

Effectiveness of a handmade shell-based substrate for the breeding of *Biomphalaria glabrata* under laboratory conditions

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ABSTRACT

Efficient snail production is essential for the proper maintenance of the *Schistosoma mansoni* life cycle in the laboratory. In order to improve the breeding of *Biomphalaria glabrata* under laboratory conditions, this study aimed to demonstrate the effectiveness of a handmade shell-based substrate on the physiological performance of *B. glabrata*. The shells used to make the substrate were cleaned, sterilized and macerated until a powder was obtained (yield of 92.3%). *B. glabrata* specimens were randomly assigned to three treatment groups: negative control group (NCG) exposed to a clay-only substrate; a positive control group (PCG) containing clay, oyster flour and calcium carbonate; and the test group (TG) with the shell-based substrate and clay. *B. glabrata* bred in the test group showed improved growth, sexual maturity, fertility, mortality rate, and shell morphology when compared to the NCG, and similar to the PCG. Therefore, the shell-based substrate proved to be efficient and has a low cost for the breeding of *B. glabrata*.

KEYWORDS: Calcium carbonate. Shell-based substrate. *Biomphalaria*. Schistosomiasis. Mollusk breeding.

INTRODUCTION

Mollusks of the genus *Biomphalaria* act as intermediate hosts for the parasite *Schistosoma mansoni*, the etiological agent of the infectious and parasitic disease known as schistosomiasis^{1,2}. Schistosomiasis is one of the most neglected tropical disease (NTDs) of significant medical importance, and is responsible for high levels of morbidity and mortality in affected individuals across different continents of the world³. Globally, it is estimated that the disease affects over 200-250 million people in 78 countries, most of them located in Africa, Latin America, the Middle East and Southeast Asia^{4,5}. In Brazil, *B. glabrata* is the most important vector in the epidemiology of schistosomiasis owing to its high susceptibility to the parasite, in addition to its wide distribution^{6,7}.

Currently, several research centers maintain the *S. mansoni* life cycle in their laboratories using experimental models of vertebrates (usually hamsters and mice) and invertebrates (mollusks of the genus *Biomphalaria* from different species and strains) in order to discover new mechanisms involved in the parasite-host interaction, screening of new drugs, immuno-physiological factors involved in the severity of the disease, and new diagnostic tools⁸. For this purpose, it is necessary that the intermediate

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hosts of *S. mansoni*, the mollusks, have favorable conditions for their development. Therefore, it is suggested that they should be properly maintained in glass aquariums containing dechlorinated water, supplied with an appropriate substrate layer (composed of clay, calcium carbonate and oyster flour), and food of plant origin, such as lettuce (*Lactuca sativa* L.)⁹.

Calcium carbonate, an essential component of the substrate needed for the effective breeding of *B. glabrata*, has been described as a limiting factor in the distribution and survival of mollusks in the environment, because it is necessary for muscle contraction, embryonic development, growth, immune responses and is also important in shell formation and strengthening^{10,11}. Despite its great importance, it is the most expensive constituent and it is hardly available when researchers intend to produce a standardized substrate according to the recommendation of the Brazilian Ministry of Health, because, in general, the main commercial sources available are from oyster shells (sold only in specialized stores) or made in compounding pharmacies. Thus, it is necessary to investigate more accessible alternative sources of calcium carbonate with low cost that can be used for the effective breeding of *B. glabrata* in the laboratory.

Alternatively, the shell of *Biomphalaria* spp. which consists of 95% to 99% of calcium carbonate can be employed as a rich source of calcium in the production of the substrate used for breeding mollusks; the shell is characterized by a rigid external structure that plays the role of supporting and protecting the soft and delicate parts of the species against predators^{12,13}. Thus, the shell of mollusks that would have been discarded can be reused to provide the calcium supply in the form of calcium carbonate in the substrate needed for their breeding.

The aim of this study was to develop a substrate with the same efficiency, low cost, and that is more accessible compared to the standardized substrate provided by the Brazilian Ministry of Health, for the breeding of *B. glabrata* under laboratory conditions.

MATERIAL AND METHODS

Obtaining the macerate of *B. glabrata* shells

The *B. glabrata* shells used in the study were obtained from the collection of the Laboratory of Human Parasitology at the State University of Maranhao (UEMA). A total of 1,000 shells (18 to 22 mm) weighing 315.8 grams were used. The shells were sterilized by immersion in distilled water and neutral liquid soap for 24 h, washed with running water, dried in an oven at 37 °C for 24 h, and then macerated using a porcelain gral and pistil to obtain the shell powder

(approximately 92.3% of the initial weight), which was stored in vacuum tubes at 28 °C for subsequent use.

Production of substrates

The standardized substrate of the Brazilian Ministry of Health¹⁴, is made with sieved clay (10 parts), oyster flour (2 parts), and calcium carbonate (1.5 parts). Thus, 1,000 grams of the standardized substrate are made up of 740 grams of sieved clay, 145 grams of oyster flour and 115 grams of calcium carbonate.

In our experiment, sieved clay and macerated shells were used only in the production of the handmade shell-based substrate, and the mix was prepared with 10 parts of clay and 3.5 parts of macerated shell according to the standardized proportion recommended by the Brazilian Ministry of Health¹⁴. Thus, 1,000 grams of shell-based substrate are made up of 740 grams of sieved clay and 260 grams of macerated shells.

The species of mollusks used for the study was *B. glabrata* (BH strain), and they were randomly assigned to three treatment groups; positive control groups PCG (standardized substrate), negative control group NCG (clay only), and test group TG (handmade shell-based substrate).

Experimental design

The study was carried out in glass aquariums (50×25×25 cm) with capacity of 25 L of water (dechlorinated and filtered), and 12 grams of the substrates: PCG, NCG, and TG were added to the various treatment aquariums, respectively. Cleaning of the experimental aquariums and the addition of new substrates to the various treatment groups took place every 15 days. Physical and chemical parameters of the water, such as temperature, dissolved oxygen and pH were evaluated using a multi-parameter probe (Thermo Scientific Orion Star A326).

The snails were fed *ad libitum* with fresh lettuce leaves (*L. sativa* L.), which were washed with dechlorinated water. The laboratory temperature was maintained between 25 °C and 28 °C, and all the experiments were carried out in triplicates.

Mollusk growth and sexual maturity

The spawns obtained from *B. glabrata* bred on the standardized substrate were counted and the viability of the embryos was confirmed through observation under a stereomicroscope. A total of 100 spawns were used for the growth and sexual maturity experiment. The growth study was carried out according to the method of Oliveira-Filho *et al.*¹⁵, with modifications. Fifteen days after

hatching, 10 mollusks were randomly selected per treatment group and the shell measurement was conducted with the aid of a stereoscopic microscope coupled to a digital camera and a computer system (NIS-Elements advanced research). The measurement was repeated at each seven-days interval for a duration of 155 days.

The time of the first spawning in the different experimental groups was recorded by observing the activities on the Styrofoam (8 cm²) placed in the different aquariums at each seven days interval for a duration of over 50 days.

Mortality and fertility of *B. glabrata*

The study was conducted in aquariums with 50 snails (5-6 mm) per experimental group. The number of dead mollusks was recorded every 15 days. Fertility studies were carried out on the mollusks according to the method by Mangal *et al.*¹⁶, with modifications. The same groups of experimental snails were used for the mortality and fertility studies. For the fertility study, three pieces of 8 cm² Styrofoam were placed in the experimental aquariums, only the number of spawns and embryos deposited on them were counted every three days with the aid of a stereoscopic microscope for a duration of 120 days. The spawns outside that place were not counted but were removed from the aquarium during each cleaning. Styrofoam pieces were replaced at each count and the values of the number of spawns and embryos were normalized by the number of live snails.

Morphological characterization of *B. glabrata* shells

The snails used in the study of the previous parameters were also used for the morphological examination, they were macroscopically analyzed to check for the presence of morphological and color changes in their shells, according to the procedures preconized by the Brazilian Ministry of Health¹⁴.

Statistical analysis

A two-way ANOVA test was used for the analysis of data obtained from the study, and the Bonferroni test for multiple comparisons was used to compare significant means obtained from the different experimental groups. Mortality was assessed using the Kaplan-Meier curve, and the Log-Rank test. Probability values less than 0.05 were considered statistically significant.

RESULTS

During the 155 days of the study, results on the growth assessment from the mollusks indicated that the test

and positive control groups showed higher growth rates, compared to the negative control group (Figure 1). At the end of the study, the mean snail size was: 18.2 ± 0.68 mm, 17.8 ± 0.56 mm, and 12.4 ± 0.45 mm for the test group, positive control group, and negative control group, respectively.

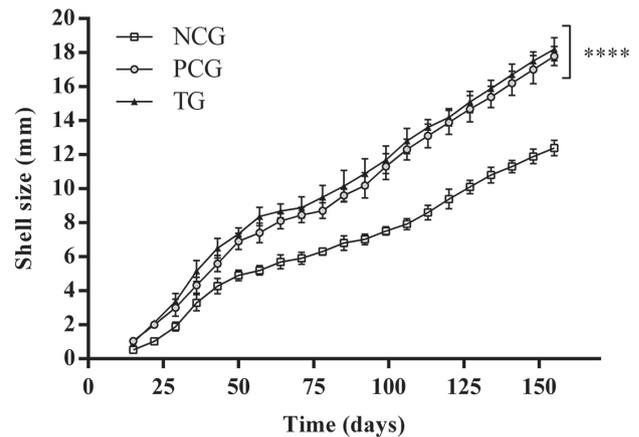


Figure 1 - Growth curve of *B. glabrata* bred using various experimental substrates: clay only in the negative control group (NCG), standardized substrate in the positive control group (PCG), and shell-based substrate in the test group (TG) for 155 days. A total of 100 *B. glabrata* per experimental group were used in the study. Statistically significant differences were obtained in comparisons with the negative control group.

Results from the assessment of sexual maturity showed that the mollusks in the test, and in the positive control groups performed oviposition earlier than mollusks in the negative group (Figure 2). The mean time (in days) of sexual maturity was: 34.9 ± 0.52, 33.3 ± 0.3, and 45 ± 0.51 for TG, PCG, and NCG, respectively.

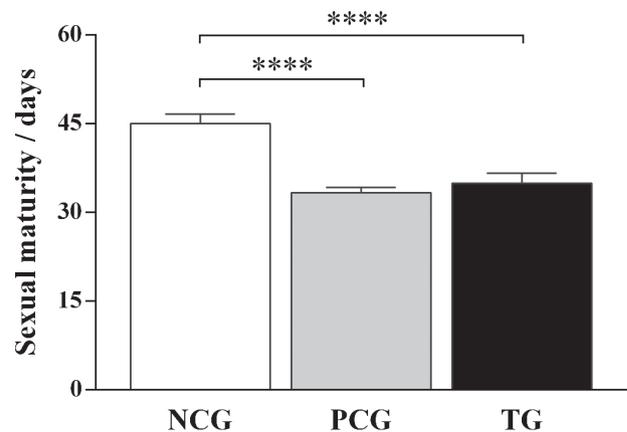


Figure 2 - Time of sexual maturity of *B. glabrata* bred using the various experimental substrates: clay only in the negative control group (NCG), standardized substrate in the positive control group (PCG), and shell-based substrate in the test group (TG). A total of 100 *B. glabrata* per experimental group were used in the study. Statistically significant differences were obtained in comparisons with the negative control group.

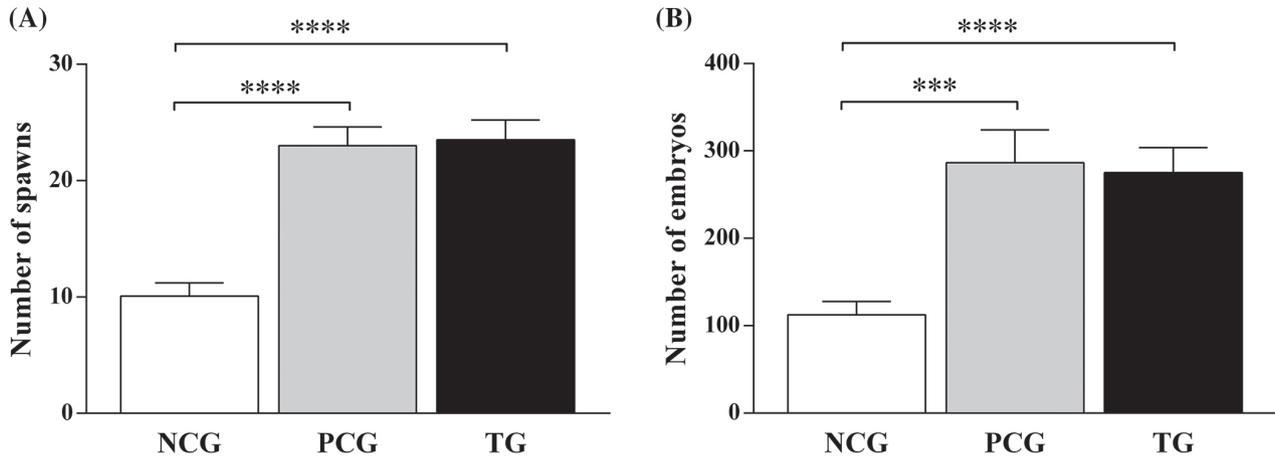


Figure 3 - Number of spawns (A) and embryos (B) of *B. glabrata* bred using the various experimental substrates: clay only in the negative control group (NCG), standardized substrate in the positive control group (PCG), and shell-based substrate in the test group (TG). A total of 50 snails per experimental group were used in the study. Statistically significant differences were obtained in comparisons with the negative control group.

During the 120 days of the study, the number of spawns (Figure 3A) and embryos (Figure 3B) in the test and positive-control groups was significantly higher than the number recorded in the negative control. The mean number of spawns and embryos was, respectively: 23.5 ± 1.7 , and 275.0 ± 28.7 in the test group; 23.0 ± 1.6 and 286.4 ± 37.6 in the positive control group; 10.08 ± 1.1 and 112.6 ± 15.1 in the negative control group.

Results on the mortality assessment indicated that the test group (TG) and positive control group (PCG) had significantly lower number of dead snails compared to the negative control group (NCG), which recorded the death of all snails assigned to it by the end of the study (Figure 4). The number of deaths per analysis time point varied as follows: test group from 2 to 8; positive control from 3 to

11 and negative control from 5 to 17. In all the experimental groups, the values of temperature ($21\text{--}25\text{ }^{\circ}\text{C}$), dissolved oxygen ($2.9\text{--}3.6\text{ mg/mL}$) and pH ($7.0\text{--}7.4$) were within the normal range.

Morphologically, the shells of snails in the test group (Figure 5C), and positive control group (Figure 5B) ranged from light brown to dark brown in color, with fewer cracks/fractures (yellow arrows) around their turns, in comparison with snails in the negative control group (Figure 5A), despite having similar shell coloration in respect to the TG, and the PCG had more cracks/fractures along the turns of their shells.

DISCUSSION

Mimicking optimal conditions in the laboratory for *B. glabrata* breeding has always been of interest to research centers, since these animals are needed on a large scale to maintain the *S. mansoni* life cycle, as well as other helminths of medical importance, such as *Angiostrongylus*¹⁷. Thus, the result of the present study showed that a simple shell-based substrate, made from a sustainable material (the shells of mollusks), can be a promising substrate to be used for the breeding of *B. glabrata* under laboratory conditions.

In general, results from the present study indicated that the handmade shell-based substrate resulted in improved survival and growth rate, shell strength, and reproductive performance (short time of sexual maturation and increase in the rate of oviposition), which is comparable to results obtained from snails bred using the standardized substrate recommended by the Brazilian Ministry of Health. Improvements of *B. glabrata* physiological performance have already been demonstrated by several researchers,

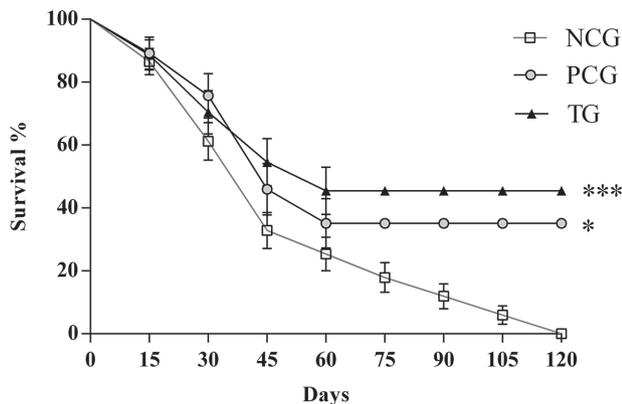


Figure 4 - Survival rate of *B. glabrata* bred using the various experimental substrates: clay only in the negative control group (NCG), standardized substrate in the positive control group (PCG), and shell-based substrate in the test group (TG) for 120 days. A total of 50 snails per experimental group were used in the study. Statistically significant differences were obtained in comparisons with the negative control group.

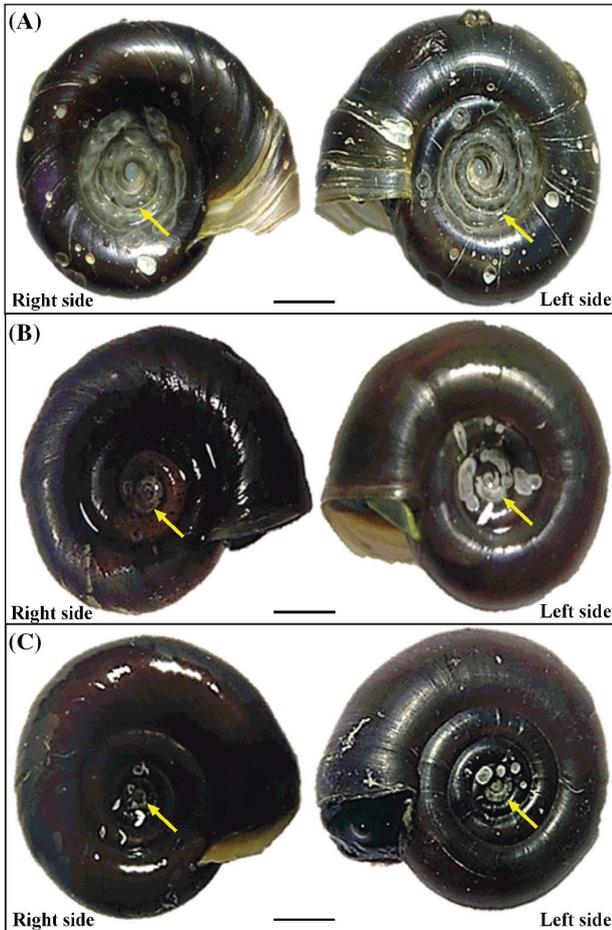


Figure 5 - Morphology of *B. glabrata* shells bred using the various experimental substrates: (A) Negative control group – NCG (with clay only); (B) Positive control group - PCG (with the standardized substrate); (C) Test group – TG (with the shell-based substrate). The yellow arrows indicate cracks/fractures on the shells. Scale: 5 mm.

under laboratory conditions, when the aquatic environment in which they are bred has the presence of optimal levels of bioavailable calcium^{10,12,18,19}. This demonstrates that *B. glabrata* shells can be an effective and low cost alternative to the calcium carbonate supplementation for the breeding of these snails in the laboratory.

Some researchers have already described the use of calcium and sources in the first stages of life of *B. glabrata*. The developing embryos obtain calcium from the perivitelline liquid and the gelatinous matrix (internal tunic) of the ovigerous mass. However, after one week, the external environment becomes the most important source of calcium for these animals¹³. In their natural environment, some studies have shown that *B. glabrata* has a preference for calcium-rich environments²⁰; calcium concentration below 20 mg/L affects negatively the breathing of mollusks and also decrease their motility²¹. Although the percentage of calcium carbonate in the shell-based substrate used in

the present study was not evaluated, some studies indicated that the shell of the mollusks contain about 95% to 99% of calcium carbonate^{12,13}; however, it is necessary to confirm this information in future studies for the better standardization and validation of shell-based substrates. Even so, due to the positive findings of the present study; improved biological and physiological parameters of *B. glabrata* bred on this shell-based substrate, it can be concluded that the calcium content of the shell-based substrate was sufficient for the proper breeding of snails under the test conditions.

In the current study, it was observed that the use of shells for the substrate production is a relatively cost-free and easy logistics process. This is because of the high reproductive capacity of *B. glabrata* when bred under optimum conditions in laboratory studies²², thus making them a high source of the raw material (shells) for the production of the substrate. In addition, encouraging the collection of these animals in the field for breeding purposes may promote an epidemiological control of schistosomiasis. Thus, the productive cycle of this shell-based substrate can be a self-sustainable, beneficial and profitable process on a large scale.

In summary, results from the present study indicated that the substrate composed of clay and macerated shells of *B. glabrata* can be used as a sustainable and an economically viable alternative for breeding *B. glabrata* under laboratory conditions, and can improve the result of researches that deal with *B. glabrata*. In addition, the use of shells from other mollusks to produce substrates requires further investigation.

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AUTHORS' CONTRIBUTIONS

Designed the study: GSM, JGMR and NSS; performed the experiments: JGMR, MGSL, RAN, GSM, GCCG, ICLL, DVM and ATR; analyzed the data: JGMR, GSM and NSS; wrote the paper: JGMR, GSM, MGSL, RAN, ICLL and DVM. All authors reviewed and approved the final version.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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