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Influence of *Echinostoma paraensei* (Lie and Basch, 1967) infection on the calcium content in *Biomphalaria glabrata* (Say, 1818)

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ABSTRACT

The calcium content in the hemolymph and shell of *Biomphalaria glabrata* (Say, 1818) was determined after exposure to different parasite burdens (5 and 50 miracidia) of *Echinostoma paraensei* (Lie and Basch, 1967). The snails were dissected 1, 2, 3, and 4 weeks after infection to collect the hemolymph and shell. An increase in calcemia was observed in snails infected with both miracidial doses. A significant decrease in the calcium ions in the shell was observed, coinciding with the calcemia peak in the hemolymph. This indicates greater mobilization of calcium between the shell and hemolymph to regulate the calcium content in the body when the snail is exposed to stress conditions, as has also been observed in some other infected snail species. The results obtained indicate that in this model, the calcium metabolism depends on the miracidial dose used.

1. Introduction

Calcium is the most important inorganic substance in the shell of gastropods, where it is stored as calcium carbonate ($CaCO_3$). Calcium carbonate provides CO_2 to enable the formation of a bicarbonate buffer that is important in the acid–base equilibrium in the hemolymph of snails submitted to stress conditions (de Witt and Sminia, 1980). Calcium ions have been referred to as a limiting factor in the distribution and adaptation of adult snails in the environment. Calcium is thus an important nutrient for shell formation, growth, and resistance to predator attacks, explaining why snails search for calcium-rich environments for their establishment and development. In parallel, studies have pointed to the involvement of this ion in the adaptive success of snails, especially for egg laying activity and development of embryos (Thomas et al., 1974; Nduku and Harrison, 1976; Appleton, 1978; Dawies and Erasmus, 1984; Tunholi et al., 2011).

Previous studies have shown changes in the inorganic metabolism of snails infected by digenetic trematodes (Pinheiro and Amato, 1995; Paschoal and Amato, 1996). However, no patterns have been established regarding the ability of parasites to induce specific changes in their hosts (Zbikowska, 2003; Pinheiro et al., 2009). For example, increases in calcium content in the shell of infected snails were shown by McClelland and Bourns (1969) studying the relationship between *Trichobilharzia ocellata* (La Valette, 1855) Brumpt, 1931 and *Lymnaea stagnalis* (Linnaeus, 1758), and by Pinheiro and Amato (1995) regarding the relationship between *Fasciola hepatica* (Linnaeus, 1758) and *L. stagnalis*. In contrast, White et al. (2005) observed a decrease in calcium in the shell of *Helisoma trivolvis* (Say, 1817), *Biomphalaria glabrata* (Say, 1818) and *Physa* sp. Draparnaud, 1801 infected by *Echinostoma trivolvis* (Cort, 1914), *Echinostoma caproni* Richard, 1964 and *Schistosoma mansoni* Sambon, 1907, respectively. In the last study, the authors proposed that the possible influence of larval trematodes on the calcium content of snail shells in laboratory or natural infections needs further investigation using more comparative and representative material for a better understanding of this phenomenon.

Echinostoma paraensei (Lie and Basch, 1967) is a Brazilian echinostomatid. The details on the morphology and biology of adult worms are described in Maldonado et al. (2001a,b). Additionally, larval intramolluscan development and metabolic alterations in the first intermediate snail host have been studied by various re-

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search groups (Dobrovolskij et al., 2000; Fujino et al., 2000; Ataev et al., 2001; Pinheiro et al., 2004a,b, 2005, 2009), but there are few studies on changes in the inorganic metabolism in *Echinostoma*-infected snails (Layman et al., 1996; White et al., 2005).

Different parasite burdens have been used to determine the impact of parasites in studies of parasite/host relationships (Sluiters et al., 1980; Vasquez and Sullivan, 2001; Tunholi et al., 2011). Previous results obtained by our group have shown that some fractions of neutral lipids in the relationship between *B. glabrata* and *E. paraensei* change in response to the miracidial dose (Tunholi-Alves et al., 2011). Thus, a better understanding of the complex balance between the host and parasite can be provided by such experiments, where the miracidial dose and/or time of infection can be experimentally manipulated (Bandstra et al., 2006). However, there is no study on the calcium metabolism in snails infected with different miracidial doses of *E. paraensei*. The aim of this study was to evaluate the alteration in calcium content in the hemolymph and shell of *B. glabrata* when exposed to different miracidial doses of *E. paraensei* during 4 weeks post-infection, an interval that corresponds to the prepatent period of this parasite.

2. Materials and methods

2.1. Collecting the E. paraensei eggs and obtaining the miracidia

Experimentally infected hamsters (*Mesocricetus auratus*) were euthanized in a CO_2 chamber and dissected to recover the adult forms of *E. paraensei* from the intestine. The adult worms were then dissected to collect the uterine eggs. These eggs were incubated in distilled water at 30 °C for 14 days, after which they were exposed to incandescent light under a 100-W bulb to stimulate eclosion of the miracidia (Pinheiro et al., 2004a,b, 2005).

2.2. Obtaining and experimentally infecting the B. glabrata specimens

The snails were obtained from the Laboratório de Esquistossomose Experimental, Instituto Owaldo Cruz, Fiocruz, RJ.

Young snails (8–12 mm) were individually placed on 24-hole plates on which miracidia had been placed previously with the aid of a micropipette, at a dose of 5 or 50 miracidia per snail (Sluiters et al., 1980; Vasquez and Sullivan, 2001; Tunholi et al., 2011). The snails from each group were individually examined under a stereomicroscope to detected miracidia in the plates. Twenty-four hours after exposure to the miracidia, there were no miracidia remaining in the plates.

The snails were then removed from the plates and transferred to aquariums. Only those snails harboring *E. paraensei* sporocysts in their circulatory system were selected for additional study, since sporocysts already are visible at 2 days post-infection (Loker and Hertel, 1987).

2.3. Maintaining the snails and forming the groups

Each aquarium was previously filled with 1,500 ml of distilled water mixed with 0.5 g of $CaCO_3$. This water was renewed once a week. Twelve groups were formed: four control groups (uninfected), four groups of snails infected with five miracidia each and four groups of snails infected with 50 miracidia each. Each aquarium contained 10 snails and the entire experiment was performed in duplicate, for a total of $n = 240$ snails. These doses (5 and 50 miracidia) were used to characterize low and high parasitemia, since in nature there is no way to control these parameters and the success of infection is related to the presence and number of snails and miracidia (Tunholi et al., 2011). The snails were fed *ad libitum* with lettuce leaves (*Lactuca sativa* Linnaeus, 1753). The aquariums were maintained every other day, when the lettuce leaves were replaced to prevent fermentation inside the aquariums and the number of dead snails was counted.

2.4. Dissection and collection of the hemolymph and shell

Weekly for four weeks post-exposure, the hemolymph was collected by cardiac puncture of randomly chosen snails from each group ($n = 10$), after which they were dissected and the shell removed, washed and left to dry at room temperature. The hemolymph was maintained in an ice bath during collection and stored at −10 °C.

2.5. Determination of the concentrations of calcium in the hemolymph and shell

The calcium content in the hemolymph of *B. glabrata* was determined by using commercial kits (Doles, Brazil) and the results were expressed as mg of calcium/dl of hemolymph. The calcium content in the shell was determined according Pinheiro and Amato (1995) and Soido et al. (2009). The calcium carbonate mass was calculated and expressed as mg of $CaCO_3$/g of ash.

2.6. Statistical analyses

The results obtained were expressed as mean ± standard deviation and the Tukey test and ANOVA were used to compare the means. A polynomial regression was calculated to analyze the relation between the values obtained and the infection time and for the parasite load to which the snails were exposed ($\alpha = 5\%$) (InStat, GraphPad, v.4.00, Prism, GraphPad, v.3.02, Prism, Inc.).

3. Results and discussion

A positive relation between the period of infection and calcium content in the hemolymph of the parasitized snails was observed in both groups (5 [$r^2 = 0.99$] and 50 [$r^2 = 0.77$] miracidia). The highest calcium levels in the hemolymph were observed in the fourth week after exposure: +50.9% (19.98 ± 0.43 mg/dl in the snails infected with five miracidia) and +20.46% (15.95 ± 0.29 mg/dl in the snails infected with 50 miracidia). This was higher than that mean observed in the uninfected group (13.24 ± 0.38 mg/dl) (Table 1 and Fig. 1a).

In the shell, a negative relationship between the calcium content and the period of infection was observed for both miracidial doses (5 [$r^2 = 0.56$] and 50 [$r^2 = 0.72$] miracidia). The ion calcium level was reduced in snails infected with five miracidia (−18.41%) and in those infected with 50 miracidia (−19.29%) at the third week post-exposure in relation to the mean control group (307.19 ± 3.4 mg of $CaCO_3$/g of ash) (Table 1 and Fig. 1b).

The increase in calcium content in the hemolymph was associated with a decrease of this ion in the shell of *B. glabrata* infected with five or 50 *E. paraensei* miracidia, evidencing the existence of a homeostatic pathway between the shell and the hemolymph. Snails exposed to stress accelerate their energy metabolism, causing a negative energetic balance due to high consumption of nutrients by the developing larval trematodes, leading to an increase in the formation of organic acids, with a resulting pH reduction in the hemolymph (Pinheiro and Amato, 1994; Pinheiro, 1996). The withdrawal of calcium carbonate ($CaCO_3$) from the shell to the hemolymph helps to maintain the acid–base balance, where it dissociates and releases CO_2, an important component of the bicarbonate buffer, vital to establish acid–base homeostasis (de Witt and Sminia, 1980; Pinheiro and Amato, 1995; Moreira et al., 2003).