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## Effects of various larval digeneans on the calcium carbonate content of the shells of *Helisoma trivolvis*, *Biomphalaria glabrata*, and *Physa* sp.

Received: 13 September 2004 / Accepted: 15 November 2004 / Published online: 28 January 2005  
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**Abstract** The calcium carbonate concentrations in the shells of *Helisoma trivolvis* and *Physa* sp. naturally infected with larval trematodes and *Biomphalaria glabrata* experimentally infected with larval trematodes were analyzed quantitatively. The larval trematode-snail relationships studied were *H. trivolvis* infected with larval *Echinostoma trivolvis* and *Physa* sp. infected with various larval digeneans, and *B. glabrata* infected with *Echinostoma caproni* or *Schistosoma mansoni*. The calcium carbonate concentrations of the shells of infected snails and uninfected cohorts and of the water in which the snails were maintained were determined by ion exchange chromatography. No significant differences in the calcium carbonate concentrations of shells of infected versus uninfected snails were found. The shells of *B. glabrata* infected with *E. caproni* contained significantly less calcium carbonate than the shells of uninfected *B. glabrata*. The hypercalcification hypothesis, i.e., larval trematodes induce an increase in the calcium concentrations in the shells of their snail hosts, was not upheld in any of the snail-larval digenean systems studied herein.

1980; Pinheiro and Amato 1995). Most of these studies were based on experimental miracidial infections of snails in the laboratory (see for example Pinheiro and Amato 1995), but others involved naturally infected snail hosts (Cheng 1971). Żbikowska (2003) tested the hypercalcification hypothesis in *Lymnaea stagnalis* naturally infected with various larval trematodes. Her work did not support the hypercalcification hypothesis, at least with the trematode-snail model she used. She stressed the need for more studies on snails naturally and experimentally infected with larval trematodes.

To assess the effects of larval trematodes on calcium in snails' shells, the calcium carbonate concentrations of the shells of wild pulmonates, i.e., *Helisoma trivolvis* naturally infected with *Echinostoma trivolvis* and uninfected *H. trivolvis*, and *Physa* sp. infected with various larval digeneans and uninfected physids, were determined. For similar studies on laboratory-infected snails, *Biomphalaria glabrata* snails experimentally infected with either *Schistosoma mansoni* or *Echinostoma caproni* were used.

### Introduction

Larval trematodes have been reported to induce an increase in the calcium levels (hypercalcification) of their first intermediate snail hosts (McClelland and Bourns 1969; Cheng 1971; Malek and Cheng 1974; Sluiter et al.

### Materials and methods

*H. trivolvis* snails naturally infected with *E. trivolvis* and uninfected snails (Schmidt and Fried 1997) were collected from Hoch Pond, Northampton County, Pa., at 40°47'20"N, 75°27'15"W on 1 June 2004 and at Amwell Lake, Wildlife Management Area, East Amwell Township, Hunterdon County, N.J. (40°26'N, 74°49'W) on 15 July 2004. *Physa* sp. containing various larval trematode infections were collected from Delaware Pond, Knowlton Township, Columbia, N.J., at 40°55'19.1"N, 75°03'49.5"W on June 23 2004. The snails were used within 1–2 days of collection; they were isolated individually in artificial spring water (ASW) prepared according to Ulmer (1970) under a light source for 1 h. Cercariae emerged from infected individuals upon isolation, and the individuals from which cercariae did not emerge were necropsied and examined for the

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presence of rediae and/or sporocysts. An individual was determined to be uninfected if cercariae did not emerge or if no rediae or sporocysts were present. Ten *H. trivolvis* individuals infected with *E. trivolvis* and ten randomly selected controls were chosen for shell analysis. The *Physa* sp. snails were infected with five species of larval trematodes, i.e., five snails with armatae cercariae, two with echinostome cercariae of the genus *Echinoparyphium*, one with a cystophorous cercaria of the genus *Halipegus*, one with an unidentified strigeid cercaria, and one with a monostome cercaria in the genus *Notocotylus*. Identification of cercariae types was based on Schell (1985). Because the prevalence of larval trematode infections in the *Physa* sp. snails was relatively low, i.e. 10 (4.4%) of 229 snails examined were infected, all of the physids with larval trematodes were considered together in a category referred to as "other infections" (see Table 1) with  $n=10$ . Ten uninfected *Physa* sp. were analyzed as the controls.

*B. glabrata* were infected with miracidia of *E. caproni* as described by Idris and Fried (1996) and used 8 weeks post-infection (PI). *B. glabrata* were infected with miracidia of *S. mansoni* as described by Fried et al. (2001) and also used 8 weeks PI. The snails were maintained in ASW and fed ad libitum on Romaine leaf lettuce. Snails, from which cercariae of either *E. caproni* or *S. mansoni* emerged, were used and matched with unexposed controls that had been maintained identically to the experimentally infected snails for 8 weeks.

At necropsy, each snail was measured with a vernier caliper to the nearest 0.1 mm. Measurements were made of the maximum snail diameter for the planorbids and maximum snail length for the physids. Each snail body was removed from its shell by dissection in deionized water, and the body discarded. The shells were rinsed at least four times with deionized water and dried overnight in an oven at 80°C, ground with a mortar and pestle, weighed, and digested individually in 5.00 ml of concentrated nitric acid (trace metal grade) by boiling to dryness in a 10-ml beaker. A sample consisted of shell

material from a single snail. The residue from each digested sample was quantitatively transferred to a 100-ml volumetric flask and diluted to volume with deionized water. Concentrated hydrochloric acid (1.00 ml) was added to dissolve any particulate matter remaining. Aliquots from samples to be analyzed were diluted 1:10 with deionized water to bring them into the range of the analytical calibration curve. For every snail-parasite combination, a blank solution was analyzed. The blank was prepared as follows: 5.00 ml of concentrated nitric acid (trace metal grade) was boiled to dryness in a 10-ml beaker that was then rinsed with deionized water into a 100-ml volumetric flask and diluted to volume with deionized water. The blank was then diluted to 1:10 as with the shell samples and analyzed by ion chromatography.

Each sample was analyzed using a Dionex DX-120 Ion Chromatograph (Dionex, Sunnyvale, Calif.) with a Dionex AS40 automated sampler, an IonPac CG12A guard column (4×50 mm), and an IonPac CS12A (4×250 mm) cation exchange column. The column was eluted isocratically with 20 mM methanesulfonic acid at a flow rate of 1.0 ml/min. A Dionex cation self-regenerating suppressor ultra (100 mA) was utilized to suppress background conductivity. Standard solutions of the calcium cation were prepared at 1 part per million (ppm), 5.00 ppm, 10.0 ppm, 25.0 ppm, 50.0 ppm, 100 ppm, and 200 ppm and used for calibration. Samples were analyzed in triplicate with an injection volume of 25  $\mu$ l. The retention time for the calcium ion was  $8.15 \pm 0.50$  min. Concentration values of the test solutions were determined by PeakNet version 5.1 software; these were converted to concentrations in the snail shell samples using the calculation: final calcium carbonate concentration (%) =  $(i \times v \times d \times z \times 100\%) / (1,000,000 \times m \times y)$ , where  $i$  is the test solution concentration from the instrument in ppm ( $\mu$ g/ml) minus the blank solution concentration from the instrument in ppm,  $v$  is the original volume of the sample (100 ml),  $d$  is the dilution factor (10),  $z$  is the molar mass of calcium carbonate (100.085 g/mole),  $m$  is the mass of the snail shell in

**Table 1** Calcium carbonate concentration (%) and snail size in infected versus uninfected snails. Snails obtained from Hoch Pond in experiment 1, Amwell Pond in experiment 2, and Delaware Pond in experiment 3; the *single asterisk* indicates a significant difference in calcium content between infected versus uninfected snails with a total of ten snails tested, but one was determined by the Q-test to be

an outlier and was thus excluded from the results, giving  $n=9$ . The *double asterisks* indicate a significant increase in snail size in the infected snails;  $n=10$  for all samples tested; the *cross* indicates water from the collection sites in experiments 1–3 and ASW in experiments 4 and 5

Experiment no.	Snail-trematode infections	Ca content in infected shells (mean $\pm$ SE)	Ca content in uninfected shells (mean $\pm$ SE)	Size of infected snails (mm) (mean $\pm$ SE)	Size of uninfected snails (mm) (mean $\pm$ SE)	Ca content in water (ppm)†
Wild snails						
1	<i>Helisoma trivolvis</i> / <i>Echinostoma trivolvis</i>	96.6 $\pm$ 0.31	95.4 $\pm$ 0.41	9.8 $\pm$ 0.4	10.5 $\pm$ 0.2	17.3
2	<i>H. trivolvis</i> / <i>E. trivolvis</i>	97.3 $\pm$ 0.22	97.3 $\pm$ 0.19	15.6 $\pm$ 0.3	15.0 $\pm$ 0.5	56.5
3	<i>Physa</i> sp./larval <i>digeneans</i>	100.2 $\pm$ 0.57	97.8 $\pm$ 0.26	8.6 $\pm$ 0.3	8.6 $\pm$ 0.2	76.3
Experimentally infected snails						
4	<i>Biomphalaria glabrata</i> / <i>Echinostoma caproni</i>	96.8 $\pm$ 0.30*	98.8 $\pm$ 0.10	13.5 $\pm$ 0.3**	11.1 $\pm$ 0.3	12.8
5	<i>B. glabrata</i> / <i>Schistosoma mansoni</i>	97.5 $\pm$ 0.21	97.8 $\pm$ 0.12	12.1 $\pm$ 0.4	12.4 $\pm$ 0.3	12.8

grams, and  $y$  is the molar mass of calcium (40.078 g/mole). Water samples, i.e., from the collection sites and the ASW, were analyzed by ion chromatography with the same parameters as the shell samples; however, the water samples were not diluted.

To verify the results of the calcium analysis, five samples were tested by flame atomic absorption spectrometry. A half ml of the 100-ml dissolved shell solution was transferred to a 10-ml volumetric flask, to which was added 1.00 ml of 10% lanthanum (III) nitrate hexahydrate to minimize interference from phosphate ions, 1.00 ml of 10% potassium chloride to act as an ionization suppressor, and deionized water to bring the final volume to 10.0 ml. The FAAS instrument was a double beam Varian SpectrAA-10 atomic absorption spectrometer (Varian, Walnut Creek, Calif.), operated with manual flame control, a Ca hollow cathode lamp (422.7 nm), and 0.2 nm spectral bandpass, and without background correction. The instrument used an air-acetylene burner. The gas supply was compressed air at a pressure of 350 kPa and flow a rate of 9 l/min, and compressed acetylene at a pressure of 63 kPa and a flow rate of 10 l/min. Sample introduction was performed manually. Five standard solutions of 5.00 ppm, 10.0 ppm, 25.0 ppm, 50.0 ppm, and 100 ppm, each with 10% lanthanum (III) nitrate hexahydrate and 10% potassium chloride, were prepared for analysis. The standards and samples were analyzed using three 30 s integrations. Concentration values of the test solutions were determined by the instrument; these were converted to concentrations in the snail shell samples using the same calculation used with the ion chromatograph values.

## Results

The calcium carbonate content of the shells, the sizes of the snails used, the calcium concentrations of the water in which the snails were maintained and the ASW are presented in Table 1.

Whereas the field-collected snails were maintained in water with varying calcium contents, none of these snail-parasite combinations showed a significant difference in calcium content between infected and uninfected snails (Student's  $t$ -test,  $P < 0.05$ ). Likewise, no significant difference in snail size was recorded in any of the field collected snail-parasite combinations.

The ASW in which the laboratory reared snails were maintained had a calcium content of 12.8 ppm (Table 1). Both the calcium content and snail size showed significant differences (Student's  $t$ -test,  $P < 0.05$ ) in uninfected *B. glabrata* versus those infected with *E. caproni*. No such differences were seen in *B. glabrata* infected with *S. mansoni* versus the controls. Thus, infection of *B. glabrata* with *E. caproni* was correlated with reduced calcium content in the shell, but also with increased size of the snail.

Data were obtained, using flame atomic absorption spectrometry, with a sample of five uninfected

*H. trivolvis* and a sample of five infected *H. trivolvis* from Hoch Pond. These data confirmed the result from ion chromatography that there was no significant difference (Student's  $t$ -test,  $P < 0.05$ ) in the calcium carbonate concentration of the shells of *H. trivolvis* infected with *E. trivolvis* ( $97.08 \pm 0.64\%$ ) versus those of uninfected *H. trivolvis* ( $96.28 \pm 0.64\%$ ).

## Discussion

We found no evidence to support the hypothesis of hypercalcification in the shells of pulmonate snails infected with larval trematodes. In spite of considerable variation in the calcium content (ppm) of the water in which the snails were maintained (from a low of 12.80 to a high of 76.25, see Table 1), all of our uninfected snails and those infected with larval trematodes showed values for calcium carbonate in the range of 95–99.9% as reported by Hare and Abelson (1965). Thus, under conditions of variable calcium concentrations in the water and larval trematode parasitism, pulmonate snails are able to maintain a high concentration of calcium carbonate in their shells.

The hypercalcification hypothesis, i.e., larval trematodes induce an increase in the calcium content of the shells of their snail hosts, was not upheld in any of the snail-larval digenean systems studied here. The possible influence of larval trematodes on hypercalcification of snail shells in laboratory or natural infections needs further study using more comparative and representative material for a better understanding of this phenomenon.

We did observe in one snail-trematode combination (*E. caproni* in *B. glabrata*) evidence for hypocalcification, i.e., *E. caproni* infection was correlated with a significant decrease in the calcium concentration in the shell of *B. glabrata*. Ponder et al. (2004) noted that the effects of *E. caproni* infection on various analytes of *B. glabrata* were quite variable, including a reduction in certain lipids and carbohydrates, but no changes in free pool amino acids were found.

Żbikowska (2003), using analysis by EDTA titration, found no differences in the calcium carbonate concentrations in pulmonate snails infected with larval trematodes, except in a case where the trematode-snail combination came from a lake with a low calcium concentration. Żbikowska suggested that, in general, the calcium content of the water was responsible for the lower calcium carbonate concentrations of uninfected snail shells, rather than the presence of larval trematodes.

Pinheiro and Amato (1995), also using EDTA titration, found a two- to threefold increase in calcium carbonate in the shells of *Lymnaea columella* infected with *Fasciola hepatica*. Both Żbikowska (2003) and Pinheiro and Amato (1995) reported their calcium carbonate concentrations in units of ppm/mg. This is not a valid unit because ppm, defined as  $\mu\text{g Ca/g shell sample}$  or  $\text{ng Ca/mg shell sample}$ , already takes the sample weight into

account and cannot be divided by the sample weight again (Kenkel 2003). After personal communications between J.S. and the authors of both of these papers about their EDTA titration techniques and calculations, we believe the values reported were, in fact, in units of weight percent. Therefore, the calcium carbonate values of  $71.22 \pm 16.15$  for uninfected and  $150.93 \pm 29.35$  for infected snails reported by Pinheiro and Amato (1995) indicate an apparent error in their analyses. Hare and Abelson (1965) and Marxen et al. (1965) state that calcium carbonate comprises 95–99.9% of molluscan shells, so the 71.22% value is much too low and the value above 100% is not possible. The values of Żbikowska (2003) correspond to the 95–99.9% range, indicating that these values really are weight percent and not ppm/mg.

We did notice a significant increase in growth of *B. glabrata* infected with *E. caproni* compared to the uninfected cohorts. As discussed by Thompson (1997), the growth of planorbid snails is a very complex issue. Some investigators have found changes in the size and growth of *B. glabrata* snails infected with larval trematodes and other workers have found contrary results with the same trematode-snail systems. Inasmuch as numerous factors may influence the growth of planorbids infected with larval trematodes, i.e., strain of parasite and snail involved, intensity of infection, nutritional status of the host, and other biotic and abiotic factors, the significance of our finding increased growth of *B. glabrata* infected with *E. caproni* awaits further study.

**Acknowledgements** We thank Dr. Jane E. Huffman and Ms. Jennifer L. Klockars, Department of Biological Sciences, East Stroudsburg University, East Stroudsburg, Pa. for providing us with *Helisoma trivolvis* and *Physa* sp. snails. We also thank Ms. Sharon R. Bandstra for help in preparing this manuscript.

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