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## INFLUENCE OF FOOD TYPE AND CALCIUM SUPPLEMENTATION ON GROWTH, OVIPOSITION AND SURVIVAL PARAMETERS OF *Biomphalaria glabrata* AND *Biomphalaria straminea*

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Wandklebson Silva da Paz<sup>1</sup>, Rosália Elen Santos Ramos<sup>1</sup>, Dharlilton Soares Gomes<sup>1</sup>, Letícia Pereira Bezerra<sup>1</sup>, Laryssa Oliveira Silva<sup>2</sup>, Tatyane Martins Cirilo<sup>1</sup>, João Paulo Vieira Machado<sup>2</sup> and Israel Gomes de Amorim Santos<sup>2</sup>

### ABSTRACT

Schistosomiasis is a parasitic disease caused by *Schistosoma mansoni* whose intermediate host is the snail of the genus *Biomphalaria*. This snail is geographically widespread, making the disease a serious public health problem. The purpose of this study was to analyze the growth, reproductive rates and mortality of *B. glabrata* and *B. straminea* in different calcium concentrations and food types. Freshly hatched snails stored in aquariums under different dietary and calcium supplementation programs were studied. Under these conditions, all planorbids survived, so there was no mortality rate and 79,839 eggs of *B. straminea* and 62,558 eggs of *B. glabrata* were obtained during the 2 months of oviposition. The following conditions: lettuce + fish food and lettuce + fish food + powdered milk resulted in the highest reproductive rates. In addition, supplementation with calcium carbonate and calcium sulfide in three different concentrations did not significantly influence the amount of eggs or ovigerous masses. Thus, this study shows that changes in diet are crucial for the survival/oviposition of these planorbids, being an important study tool for population control. Calcium is also a key factor in these conditions, but more work is necessary to better assess its effect on snail survival.

KEY WORDS: Laboratory breeding; *Biomphalaria glabrata*; *Biomphalaria straminea*; food type; calcium concentration.

### INTRODUCTION

In Brazil, Schistosomiasis is a disease caused by *Schistosoma mansoni* a digenetic trematode transmitted to humans (definitive hosts) from snails of the *Biomphalaria* genus (Carvalho et al., 2018).

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1. Programa de Pós-Graduação em Biologia Parasitária, Universidade Federal de Sergipe (UFS), São Cristóvão, SE, Brazil.

2. Laboratório de Parasitologia Humana e Malacologia, Universidade Estadual de Alagoas (UNEAL), Santana do Ipanema, AL, Brazil.

Corresponding author: Wandklebson Silva da Paz. Laboratório de Parasitologia Humana e Malacologia, Universidade Estadual de Alagoas, BR 316, km 87.5, Santana do Ipanema, AL, CEP: 57.500-000. E-mail: wandklebson.paz@gmail.com

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Until now, three species of these naturally infected planorbids have been found, namely: *Biomphalaria glabrata*, *Biomphalaria straminea* and *Biomphalaria tenagophila* (Katz, 2018).

*B. glabrata* is the most susceptible specie to *S. mansoni* infection and its presence is usually associated with the occurrence of the disease; *B. straminea* presents low susceptibility, but this specie is well adapted to the country's environment; and *B. tenagophila* is of epidemiological importance only in some cities in the South and Southeast of Brazil (Teles, 1996; Brasil, 2008; Carvalho et al., 2018).

Physiologically, several environmental factors may affect these snails, favorably or not. One of the factors influencing on their physiology is food and calcium availability, which are essential to their metabolic regulation and shell formation. These snails absorb calcium carbonate dissolved in water or aquatic macrophytes on which they feed (Marxen, 2003; Silva et al., 2006; Magalhães et al., 2011).

Shell size is an important determinant of the snails' fertility, so calcium and food type are crucial factors. They can survive on a lettuce only diet, but growth and the number of eggs both increase with diet supplementation, mainly with protein (Eveland & Ritchie, 1972).

Chieffi (1975), tested several food combinations on the fertility and size of *B. glabrata* and found that a protein-rich diet increased growth and fertility rates in adult snails. The same pattern was reported in laboratory-bred snails of the genus *Helisoma* (Andrade et al., 1978).

Augusto et al. (2009) demonstrated that the amount of food offered, also influences fertility, even when there is no protein supplementation. Snails of the species *B. glabrata* had better reproductive rates in aquariums when offered higher amounts of lettuce and concentration of calcium carbonate.

The standardization of methods that recreate the environmental conditions in the laboratory in which these snails live, will allow to better understand their standards of living and behavior. This can optimize techniques that favor the population increase of these molluscs, aiming to carry out a greater number of experiments, such as testing planorbid susceptibility to *S. mansoni*, or population control methods, such as molluscicide breeding and techniques for interrupting the biological cycle of the parasite. Further studies along these lines are evidently necessary.

In the present study, we analyze the growth, reproductive rate and mortality of *B. glabrata* and *B. straminea* with different calcium concentrations and food types.

## MATERIAL AND METHODS

### *Mollusks*

We performed the studies with two species of planorbids: *B. glabrata* and *B. straminea*. These snails were provided by the Laboratory of Immunopathology Keizo Asami (LIKA), at the Federal University of Pernambuco. The snails were collected in the Sotave - Jaboatão dos Guararapes/PE neighborhood and kept at LIKA. Samples of these animals were transferred to the molluscary in the Laboratory of Human Parasitology and Malacology (LAHUPAMA) at Campus II of the State University of Alagoas, and the descendants of these snails were used for the experiments.

### *Experimental conditions*

Two experiments were carried out over a 90-day period, starting from the hatching of the planorbids. Newly hatched snails were acclimatized in 2L polyethylene aquariums, containing dechlorinated water and kept under laboratory conditions: 25°C ± 2°C, 50-60% relative humidity, pH 7.5 and luminosity in alternating 12 hours light – dark cycles.

The snails were fed according to a specific diet, supplemented with different concentrations of calcium carbonate, based on the methodology of Augusto et al. (2009). We also tested calcium sulfide as a possible equivalent to calcium carbonate, due to accessibility and handling reasons, as well as having been previously tested in other experiments by the authors.

The first experiment was performed according to a calcium supplementation program. To this, 180 snails were divided into 2 groups and distributed in aquariums filled up with 1.5 L of water. The first group was distributed equally in six aquariums (15 animals each), three for each species. In each pair of aquariums, the snails were submitted to the following calcium carbonate concentrations: 20mg/L, 50mg/L and 80mg/L.

For the second group, we established the same experimental conditions, however, using calcium sulfide for supplementation. Once per week, the snails were exposed to these different calcium concentrations and fed every two days with lettuce and ornamental fish food *ad libitum*.

The second experimental condition occurred according to a food program. Thus, 120 planorbids divided into 3 groups and distributed equally in different aquariums with 10 animals each, containing 1L of water. The first group distributed in four different aquariums, 2 for each specie received only lettuce as a diet. In addition, the snails from each pair of aquariums were fed daily (6mg/animal) and on alternate days (12mg/animal) respectively.

Using the same parameters as established before, the remaining two groups received a diet composed by: lettuce + ornamental fish food; and lettuce + fish + powdered milk. Due to the need for calcium for these planorbids, we supplemented with the minimum concentration analyzed in this study (20 mg/L) to avoid changes in the experiment.

### *Parameters analyzed*

Three parameters were evaluated based on the conditions described. The first was mortality, considering the number of snails that died over the 90 days of the experiment.

The second was the snail spawn count, which occurred on approximately 7 cm x 7 cm Styrofoam dishes placed in the aquariums. Eggs and ovigerous masses present in the Styrofoam dishes were counted twice a week before hatching, using a Stereoscopic Microscope, Global Optics, NO106 series, Ningbo Tianyu Optoelectronic Technology CO. LTD – China.

In order to avoid egg hatching and sudden changes in the environment of the snails, once per week, after the cleaning of the aquariums, we replaced the water and as well the content of calcium. After counting, the eggs were placed in other aquariums for further studies. Finally, the third parameter was the evaluation of planorbid diameter. This measurement took place at the end of the experiment in order to correlate the type of diet and calcium supplementation to their respective sizes. This experiment was performed using a circle ruler with diameters ranging from 2mm to 40mm.

### *Data analysis*

The statistical analyzes were performed using BioEstat 5.3. For analysis of variance, one-way ANOVA and Tukey post-test were applied to analysis which programs influenced the parameters used.

The T test was used to compare the significance of the binary experiments of the daily food plan and every two days in a few comparative analyzes between the two species. The analyzes were considered significant when  $p < 0.05$ .

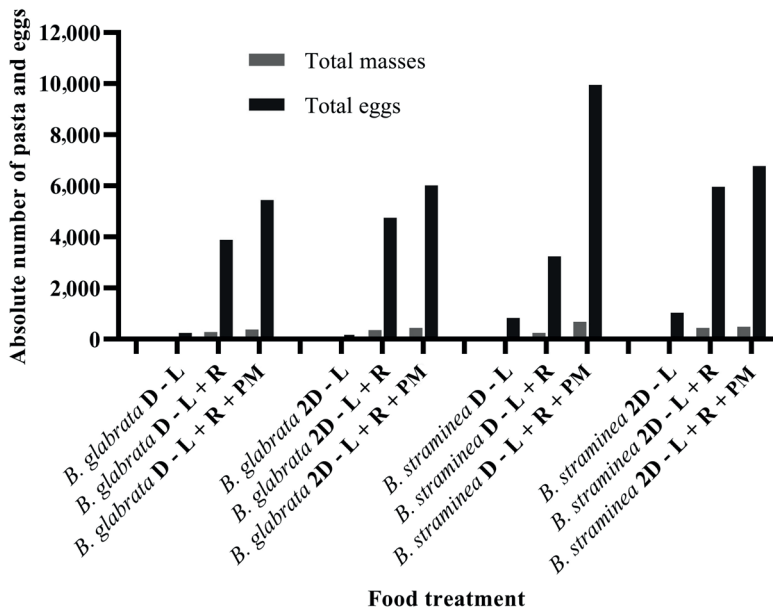
## RESULTS

The first visible ovigerous masses occurred in the 5<sup>th</sup> week of the three months of study in almost all aquariums in both experiments, and continued until the end of the research, totaling eight weeks of oviposition. Only snails fed with lettuce and lettuce + fish food, for both species, presented the first ovigerous masses as of the 7<sup>th</sup> week.

During the two months of oviposition, we counted 79,839 eggs for all *B. straminea* individuals and 62,558 eggs for all *B. glabrata* individuals. However, we observed that many ovigerous masses were stuck in the walls of the aquariums, occasionally reducing the normal amount in the Styrofoam plates, especially in the experiment for the type of food offered only lettuce for both species.

In the feeding plan experiment, the oviposition behavior of planorbids increased according to the type of food offered to both species, both for those fed daily and on alternate days (Figure 1).

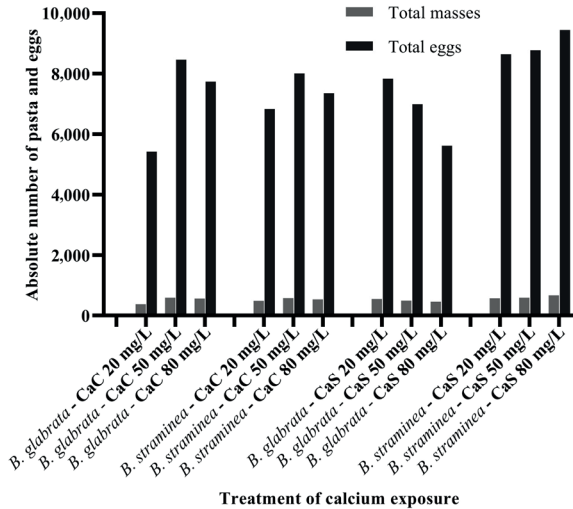
Figure 1. Quantity of eggs and ovigerous masses laid by snails of the species *B. glabrata* and *B. straminea* under different feeding programs after 60 days of oviposition in the laboratory.



D: Daily; 2 D: Every two days; L: lettuce; R: Ration; PM: Powdered Milk.

In the second experiment using calcium, *B. straminea* presented larger amounts of eggs and ovigerous masses in all programs using calcium sulphide, and in only one of the programs using 20mg/L calcium carbonate in comparison with *B. glabrata* (Figure 2).

Figure 2. Quantity of eggs and ovigerous masses laid by snails of *B. glabrata* and *B. straminea* species when submitted to different concentrations of calcium carbonate and calcium sulphide after 60 days of oviposition in the laboratory.



CaC: Calcium carbonate/CaS: Calcium sulphide

For both the dietary plans and the calcium exposure experiment, *B. glabrata* and *B. straminea* presented similar behavior in relation to the smaller amounts of eggs and ovigerous masses in the second weekly count when compared to the first weekly egg count (Figure 3).

There were no statistically significant differences between any concentrations of calcium carbonate or calcium sulphide regarding numbers of ovigerous masses and numbers of eggs deposited. A margin of error was obtained ranging from  $p=0.52$  to  $p=0.9$  in the parameters mentioned.

In the results related to the food type, all parameters for both species were significant for the variance test in relation to the number of eggs and number of ovigerous masses.

Feeding *B. glabrata* with lettuce + fish food or lettuce + fish food + powdered milk had a positive effect on the number of egg masses deposited and egg numbers when compared to those animals fed only lettuce. We also observed this relationship in the two dietary plans: daily feeding and every two days (Table 1).

For *B. straminea*, however, feeding the snails daily was only significant when used lettuce + fish food + powdered milk for the number of egg masses and the number of eggs. If only lettuce or lettuce + fish food were used, the number of egg masses and number of eggs was lower than when the first diet was used (Table 1).

Figure 3. Comparison of the two weekly *B. glabrata* and *B. straminea* egg counts.

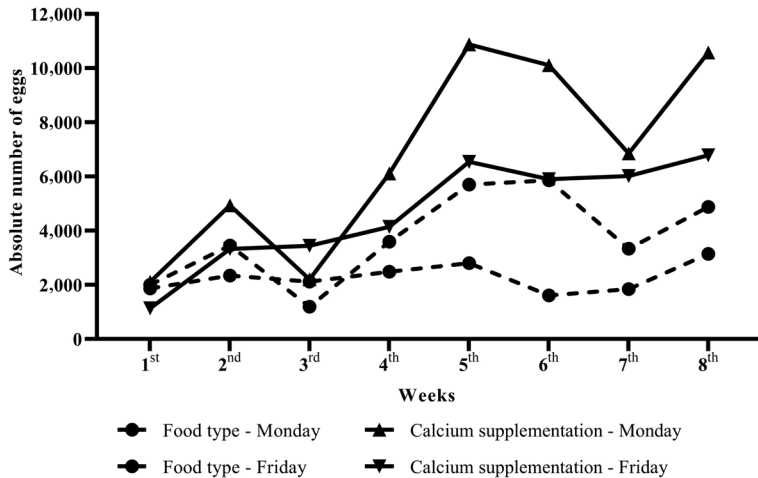


Table 1. Statistical analysis of the eating plan on the parameters regarding the number of deposited ovigerous masses and eggs of *B. glabrata* and *B. straminea*.

Groups	Daily food				Every two days			
	<i>B. glabrata</i>		<i>B. straminea</i>		<i>B. glabrata</i>		<i>B. straminea</i>	
	p-value							
	Eggs	Masses	Eggs	Masses	Eggs	Masses	Eggs	Masses
L – LR	< 0.05	< 0.05	ns	ns	< 0.05	< 0.05	< 0.05	< 0.01
L – LRPM	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
LR – LRPM	ns	ns	< 0.01	< 0.01	ns	ns	ns	ns

L: Lettuce; R: Ration; PM: Powdered Milk; ns: not significant ( $p > 0.05$ )

Average snail size in relation to the type and concentration of calcium offered did not show significant results for either species, whether descriptively or for the analysis of variance test, with values ranging between  $p=0.06$  and  $p=0.97$ .

Regarding the size of the planorbids, the largest diameters occurred in aquariums fed with fish food and/or milk, both when fed daily and every two days. When compared daily and alternate day dietary patterns by means of the t test, only *B. straminea* aquariums fed daily had a significant influence on the diameter (Table 2).

In the case of the largest average diameter of the snails in each of the three eating plans of each species using ANOVA/Tukey, those fed only with lettuce presented a significant difference in diameter when compared with the other two groups. However, when comparing these two groups with each other, there are no significant differences among them (Table 2).

Table 2. Verification of the size of *B. glabrata* and *B. straminea*, influenced by the three types of food offered daily and every two days, using T - Test and ANOVA/Tukey.

Groups	Average Diameter (cm)	T - Test	ANOVA/Tukey
<i>Biomphalaria glabrata</i>			
Daily - L	8.8	0.22	*
Every two days - L * <sup>1</sup>	9.2		1 ≠ 2 (p < 0.01)
Daily - L + R	12.3	0.21	1 ≠ 3 (p < 0.01)
Every two days - L + R * <sup>2</sup>	12.8		2 ≠ 3 (ns)
Daily - L + R + PM * <sup>3</sup>	13.3	0.07	(ns)
Every two days - L + R + PM	12.5		
<i>Biomphalaria straminea</i>			
Daily - L ** <sup>4</sup>	7	0.11	**
Every two days - L	6.5		4 ≠ 5 (p < 0.01)
Daily - L + R ** <sup>5</sup>	8.9	0.42	4 ≠ 6 (p < 0.01)
Every two days - L + R	8.8		5 ≠ 6 (ns)
Daily - L + R + PM ** <sup>6</sup>	9.6	0.04***	(ns)
Every two days - L + R + PM	9		

L: lettuce; R: Ration; PM: Powdered Milk; ns: not significant (p > 0.05)

\* / \*\* Treatments used for comparison in the variance test (ANOVA/Tukey).

\*\*\* Significant treatment for t-test

## DISCUSSION

*B. straminea*, compared to *B. glabrata*, had the higher reproductive rates in all experiments. This can be explained by the fact that this species is the best adapted to the climate and ecological variations in the country (Carvalho et al., 2008). Therefore, this specie is well adapted to develop under the artificial conditions created and to present higher egg laying numbers.



Costa et al. (2004), compared the ovipository capacity of these two species of *Biomphalaria*, and found higher reproductive rates in *B. straminea* compared to *B. glabrata*, corroborating the data of this research.

This may explain why this species seems to maintain the disease in the Northeast region of Brazil, as it acclimatizes easily to adverse environmental conditions, guaranteeing its reproductive efficacy. Besides, the region is ideal for the development of the disease, due to factors such as the lack of adequate sanitary conditions and the high number of houses, favoring the contact of snails with human feces and allowing the continuity of the biological cycle (Ponce-Terashima, 2014).

Several studies highlight the importance of a diet supplemented with proteins, in addition to traditional lettuce for snails. This feeding program results in greater reproductive capacity, an increase in shells sizes, in addition to an improved survival rate (Chieffi, 1975; Andrade et al., 1978).

Fresh lettuce is widely used for *Biomphalaria* feeding, however, many food combinations can present better rates (Rosa et al., 2013). Feeding snails with wheat, barley and rye, mixed with wheat germ and powdered milk, has resulted in snails almost 3 times larger and depositing 7 times more ovigerous masses than those fed with lettuce (Eveland & Ritchie, 1972).

Our results showed that the snails presented the highest amount of eggs and ovigerous masses and the largest shell sizes according to the type of food offered, therefore, the programs using the three types of food showed the highest values.

Some specific requirements are necessary to optimize the growth and conservation of these snails. Fertility, growth, mortality rates and shell size are essential indicators of the snails' physiological status (Eveland & Haseeb, 2010). Furthermore, many other aspects of correct snail rearing are required in addition to feeding and supplying calcium, such as temperature, oxygen, pH, conductivity and partner selection (Tallarico, 2015), which explains the fact that in all aquariums, the mollusks showed lower reproductive rates in the second weekly egg count.

This may have been due to water turbidity as a result of residual organic matter from the food placed in the aquariums throughout the week and the accumulation of planorbid feces. The pH of the water had been altered hindering oxygenation, essential for the survival and reproduction of these animals (Katz, 2008).

Individuals on the feeding program often presented whitish shells. However, pigmentation was recovered days after the weekly calcium exposure, a fact that was not observed in the other experiment, as the amount of calcium offered was greater.

*B. glabrata*, growth rates, relative weight of shells and fertility correlates with increased calcium concentrations (Thomas et al., 1974). Planorbid mortality is significantly higher when calcium levels are very low (1.5 mg/L) or very high (75 mg/L), with 30 mg/L being the ideal calcium concentration for the highest fertility of snails (Mishkin & Jokinen, 1986).

Augusto et al. (2009), stated in their work that the higher the calcium concentration, the higher the survival rate. We confirmed these results, as there was no mortality from planorbids during the study period.

In this work, we showed that snails fed with lettuce + fish food and/or powdered milk, had the best reproductive rates and largest shell sizes, in addition, the calcium concentration did not significantly influence our results. Thus, alterations in water and food quality are crucial for the survival/oviposition of these planorbids. Therefore, presenting an important study tool for population control, as small changes in these parameters contributed to significant variations in the number of eggs. Calcium is also a key factor for this, but further research is needed to better evaluate its effect on snail survival.

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