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Behavioral response of *Biomphalaria glabrata* exposed to a sublethal concentration of *Euphorbia milii* var. *hislopii* latex

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Abstract: The *Euphobia milii* var. *hislopii* latex has been tested in the control of schistosomiasis but its action in the locomotor activity of *Biomphalaria glabrata* is unknown. The objective of this work was to study the locomotor and reproductive behaviors of *B. glabrata* exposed to *E. milii* var. *hislopii* latex. For this, 96 snails were individually exposed to the latex (LC_{50} - 0.53 mg / L) for 24 hours. The specimens were submitted to biomonitoring for image analysis to record the locomotor parameters at different times: before exposure (control), one day post exposure (group 1 d-p-e) and 30 days post exposure (group 30 d-p-e). The reproductive parameters were recorded weekly for 10 weeks. All locomotor activities of group 1 d-p-e decreased significantly. The egg/egg mass ratio decreased the week after exposure while there was an increase in the hatching rate. After 30 days, these reproductive parameters were similar to those observed in these same snails before exposure. However, the number of hatched snails declined after exposure until the end of the experiment. The influence of the latex in the parameters of *B. glabrata* added further evidence that this natural water-soluble product can be an important tool for the control of schistosomiasis.

Key words: behavior, biomonitoring, *Biomphalaria glabrata*, control of schistosomiasis, *Euphorbia milii* var. *hislopii*, freshwater environments.

INTRODUCTION

Alterations of freshwater environments are caused by contamination by organic and inorganic wastes, environmental catastrophes and climatic changes, interfering in the trophic relations and the flow of energy and biomass (Perkins et al. 2010, Ledger & Milner 2015, Woodward et al. 2016). Freshwater organisms are influenced by these environmental factors, so they are often used as bioindicators of contamination (Hook et al. 2014, Habib et al. 2016). Among the aquatic organisms used in biomonitoring programs, mollusks are relevant because they occupy intermediate trophic levels in the ecosystem and act as fundamental links in the food chain (Abílio et al. 2007). Aquatic snails have been the subject to toxicological studies because despite being tolerant to organic pollution, they are vulnerable to other pollutants even at low concentrations (Oliveira-Filho & Paumgartten 1997, Abd Allah et al. 2003).

Schistosomiasis is the main helminth-borne disease in the world and *Biomphalaria glabrata* (Say, 1818), the main intermediate host of *Schistosoma mansoni* (Sambon 1907) in Brazil, has been used as a study model due to its wide geographical distribution, long life cycle

and easy maintenance in the laboratory (Hotez et al. 2009, Scholte et al. 2014). The chemical compound most commonly used in campaigns to control these vectors is niclosamide, which causes high mortality of fish, among other non-target organisms (Vega et al. 1988). Due to the impact of this product on the environment, the Ministry of the Environment recommends its use in Brazil only for areas with high prevalence (Brasil 2006). As regards natural molluscicides, *Euphorbia milii* var. *hislopii* satisfies the recommendations of the WHO for efficiency in the control of mollusks at lethal concentrations (LC_{90}) between 1.5 mg/L and 5.0 mg/L. The latex photo degrades over a period of 30 days (Schallet al. 1992, Oliveira-Filho & Paumgartten 1997) and has no carcinogenic, mutagenic or embryotoxic effects on mammals at lethal concentrations to snails (Vasconcellos & Amorim 2003). The latex significantly reduces the elimination of *S. mansoni* cercariae by snails and has been tested as a natural product to control the transmission of schistosomiasis (Augusto et al. 2015). Recently, Augusto et al. (2016, 2017) analyzed the chemical composition of the latex and confirmed the efficacy of this latex as a natural molluscicide for schistosomiasis vector control using comparative transcriptomics and proteomics. It was proved that the latex changes the morphological structure of adult parasites obtained from cercariae exposed to the latex, thus impeding the normal development of adults.

Behavioral, reproductive and physiological changes which may occur in *B. glabrata* have been reported in different situations, such as parasitic infection, starvation, aestivation and exposure to molluscicides (White et al. 2007, Faro et al. 2013, Mello-Silva et al. 2011). In our previous paper we video-tracked the locomotor activity of *B. glabrata* before and after infection by *S. mansoni*. Their reproductive parameters were also observed the infected snails, which were eliminating cercariae were less motile and the number of cercariae shed was directly associated with the reduction/interruption in egg-laying with an increase in random movement (Alberto-Silva et al. 2015). However, these experiments did not include the use of the natural moluscicide *E. milli* var. *hislopii* latex.

The *E. milii* var. *hislopii* latex has been tested in the control of schistosomiasis but its action in the locomotor activity of *Biomphalaria glabrata* is unknown. The objective of this work was to study the locomotor and reproductive behaviors of *B. glabrata* exposed to *E. milii* var. *hislopii* latex.

MATERIALS AND METHODS

Ethics

This study was approved by the Animal Ethics Committee of Oswaldo Cruz Foundation (CEUA/IOC 016/2015), in accordance with the guidelines of the Brazilian Society of Laboratory Animal Science (COBEA). This study is registered in the National System of Management of Genetic Heritage and Associated Traditional Knowledge - SisGen (no. A9666E5).

Euphorbia milii var. *hislopii* latex

The *E. milii* var. *hislopii* latex was collected in the Ilha do Governador district (22°48´09´´S/43°12´35´´W), Rio de Janeiro, Brazil, on May 4, 2015 by the first author.

The latex *in natura* was lyophilized on 6th May of the same year and used in the trial in the months of July and August 2015, according to the method described by Augusto et al. (2016). The lyophilized pellet obtained was diluted in distilled water and homogenized by sonication (UltraSonic Clean S-1600/HM 230V, frequency of

40 kHz) for 20 min. After this, the stock solution at a concentration of 100 mg/L was prepared and from this solutions at different concentrations were prepared for use in the bioassays (0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 5.0 and 10.0 mg/L). This procedure followed the method described and employed by Vasconcellos & Amorim (2003). The sublethal concentration (LC_{50}) in this experiment was 0.53mg/L.

Raising *Biomphalaria glabrata* and exposure to *E. milii* var. *hislopii* latex

In this experiment, we used 96 specimens of *B. glabrata* (BH lineage) aged six weeks, raised in the Schistosomiasis Laboratory of Fiocruz, which has a quality policy with traceability system of all parameters of their lyfe-cycle. Therefore, ween sure that the snails used in this study had the same age, since they came from egg-laying masses of the same week. For this experiment, we monitored this group of mollusks until they reached sexual maturity around five weeks of age (young). The mollusks of this group measuring 8 to 12 mm were selected to be tested. In this 60-day period (observation time of this experiment), the mollusks are still considered young. Under optimal laboratory conditions, the mollusks have a longer life spanup to 24 months (Brasil 2008). The mean weight of the specimens was 0.34g (SD: 0.09, range 0.10 -0.27g) and the shell diameters ranged from 8 to 12 mm. The snails were individually kept in beakers (100 mL) with dechlorinated water, which was changed weekly. Pieces of styrofoam were put on the water for egg laying and also replaced weekly. The temperature remained between 25 and 28°C during the experiment. The mollusks were fed daily *ad libitum* with fresh lettuce leaves (*Lactuca sativa* L.), except on the day of exposure to *E. milii* var. *hislopii* latex and the day of analysis of locomotor activity. The snails were individually exposed to the sublethal concentration (LC_{50} - 0.53 mg / L) of the latex for 24 hours. After this period, they were placed back in the dechlorinated water.

Locomotor activity of *Biomphalaria glabrata*

The analyses of the snails' locomotor activity were performed before and after exposure to the latex, using the image analysis biomonitoring system (IABS) (Columbus Instruments, Ohio, USA) with the Travelled Distance of Multiple Objects software, according to Alberto-Silva et al. (2015). The biomonitoring was realized with the same snails at three times: before exposure (control group), one day post-exposure (1 d-p-e) and 30 days post-exposure (30 d-p-e.). Each analysis was performed for 1 hour 20 minutes, with the first 20 minutes being for acclimation and 1 hour of video analysis recorded at 60 intervals of 1 min each. All values for each interval of five parameters of locomotor activity were used for statistical analysis: distance traveled, ambulatory time, stereotypic time, resting time, and average speed (Alberto-Silva et al. 2015).

Reproductive parameters of *Biomphalaria glabrata*

The reproductive parameters were also analyzed weekly, five weeks before exposure (control group) and five weeks afterward (1 d-p-e and 30 d-p-e groups). We analyzed the following reproductive parameters: fecundity- total number of egg masses, total number of eggs, number of eggs/egg mass; and fertility- number of hatched eggs and hatching rate. All parameters were analyzed using a stereomicroscope. Styrofoam plates containing the egg masses and eggs were transferred to Petri dishes identified with the laying date, containing dechlorinated water from the original beaker where the snails were reared. The hatching rate was measured 15 days after the date of separation of the egg masses, as described by Alberto-Silva et al. (2015).

Statistical analysis

All statistical analyses were performed using the R program (R Development Core Team 2016), using the 'geepack' and 'ggplot2' packages (Højsgaard et al. 2006; Wickham 2009). The snail weight data were not normally distributed, so transformation was necessary (Shapiro-Wilk test =0.96; p<0.001). Therefore, the nonparametric Wilcoxon test was used to determine the difference in weight of the snails before and after exposure. The generalized estimation equation (GEE) was used to check the difference between the locomotor parameters of the groups. The first model (1) used each locomotor parameter as a response variable and its interaction with the individual snail weight. The second model (2) calculated the differences in the reproductive parameters of the mollusks (number of egg masses, eggs, hatched snails) before and after exposure to *E. milii* var. *hislopii* latex, using each parameter as a response variable and its interaction with individual weight. Both models were adjusted to control for excess dispersion and normalization of residuals. Finally, the goodness-of-fit statistic and quasi-likelihood information criterion were used to evaluate the models (Pan 2001). The level of significance assumed for the statistical tests was 5%.

RESULTS

The biomonitoring study started with 96 snails. After 24 h of exposure to the LC_{50} of *E. milii* var. *hislopii* latex, 54 snails died (56.25%) and 42 survived (43.75%). This experiment finished with 27 animals. The statistical analysis (GEE) did not show a relationship between weight and locomotor activity for any of the parameters analyzed, although weight gain was found in the snails between the periods before and after latex exposure (Wilcoxon test: 6,237.5, P-value < 0.001).

Before *E. milii* var. *hislopii* latex exposure (control group – n= 96), the snails had the following mean values for locomotor parameters: distance traveled 22.39 (± 13.25) mm; ambulatory time 2.03 (± 1.18) s; stereotypic time 42.72 (± 9.14) s; resting time 15.24 (± 9.55) s; and average speed 9.81 (± 3.04) mm/s. One day post latex exposure (1 d-p-e – n= 42), the snails presented the following mean values: distance traveled 1.02 (± 3.57) mm; ambulatory time 0.09 (± 0.32) s; stereotypic time12.97 (± 17.97) s; resting time 46.93 (± 18.12) s; and average speed 0.91 (± 3.44) mm/s. Comparing the two groups (control and 1 d-p-e), there was a 95.44% reduction in distance traveled, 95.5% in ambulatory time, 69.6% in stereotypic time, 90.7% in average speed and increasing 307.9% in resting time. All differences were significant (p <0.05).

The same mollusks from the 1 d-p-e group were reanalyzed in relation to locomotor activity after 30 days (group 30 d-p-e – n= 27). The mean values of the locomotor parameters were: distance traveled 25.14 (± 15.86) mm; ambulatory time 2.17 (± 1.37) s; stereotypic time 42.54 (± 11.53) s; resting time 13.93 (± 10.67) s; and average speed 9.54 (± 3.94) mm/s. Comparing the two exposed groups (1 d-p-e and 30 d-p-e), the majority of the parameters increased significantly in the 30 d-p-e group. When the movement declined, the resting time increased. However, when we compared the locomotor parameters of the 30 d-p-e group with those parameters of the same snails before exposure (control group), we did not observe a significant difference. Thirty days after exposure, the surviving snails recovered their normal locomotion ability (p > 0.005) (Fig. 1).

Regarding the reproductive parameters, no influence of the snail weight was observed (p> 0.005). Before latex exposure, the snails had the following mean values analyzed for five weeks: 2.69 (± 1.80) egg masses/snail; 29.84 (± 23.88)

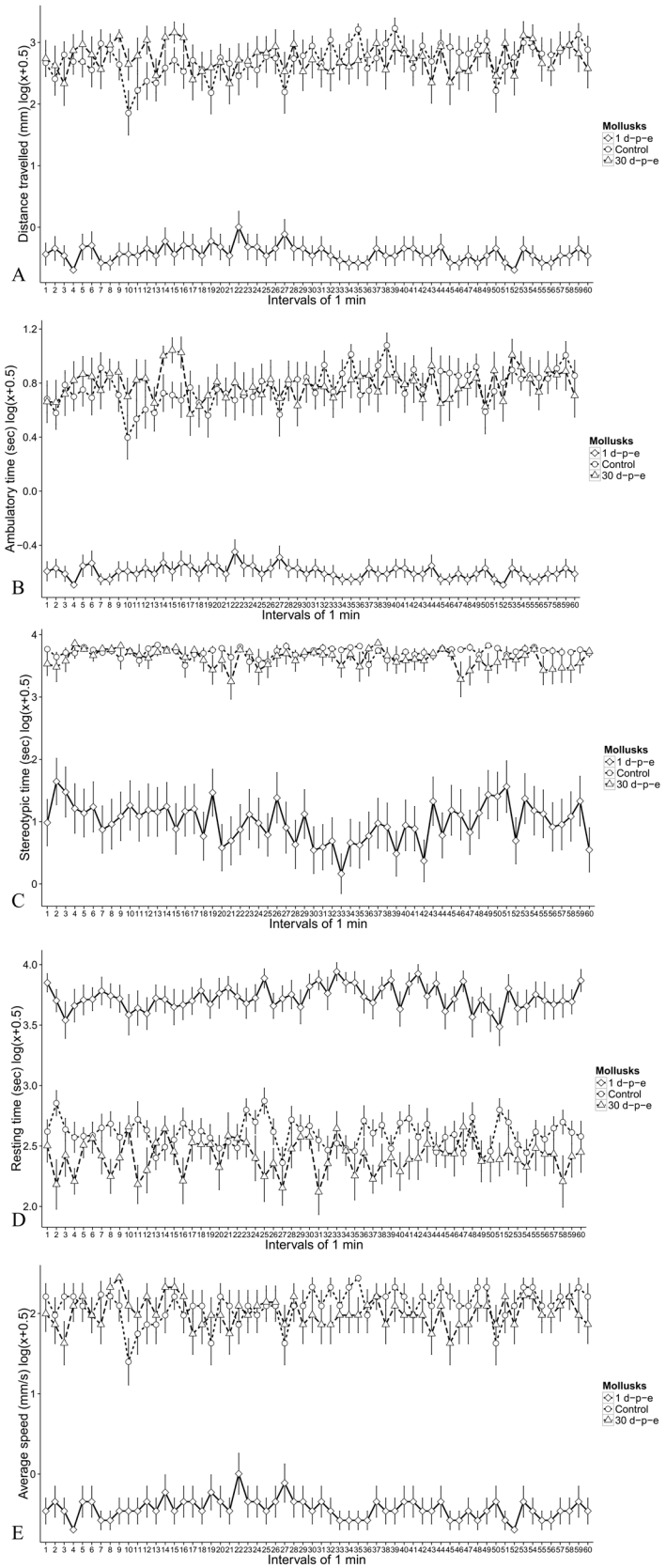

Figure 1. (a-e) Comparison between the biomonitoring graphics of *Biomphalaria glabrata* (control, 1 d-p-e. and 30 d-p-e) exposed to *Euphorbia milii var. hislopii* latex during 60 intervals of one minute: (a) distance traveled (mm), (b) ambulatory time (s), (c) stereotypic time (s), (d) resting time (s) and (e) average speed (mm/s).

eggs/snail; 11.14 eggs/egg mass; and 10.88 (± 13.54) hatched snails. There were no significant differences among reproductive parameters between first and fifth week before exposure (Egg masses: estimative: -0.00, Wald: 0.00, p >0.005; Eggs: estimative: 0.04, Wald: 0.96, p =0.33; Hatched snail: estimative: -0.04, Wald: 3.96, p =0.05).Thirty days after exposure, the mean values of the reproductive parameters were: 2.60 (± 1.78) egg masses/snail; 27.64 (± 26.48) eggs/snail; 9.99 eggs/egg mass; and 7.00 (± 9.51) hatched snails. The lowest values of reproductive parameters were found one week after exposure: 1.25 egg masses/snail; 8.62 eggs/snail; and 6.89 eggs/egg mass, in all cases significantly different from the last week before exposure and the second week after exposure. This week was also when the number of eggs laid was lowest, but when the hatching rate was highest (62%) (Fig. 2).

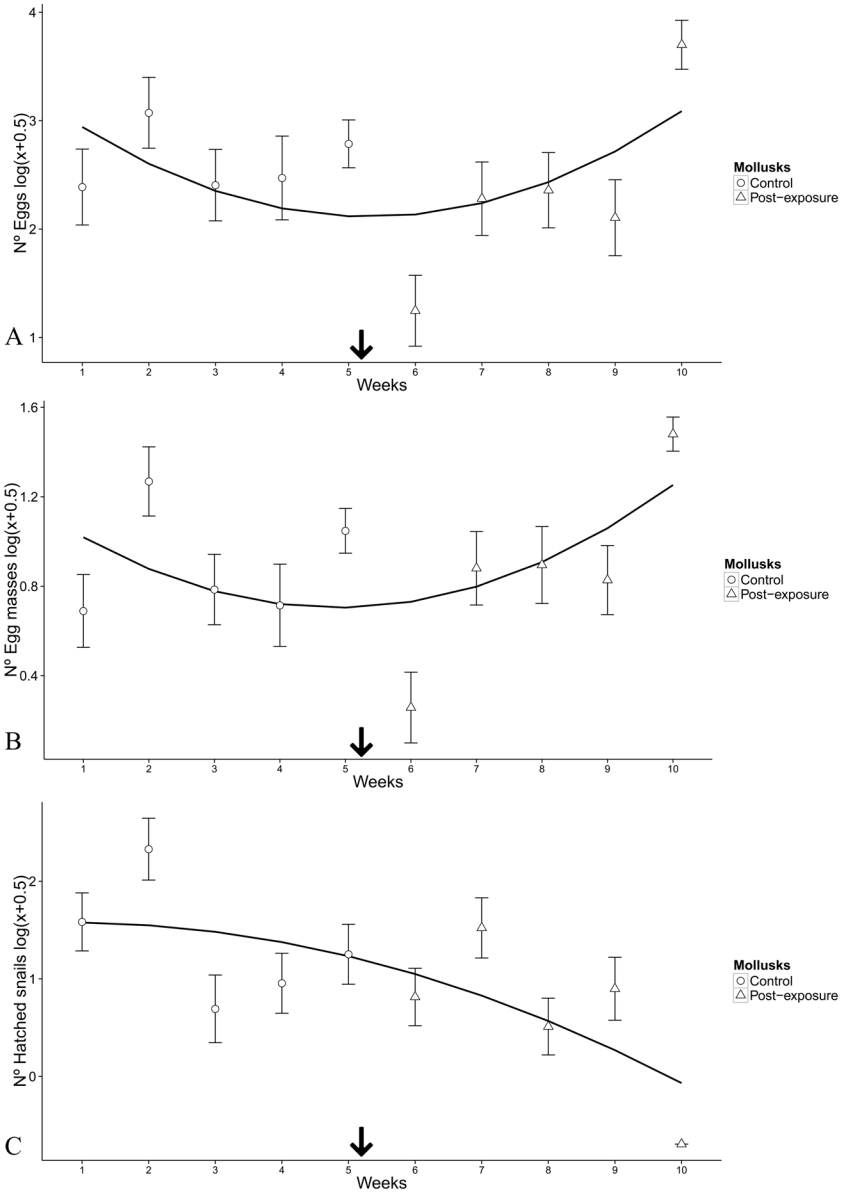

Figure 2. **(a-c) Weekly analysis of the reproductive parameters of *Biomphalaria glabrata*; Control and post-exposure to *Euphorbia milii* var. *hislopii* latex: (a) number of the eggs, (b) number of egg masses, and (c) number of hatched snails. Arrow represent day of exposure to *Euphorbia milii* var. *hislopii* latex.**

Discussion

Toxicological analysis of the effects of a molluscicidal product on the locomotor parameters of snails as intermediate hosts was first described by Sarquis et al. (1998). They verified the effect of the exposure to Bayluscide WP 70 (niclosamide) on *Biomphalaria straminea*, an intermediate host responsible for the transmission of schistosomiasis in northeastern Brazil. They observed effects on locomotor activity using only two parameters (average speed and ambulatory time). In the first three hours of action of the product, they observed a significant reduction in both parameters in the exposed group in comparison with the control group. In our study using the same method, exposure to *E. milii* var. *hislopii* latex at LC_{50} of group 1 d-p-e caused a decrease of all the locomotor parameters while resting time increased. Thus, both niclosamide and *E. milii* var. *hislopii* latex have the same effect on the locomotor system of the intermediate host, influencing its behavior. A behavioral response to *E. milii* var. *hislopii* latex has also been observed in other species of snails of medical and economic importance, as *Indoplanorbis exustus* (Deshayes, 1834) species widely distributed in the tropics and host of several trematodes (Sermsartet al. 2005), *Biomphalaria tenagophila* (d'Orbigny, 1835) intermediate host of *S. mansoni*, *Helisoma duryi* (Wetherby, 1879), *Pomacea* sp. (Oliveira-Filho & Paumgartten 2000) and *Achatina fulica* (Ferussac, 1821) (Crignis et al. 2012).

The behavior of the exotic species *Achatina fulica*, intermediate host of the nematode *Angiostrongylus costaricensis* (Morera & Cespedes 1971), which causes angiostrongyliasis, and *Angiostrongylus cantonensis* (Chen 1935), which causes eosinophilic meningoencephalitis, were evaluated in laboratory conditions 96 hours after spraying with *E. milii* var. *hislopii* latex. Contraction of cephalopedal mass, random movements and large release of mucus were observed (Crignis et al. 2012). Similar behavior was observed in our study with *B. glabrata*, where the exposed snails showed an increase the resting time, probably because they were retracted inside the shell, with consequent reduction of ambulatory and stereotypical time.

Many products released in water bodies not only cause locomotor changes in freshwater snails, they also affect their ability to produce eggs. Schall et al. (1998) observed the direct action of *E. milii* var. *hislopii* latex on *B. glabrata* egg masses, showing a lethal effect on the eggs from 870 mg/L, eight times more than recommended by the WHO as the lethal concentration for mollusks. Mello-Silva et al. (2007) studied the action of this same product in sublethal concentrations (LC_{50}) on *B. glabrata* exposed for 24 hours. In the present study, we observed a temporary reduction in egg laying in *B. glabrata* caused by latex exposure in the first week after exposure in accordance to Mello-Silva et al. (2007) which also reported the reduction of the egg laying in the first week after exposure to the *E. milii* var. *hislopii* latex. In addition, there both studies observed a reduction in the hatching rate five weeks after exposure. We evaluated the same snails before and after latex exposure, so we minimized the question of differences in egg-laying patterns among specimens of the same species, to the results are closer to what happens in freshwater habitats. Thus, the decrease in number of eggs, irregular laying parameters and formation of infertile eggs can help control the snail population, especially immediately after application of the product. This is an important fact that should be considered for the use of this product in freshwater ecosystems.

Environmental conditions directly influence the reproductive potential of *B. glabrata*. Several factors may alter the reproductive biology of

snails such as parasitic infection, starvation, and exposure to toxic products, since these factors may influence carbohydrate reserve, hemolymphatic glucose alteration, glycogen levels, and mobilization of galactogen reserves (Mello-Silva et al. 2007, Augusto et al. 2015, Faro et al. 2013). However, the snails can restore the population rapidly, from only one specimen, when the environmental conditions become favorable (Barbosa & Barbosa 1994). In our experiment, the snails were kept isolated, laying eggs by self-fertilization, an unusual feature, but important in studies aimed at control, in order to evaluate the capacity for repopulation under extreme conditions. Costa et al. (2004) compared the reproductive strategies of self-fertilization and cross-fertilization in *B. glabrata* and *B. straminea*, observing that *B. glabrata* presented higher reproductive capacity in cross-fertilization than self-fertilization. Comparing self-fertilization in both species, *B. glabrata* showed lower reproductive potential than *B. straminea*. In our study, *B. glabrata* in self-fertilization showed, on average, a greater number of egg masses and eggs but a lower hatching rate than that reported by Costa et al. (2004) in all observations except for the first week after latex exposure. At this time, egg and egg mass values were lower than in other weeks and hatching rate was 5% higher in comparison to the other weeks.

The action of *E. milii* var. *hislopii* latex resembles physiologically the parasitism by *S. mansoni* in *B. glabrata*. The two physiological stress processes, latex action of *E. milii* var. *hislopii* and parasitism by *S. mansoni*, reduce the snails' energy reserves (glucose and galactose) and temporarily prevent egg laying (Mello-Silva et al. 2007, 2011, Faro et al. 2013, Augusto et al, 2015). Regarding changes in the energy reserves, El-Ansay & Al Daihan (2006) observed that a metabolic decrease can influence locomotor parameters. The results of our experiments suggest that the reduction of the locomotor and reproductive parameters of the mollusks one day after *E. milii* var. *hislopii* latex exposure is related to changes in carbohydrate metabolism in the reserve sites. Mollusks move less and lay fewer eggs when they have less energy reserves, especially during the action of the product. When the effect of the product ends, the locomotor and reproductive parameters return to normal. Reproductive and locomotor behaviors are among the factors that interfere in the distribution and abundance of snails in freshwater environments. Changes in these behaviors are adaptations of the snails to maintain homeostasis when they are subject to a stress agent. In conclusion, exposure to the LC_{50} of *E. milii* var. *hislopii* latex temporarily altered the locomotor and reproductive parameters of *B. glabrata*, which can interfere in the balance of this population and in populations of other species of the trophic chain. However, the short time of the effects of *E. milii* var. *hislopii* latex on the snails indicates that the latex's toxic effects fade quickly, reducing the risk of environmental contamination by reestablishing the normal water quality.

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ACAS, CCMS, MCV and CPS conceived and designed the study. ACAS, VAC and RAC performed the behavioural experiments and EGNS performed the statistical analysis. CCMS, MCV and CPS revised the manuscript. All authors wrote the paper and approved the final version of the manuscript. This study is part of the Master dissertation of Anna Carla Alberto da Silva at the Postgraduate course in Ciências Veterinárias at Universidade Federal Rural do Rio de Janeiro.