glabrata* significantly in relation to the snails in the control group (unexposed). In turn, infection of the snail with *A. cantonensis* without latex led to a significant reduction in host survival at the fourth week, similarly to the exposed mollusks after 21 and 28 days.

After four weeks, the combination of infection with *A. cantonensis* and exposure to *E. milii* var. *hislopii* latex resulted in the lowest survival rates, strengthening the idea already reported by other authors that *B. glabrata* is a good host of *A. cantonensis* under laboratory conditions (Tunholi-Alves et al., 2011; Bonfim et al., 2014).

We can infer from these results that exposure to *E. milii* var. *hislopii* latex has a more pronounced effect on *B. glabrata* snails in the final weeks of infection with *A. cantonensis*, when it is already possible to find L3 larvae formed and encapsulated in the snail tissues. These findings are important for the possible use of *E. milii* var. *hislopii* latex in areas where neural angiostrongyliasis occurs, aiming at the integrated control of this disease, due to its action on the viability of infected snails in the periods when they host larvae able to infect the definitive rodent hosts and humans.

Sorensen and Minchella (2001) pointed out the intimate association between parasites and hosts, whereby the former need to optimize their ability to utilize the resources and energetic substrates of the host, while the host needs to mitigate the negative effects of this action of the parasite. Such pressures shape the co-evolutionary pathways in this parasite-host pair (Davis et al., 2021).

In this same vein, our group, led by Denis J. Minchella, recently published a paper highlighting that despite the occurrence of increased reproductive effort in the process of fecundity compensation, altering aspects of the life cycle of the parasite-host pair as a response to parasitism, it is unclear whether there is also a tradeoff between the quantity and quality of host snails' offspring (Duffield et al., 2017).

Although parasitic castration has been known since the first description by Baudoin (1975), it still lacks satisfactory elucidation. The way in which the compensation by greater fecundity in response to parasitic castration occurs, resulting in increased quantity and/or quality of offspring, is one of the points that Duffield et al. (2017) mentioned as needing further elucidation.

Alberto-Silva et al. (2020), evaluating the reproductive and locomotor parameters of *B. glabrata* when exposed to the LC_{50} (0.53 mg/L) of *E. milii* var. *hislopii* latex, also observed a significant reduction in the number of eggs and egg masses in the first week after exposure, followed by a return to normal oviposition patterns. However, hatchability continued to decline. Augusto et al. (2015) also found similar results, observing a 95% reduction in the number of eggs and even total castration in *B. glabrata* infected by *S. mansoni* and exposed to the LC_{50} (1.4 mg/L) of *E. milii* var. *hislopii* latex.

Our results allow concluding that infection by *A. cantonensis* causes a significant process of parasitic castration in *B. glabrata*, without terminal investment, as opposed to reproductive losses. However, exposure to *E. milii* var. *hislopii* latex in both exposed and uninfected (E) and exposed groups after infection with *A. cantonensis* led to a marked compensatory process, evidencing terminal investment, especially in the final observation periods used in the present study (21 and 28 days). One of the consequences of this large investment in reproductive activity was that the snails infected and exposed to the latex had the lowest survival rates at the end of the study. Selectively, latex then is able to act on snails infected by *A. cantonensis* with greater effectiveness at the end of the pre-patent period, leading to the desired effect in future integrated programs to control neural angiostrongyliasis.

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