ARTIGO ORIGINAL

THE INFLUENCE OF POPULATION DENSITY AND FOOD INTAKE ON THE REPRODUCTIVE BIOLOGY OF *Biomphalaria glabrata* (MOLLUSCA) AND CALCIUM PROPORTION IN SNAILS EXPERIMENTALLY INFECTED WITH *Schistosoma mansoni* (TREMATODA)

Ronaldo de Carvalho Augusto, [1 e 2] *Aline Cristina da Silva Magalhães* [2] *and Clélia Christina Mello-Silva* [2]

ABSTRACT

The aim of this study was to evaluate the influence of population density and food intake on the survival and reproduction of uninfected *Biomphalaria glabrata* and the effect of calcium carbonate availability on cercarial emergence from experimentally infected snails, to define conditions to maximize snails' breeding and cercarial production for future studies about this model. The results observed in this study indicate that increased population density has a negative effect on the survival and reproductive activity of *B. glabrata.* In addition, the quantity of lettuce offered to the snails altered the number of eggs laid per snail. There was a significant relation between the amount of lettuce eaten per day and the number of eggs produced per snail, as well as the number of egg masses per snail, and eggs per egg mass. Furthermore, the snail's survival was directly associated with the amount of calcium carbonate and the cercarial emergence was inversely related to the calcium carbonate amount. This study might help to understand the influence of population density and food intake on the reproductive biology of captive snail populations. In relation to cercarial emergence, calcium supplies must not be provided to *Schistosoma mansoni*-infected snails while maintaining *S. mansoni* under laboratory conditions because it decreases cercarial emergence.

KEY WORDS: *Biomphalaria glabrata. Schistosoma mansoni.* Survival rate. Egg-laying rate. Cercarial shedding.

RESUMO

A influencia da densidade populacional e a ingestão de alimentos na biologia reprodutiva da *Biomphalaria glabrata* (Mollusca) e da quantidade de cálcio em caramujos experimentalmente infectados com *Schistosoma mansoni* (Trematoda)

O presente trabalho teve por objetivo avaliar a influência da densidade populacional e da ingestão de alimento na sobrevivência e atividade reprodutiva de *Biomphalaria glabrata* não infectada e o efeito

1 Curso de Pós-Graduação em Ciências Veterinárias, Departamento de Parasitologia Animal, Instituto de Veterinária, Universidade Federal Rural do Rio de Janeiro, Km7, BR 465, Antiga Estrada Rio - São Paulo, 23890-000 Seropédica, RJ, Brazil.

2 Laboratório de Esquistossomose Experimental, Instituto Oswaldo Cruz, Fiocruz, Brazil.

Corresponding author: Clélia Christina Mello-Silva. E-mail: clelia@ioc.fiocruz.br

Recebido para publicacao em: 23/11/2011. Revisto em: 20/3/2011. Aceito em: 25/3/2012.

do carbonato de cálcio na emergência de cercárias de caramujos experimentalmente infectados, a fim de definir condições para maximizar a criação e a produção de cercárias em futuros estudos sobre esse modelo. Os resultados observados nesse estudo indicam que o aumento da densidade populacional tem efeito negativo sobre a sobrevivência e atividade reprodutiva de *B. glabrata* e a quantidade de alface fresca oferecida aos caramujos altera o número de ovos postos por molusco. Foi observada correlação significativa entre a quantidade de comida ingerida por dia e o número de ovos produzidos por molusco, bem como o número de massas ovígeras e ovos por massa ovígera. Além disso, a sobrevivência dos caramujos infectados foi diretamente associada a quantidade de carbonato de cálcio e a emergência de cercárias foi inversamente proporcional a quantidade de carbonato de cálcio. Esse estudo auxilia na compreensão da influência da densidade populacional e a ingestão de alimento na biologia reprodutiva de moluscos mantidos em colônias. Em relação à emergência de cercárias, suplementos de cálcio não devem ser adicionados nos criadouros de caramujos infectados com *Schistosoma mansoni*, tendo em vista que essa ação diminui a quantidade de cercárias eliminadas por caramujo.

DESCRITORES: *Biomphalaria glabrata. Schistosoma mansoni.* Taxa de sobrevivência. Taxa de oviposição. Eliminação de cercárias

INTRODUCTION

Among the parasites transmitted by species of the *Biomphalaria* genus, the most often investigated is *Schistosoma mansoni* (Sambon, 1907), due to its medical and veterinary importance. *Biomphalaria glabrata* (Say, 1818), *B. tenagophila* (Orbigny, 1935) and *B. straminea* (Dunker, 1848) are the intermediate host species found naturally infected in Brazil. *Biomphalaria glabrata* is commonly used in experimental studies due to its favorable characteristics, such as high reproductive potential (Costa et. al., 2004), ability to survive under different stress conditions (Schall et al., 1998; Mello-Silva et al., 2010; 2011), susceptibility to isolated and/or mixed infections (Coelho et. al., 2008), high rate and prolonged elimination of infective trematode larvae and tolerance to broad ranges of water conductivity, calcium, sodium and potassium chloride ions, carbon dioxide, nitrogen (ammonia), dissolved oxygen, turbidity, temperature and pH (Silva et. al., 2006). All of these factors facilitate the maintenance of experimental populations in the laboratory, as well as the use of this species as an intermediate host to parasites of the Strigeidae, Echinostomatidae, Derogenidae (Souza et. al., 1998), Angiostrongylidae (Richards, 1967) and Schistosomatidae families.

Studies of the biological aspects and behavior of snails in the laboratory are important to improve breeding and management techniques so these animals may be used in experimental studies with parasites (Almeida & Bessa, 2001). Among the important aspects for maintenance of productive snail colonies, two factors – survival and reproduction – should be particularly considered, because of their influence on the maintenance cost and viability of the specimens raised. In infected snails, the cercariae production is considered as indicative of the quality of maintenance of the *S. manson*i life cycle under laboratory conditions.

Nevertheless, studies of the bio-ecological factors associated with optimization of the resources employed in managing snails infected and not infected with *S. mansoni* in the laboratory are scarce in the literature. The aim of this work was to assess how population density and food intake affect the survival and reproduction of *B. glabrata* and to evaluate the influence of the availability of calcium carbonate on cercariae production by infected snails, under laboratory conditions.

MATERIAL AND METHODS

Description of the breeding conditions

This study was carried out in the Schistosomiasis Experimental Laboratory, Instituto Oswaldo Cruz (Fiocruz, Rio de Janeiro, Brazil). The uninfected adult snails were maintained in polyethylene aquariums measuring 30 x 5.5 x 19 cm with capacity of 6 liters and the infected snails were kept in polyethylene boxes measuring 30 x 20 x 12cm with capacity of 3 liters. All the aquariums and boxes were cleaned weekly and the snails were counted. The laboratory temperature was maintained between 25º and 28 ºC. The water was dechlorinated by decantation, as suggested by the Brazilian Health Ministry (Ministério da Saúde, 2007).

Maintenance of Schistosoma mansoni *life cycle under laboratory conditions*

Biomphalaria glabrata snails, measuring 8-10 mm in shell diameter, were experimentally infected with *S. mansoni* by individual exposure to 6 – 8 miracidia per snail, as described by Mello-Silva et al. (2007). Screenings for positive snails started on the 35th day after exposure to the miracidia and was repeated once a week for five weeks thereafter. For screening, the snails were isolated in vials with 5 mL of dechlorinated water and exposed to light (incandescent lamp, 60 watts for 1 hour) to induce cercarial emergence. Three aliquots (0.5 mL of water with cercariae) were distributed into glass plates with lugol and counted with the aid of a stereoscopic microscope. The negative snails, after the observation period, were sacrificed by crushing between glass plates.

Experiments to evaluate reproductive fitness

Two experiments were performed to determine the effect of population density and food intake on live snails and reproductive fitness to optimize the breeding of this snail species.

Experiment 1- Population Density

In this experiment, 2,080 *B. glabrata* specimens were used, separated into eight groups, with four repetitions for each. All the snails were maintained in

the same volume of dechlorinated water (6L) and fed with fresh lettuce leaves *ad libitium* (*Lactuca sativa*, Linnaeus) three times a week. The relation of density by group was: Group A- 5.0 snails per liter (30 snails) ; Group B- 6.6 snails per liter (40 snails); Group C – 8.3 snails per liter (50 snails); Group D- 10.0 snails per liter (60 snails); Group E – 11.6 snails per liter (70 snails); Group F- 13.3 snails per liter (80 snails); Group G- 15 snails per liter (90 snails) and Group H- 16.6 snails per liter (100 snails). This experiment was conducted during four months.

Experiment 2- Food intake

Five aquariums with 30 snails each were provided daily with controlled quantities of lettuce (*Lactuca sativa L.*). The following proportions of lettuce per snail were used: Aquarium 1 = 0.06g/snail; Aquarium 2 = 0.1g/snail; Aquarium 3 = 0.13g/snail; Aquarium 4 = 0.16g/snail; and Aquarium 5 = 0.2g/snail. The unconsumed lettuce leaves were removed and disposed in appropriate containers. All the snails were reared in the same volume of dechlorinated water (6L). This experiment lasted four months.

Experiment to evaluate cercarial emergence from infected snails

The experiment was carried out using different amounts of calcium carbonate to assess the influence of the calcium ion concentration on cercarial emergence. Seven hundred and twenty one-day-old snails were divided into three groups, two groups with 210 snails each, raised with 60 and 80 mg/L calcium carbonate supplement, and one group maintained as control without calcium carbonate supplement. All snails were maintained for two months and the cercarial emergence was observed weekly for three weeks to count the cercariae. The quantification of cercariae per snail was performed by exposure to artificial light (60 watts) for one hour. The cercariae were collected in aliquots of 10% (0.5ml) of the total sample (5mL), stained and fixed with lugol and the results were recorded.

Statistical analysis

The results were expressed as mean with standard deviation and submitted to one-way ANOVA and the Tukey-Kramer test (α=5%). (Instat, GraphPad, v.4.00, Prism, GraphPad, v.3.02, Prism Inc.).

RESULTS

Experiments to evaluate reproductive fitness

In general, there was a negative relationship between the population density and reproductive rate in snails from different groups: the groups with lower

population density produced more eggs per snail (Figure 1). However, there was a significant difference in relation to eggs laid per snail: the groups with lower population densities (Groups B and C) differed significantly from those with higher population density (Groups G and H).

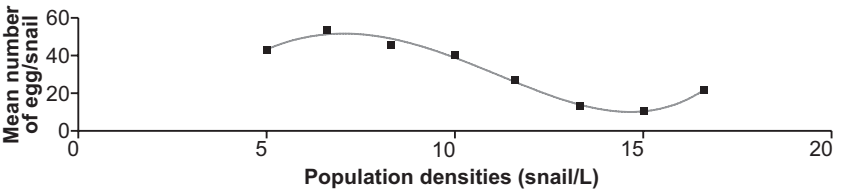

Figure 1. Relation between the mean number of egg laid by *Biomphalaria glabrata* and the different population densities, expressed as number of snail/ liters. Polynomial: Fourth Order Y= -59.1+32.3x-2.1x²-0.06x³0.004x4, $R^2 = 0.99$.

The relation between population density and number of eggs per egg mass indicates a possible negative influence of crowding on the snails' reproduction. These results reinforce the observations on egg production, because the snails in the groups with lower density had higher results than those with greater density. Group A (5.0 snails/liter) produced 13.19 eggs/egg mass, 10.6% higher than Group B (6.6 snails/liter) and 30.6% more than Group H (16.6 snails/liter) (Table 1).

Table 1. Reproductive parameters of *Biomphalaria glabrata* maintained in different population densities lasted four months. Data expressed as mean and standard deviation.

Snails/ L	Eggs/Snails	Egg masses/Snails	Eggs/egg masses
Group A 5,0 snails/L	10.6±1.1[a,b]	3.2±2.2[a]	13.3±1.9[a]
Group B 6.6 snails/L	29.8±4.6[a]	10.1±2.9[b]	5,3±1.5[a,c]
Group C 8.3 snails/L	11.4±0.4[a,c]	4.6±6.6[a]	9.9±0.7[a,b]
Group C 10.0 snails/L	10.0±0.4[a,b]	4.3±5.6[a]	9.2±0.5[a,b]
Group D 11.6 snails/L	6.7±0.4[a,b]	3.2±5.4[a]	8.3±0.2[b]
Group E 13.3 snails/L	3.2±0.3[b,c]	1.3±3.1[a,c]	9.7±1.4[a,b,c]
Group F 15.0 snails/L	2.6±0.4[b]	1.2±5.1[a,c]	8.4±0.4[c]
Group G 16.6 snails/L	5.4±0.2[b]	2.4±7.4[a]	9.1±0.7[a,b,c]

a, b, c Means differ significantly (a = 5%).

The snails' survival was also directly affected by the population density. In the groups with the two lowest densities (Groups A and B), the survival rate was 90% at the end of the experiment, while in Group H, with the highest density, only 21% of the snails survived.

The quantity of lettuce given to the snails altered the number of eggs laid per snail. There was a significant relation between the amount of lettuce eaten per day and the number of eggs produced per snail, as well as the number of egg masses per snail and eggs per egg mass (Figure 2). However, there was a significant increase in relation to egg laid by snail between Group 1 (0.06g/ snail) and the other groups. The snails in Group 1 produced an average of 0.47 eggs for 0.06 eggs mass per snail, while in Group 5 – 0.2g/snail; the snails produced 22.95 eggs in 1.1 egg masses per snail. The number of egg masses per snail increased in Groups 1 to 5, for the remaining groups it declined 7.7% (Table 2). There was no significant difference between the amount of lettuce offered to the snails and food intake, throughout the observation period (Figure 3). The snails' survival was not affected by the quantity of lettuce given. In the group with the lowest proportion of lettuce per snail (0.06g/ snail), the survival rate was 28% at the end of the experiment, while in the group with the highest proportion lettuce per snail, the survival rate was 29.5%.

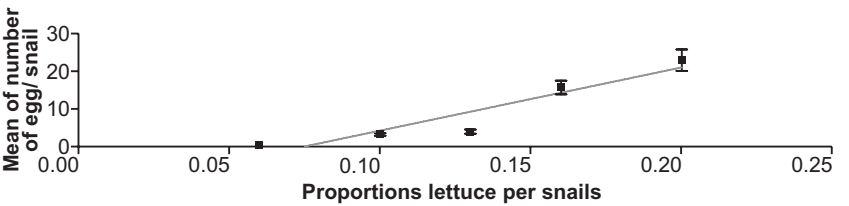

Figure 2. Relation between the mean number of egg laid by *Biomphalaria glabrata* and the different quantities of lettuce (Lactuca sativa L.), expressed as proportions lettuce per snails (g/snail). Polynomial : First Order: $Y = 0.69+5.01x$, $R^2=0.88$

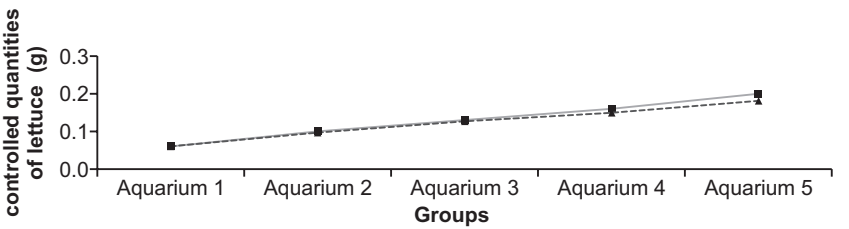

Figure 3. Proportions available of lettuce (*Lactuca sativa L.*) per snails (▪ , line) and food intake (▲ , dotted line).

Table 2. Reproductive parameters of Biomphalaria glabrata exposed to differents quantities of lettuce (Lactuca sativa L.) lasted four months. Data expressed as mean and standard deviation.

Amount lettuce/snails/Day	Eggs/snail	Egg masses/snail	Eggs/egg mass
Group 1 0.06g/ snail	0.47±0.6[a]	0.06±2.7[a]	4.8±3.3[a]
Group 2 0.1g/snail	3.24±1.8[a,b]	0.26±4.9[a]	12.3±4.6[a]
Group 3 0.13g/snail	3.99±3.0[a,b]	0.35±9.4[a]	12.1±2.7[a]
Group 4 0.16g/snail	15.7±1.0[b]	1.19±2.7[b]	13.0±0.8[a]
Group 5 0.2g/snail	22.95±1.5[b]	1.1±35.6[b]	12.0±0.1[a]

a, b Means differ significantly (a = 5%).

Table 3. Effects of cercariae shedding by Biomphalaria glabrata infected with Schistosoma mansoni and exposed a 60 and 80 mg CaCO3/liter in three analyses. Data expressed as mean ± standard deviation

Quantities of CaCO3	First analysis (40 days after infection)	Second analysis (47 days after infection)	Third analysis (54 days after infection)
Control	490±41.03[a]	345.0±15.08[a]	945±17.18[a]
60mg CaCo3/ liters	101.23 ±7.76[a]	184.74±7.3[a]	184.48±8.04[b]
80mg CaCo3/ liters	229.74±5.4[a]	179.48±10.29[a]	195.48±17.42[b]

a, b Means differ significantly (a = 5%).

Experiment to evaluate cercarial emergence from infected snails

Changes in the cercarial emergence by *B. glabrata* infected with *S. mansoni* were probably influenced by calcium carbonate amount in relation to the control group (without $CaCO_3$) throughout the period analyzed (Figure 4).

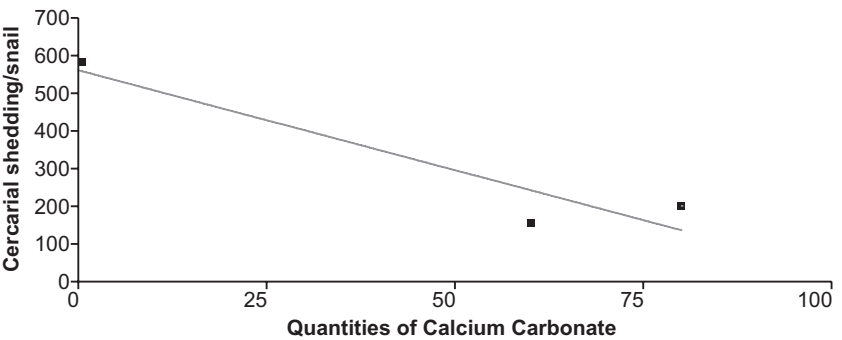

Figure 4. Relation between the mean number of cercarial emergence of *Schistosoma mansoni* and the different quantities of calcium carbonate.

The number of cercariae was 75.5% higher compared to the control group and the group submitted to 60mg/liter and 39% greater than the group submitted to 80mg/liter in the first week of analysis (Table 3). In the third week of analysis, the difference in cercarial production between the control group and those maintained with 80 mg of $CaCO_3$/liter was 73%. Significant differences between the cercarial shedding by snails in the control group and the other groups were observed in the third week of analysis.

The snails' survival was directly associated with the concentration of calcium carbonate. At the end of this experiment, in the control group (without $CaCO_3$), 60 infected snails survived (85.7%) and they were all positive (100%). In the group maintained with 60mg $CaCO_3$/liter, 26.6% died and 88.6% (39 snails) were positive, whereas in the group maintained with 80 mg $CaCO_3$/liters there were 44 snails remaining (26.6% mortality) and 97.7% (43 snails) positive.

DISCUSSION

Studies of the improvement of techniques to breed snails in the laboratory are important to clarify what conditions allow these organisms to best allocate their energy reserves and thus maximize their reproductive fitness. The storage and mobilization of resources for reproduction in snails are associated with different life history strategies, selected in the course of evolution (Antkowiak & Chase, 2003). After hatching, the energy stores are mainly used to maintain vital functions and the excess is directed to growth. After sexual maturity, growth slows down so that all the excess energy can be allocated to reproductive functions (Carvalho et al., 2008).

The results observed in this study indicate that increasing population density has a negative effect on the survival and reproductive activity of *B. glabrata*. According to Lande et al. (2002), in nature the intra-specific population density is a critical ecological factor that directly affects the growth, survival and fecundity of snails, with rising density having negative consequences on the population dynamic and maintenance in the environment. Mangal et al. (2010) suggested that the effects observed in *B. glabrata* maintained at high densities can be a result of competition for food resources, oxygen and/or calcium depletion and the production of products that inhibit growth or of toxic products dispersed in the water. However, Daguzan & Verly (1989), studying land snails, suggested that the large quantity of mucus produced when snails are subject to high densities is responsible for inhibiting the growth and reproduction of *Bradybaena similaris*.

Some authors have shown the preference of the snails for lettuce compared to other types of food, like fish food or synthetic compounds. Selck et al. (2006) in order to determine the highest growth rates in the colony, suggested fresh lettuce *ad libitum* instead of other food types. This preference for lettuce appears to be associated with preference of invertebrate herbivores for foods that contain higher amounts of nitrogen in their composition (Barilie et al., 2004).

In *B. glabrata*, calcium has a direct influence on shell growth, fecundity, oviposition, survival and maintenance of the internal metabolism, acting as a buffer system. Calcium is mainly located in the shell and digestive gland (Wilbur & Tompa, 1979). Snails obtain calcium ions from their food and lack of calcium resources in the environment can limit the growth of populations because this ion is essential to the egg shell formation and embrionary shell (Davies & Erasmus, 1984). When snails are maintained under physiological stress factors, calcium is mobilized from the shell to the hemolymph in the form of calcium carbonate ($CaCO_3$), which breaks down, leading to the formation of a bicarbonate buffer system, thus preserving the organism's homeostasis (De Witt & Sminia, 1980; Magalhães et al., 2011). This ion also affects the immune response of gastropods, since the phagocytic activity of the hemocytes and other defense cells depends on the presence of these ions in the hemolymph (Souza and Andrade, 2006). Calcium is often added to the diets of snails raised in the laboratory, but there is no study about the ideal proportion of calcium per liter of water to optimize their survival.

In these snails, calcium directly influences the shell composition, fecundity, oviposition, survival, development of eggs and embryos, mortality and maintenance of internal homeostasis (Thomas et al., 1974, Dawies & Erasmus, 1984, Magalhães et al., 2011). This ion may influence intramoluscal larval development so there is lower emergence of cercariae, as observed in this experiment, due to its influence on the production of defense cells of mollusks, the hemocytes, interfering with the susceptibility and resistance to infection by *S. mansoni*.

CONCLUSION

The success of snails reared under laboratory conditions for research purposes is directly related to their health and number of sexually mature specimens, because the younger and more fecund, the fitter they are for use in experiments and the more animals are produced. This study can help to estimate how the population density and food intake affect the reproductive age and survival in the laboratory of snail populations. In relation to cercarial emergence, the shedding was inversely associated with the number of cercariae. Therefore, in line with the aim of laboratory breeding, calcium should not be used as a supplement for infected snails, because the number of cercariae released decreases.

REFERENCES

1. Almeida MN, Bessa ECA. Estudo do crescimento e da reprodução de *Bradybaena similaris* (Férussac) (Mollusca, Xanthonychidae) em laboratório. *Rev Bras Zool 18*: 1115-1122, 2001.
2. Antkowiak T, Chase R. Sensory innervation of the ovotestis in the snail *Helix aspersa*. *J Exp Biol 206*: 3913-3921, 2003.
3. Barile PJ, Lapointe BE, Capo TR. Dietary nitrogen availability in macroalgae enhances growth of the sea hare *Aplysia californica* (Opisthobrancia: Anaspidea). *J Exp Mar Biol Ecol 303*: 65-78, 2004.

4. Carvalho OS, Amaral RS, Dutra LV, Scholte RGC, Guerra MAM. Distribuição espacial de *Biomphalaria glabrata, B. straminea e B. tenagophila*, hospedeiros intermediários do *Schistosoma mansoni* no Brasil. In: OS Carvalho, PM Zech Coelho, HL Lenzi (eds.), *Schistosoma mansoni e esquistossomose: uma visão multidisciplinar*. Editora Fiocruz: Rio de Janeiro, 2008. p. 1124.
5. Coelho PMZ, Andrade ZA, Borges CMC, Ribeiro F, Barbosa L. Evolução do *Schistosoma mansoni* no hospedeiro intermediário. In: Carvalho, O. (org.) *Schistosoma mansoni & Esquistossomose: Uma visão multidisciplinar*. Rio de Janeiro: Editora FIOCRUZ, 2008. p.147-160.
6. Costa MJFS, Grault CE, Confalonieri UEC. Comparative study of the fecundity and fertility of *Biomphalaria glabrata* (Say, 1818) and *Biomphalaria straminea* (Dunker, 1848) in a laboratory through self-fertilization and cross-fertilization. *Rev Inst Med Trop São Paulo 46*: 157-163, 2004.
7. Daguzan J, Verly D. Etude experimentale de léffet de la densite sur la reproduction de l'escargot petit-gris (*Helix aspersa* Muller). *Haliotis 19*: 105-115, 1989.
8. Davies TW, Erasmus DA. An ultrastructural study of the effect of parasitism by larval *Schistosoma mansoni* on the calcium reserves of the host, *Biomphalaria glabrata*. *Cell Tissue Res 236*: 643-649, 1984.
9. De Witt ND, Sminia T. The effects of the nutritional state and the external calcium concentration on the ionic composition of the hemolymph and on the calcium cells in the pulmonate freshwater snail *Lymnaea stagnalis*. *Proc K Ned Akad Wet 83*: 217-227, 1980.
10. Lande R, Engen S, Saether BE. Estimating density dependence in time-series of age-structured populations. *Phil Trans R Soc B 357*: 1179-1184, 2002.
11. Magalhães AC, Pinheiro J, Mello-Silva CC. A mobilização do cálcio em Biomphalaria glabrata exposta a diferentes quantidades de carbonato de cálcio. *Rev Patol Trop 40*: 46-55, 2011.
12. Mangal TD, Paterson S, Fenton A. Effects of Snail Density on Growth, Reproduction and Survival of *Biomphalaria alexandrina* Exposed to *Schistosoma mansoni*. *J Parasitol Res 1*: 1-6, 2010.
13. Mello-Silva CC, Vilar MM, Bezerra JCB, Vasconcellos MC, Pinheiro J, Rodrigues MLA. Reproductive activity alterations on the *Biomphalaria glabrata* exposed to *Euphorbia splendens* var. *hislopii* latex. *Mem Inst Oswaldo Cruz 102*: 671-674, 2007.
14. Mello-Silva CC, Vilar MM, Bezerra JCB, Vasconcellos MC, Rodrigues MLA, Pinheiro J. The influence of exposure to *Euphorbia splendens* var. *hislopii* látex on the concentrations of total proteins and nitrogen products in *Biomphalaria glabrata* infected with *Schistosoma mansoni*. *Acta Trop 117*: 101-104, 2011.
15. Mello-Silva CC, Vilar MM, Vasconcellos MC, Pinheiro J, Rodrigues MLA. Carbohydrate metabolism alterations on the *Biomphalaria glabrata* infected with *Schistosoma mansoni* and exposed to *Euphorbia splendens* var. *hislopii* latex. *Mem Inst Oswaldo Cruz 105*: 492-495, 2010.
16. Ministério da Saúde. *Vigilância e Controle de moluscos de Importância Epidemiológica*. 2nd edition. Brasília-DF. 2007.
17. Richards CS. Estivation of *Biomphalaria glabrata* (Basommatophora: Planorbidae): associated characteristics and relation to infection with *Schistosoma mansoni*. *Am J Trop Med Hyg 16*: 797-802, 1967.
18. Schall VT, Vasconcellos MC, Souza CP, Baptista DF. The molluscicidal activity of Crown of Christ (*Euphorbia splendens var. Hislopii*) latex on snails acting as intermediate hosts of *Schistosoma mansoni* and *Schistosoma haematobium*. *Am J Trop Med Hyg 58*: 7-10, 1998.
19. Selck H, Aufderheide J, Pounds N, Caspers CSN, Forbes V. Effects of food type, feeding frequency, and temperature on juvenile survival and growth of *Marisa cornuarietis* (Mollusca: Gastropoda). *Invert Biol 125*: 106-116, 2006.
20. Silva PB, Barbosa CS, Pieri OS, Travassos A, Florencio L. Aspectos físico-químicos e biológicos relacionados à ocorrência de *Biomphalaria glabrata* em focos litorâneos da esquistossomose em Pernambuco. *Quim Nova 29*: 901-906, 2006.
21. Souza CP, Lima LC, Jannoti-Passos LK, Ferreira SS, Guimarães CT, Vieira IBF, Junior RM. Moluscos límnicos da microrregião de Belo Horizonte, MG, com ênfase nos vetores de parasitoses. *Rev Soc Bras Med Trop 31*: 449-456, 1998.
22. Souza SS, Andrade ZA. On the origin of the *Biomphalaria glabrata* hemocytes. *Mem Inst Oswaldo Cruz 101*: 213-218, 2006.
23. Thomas JD, Benjamin M, Lough A, Aram RH. The effects of calcium in the external environment on the growth and natality rates of *Biomphalaria glabrata* (Say). *J Anim Ecol 43*: 839-860, 1974.
24. Wilbur K, Tompa AS. Physiological changes in Gastropods during egg shell calcification. *Malacologia 18*: 561-562, 1979.