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Effects of Estivation on the Concentrations of Selected Carboxylic Acids of Two Strains of *Helisoma trivolvis*

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Abstract. High-performance ion exclusion column liquid chromatography was used to analyze the effects of estivation on certain carboxylic acids in the digestive gland-gonad complex (DGG) of a Pennsylvania (Pa) and Colorado (Co) strain of *Helisoma trivolvis*. The DGG samples were extracted using 50% Locke's solution, followed by cleanup using anion-exchange solid phase extraction and analysis by ion exclusion high performance liquid chromatography with ultraviolet detection. Succinic, pyruvic, malic, and fumaric acids were detected and quantified in the DGGs of both estivated and unestivated snails at concentrations ranging from 0.91 to 2200 ppm. There was a significant (Student's *t*-test, $P \leq 0.05$) reduction in the concentrations of succinic, pyruvic, and malic but not of fumaric acid in unestivated versus estivated *Helisoma trivolvis* (Pa). For *Helisoma trivolvis* (Co), there was a significant increase in succinic but not in fumaric, pyruvic, or malic acids in unestivated versus estivated snails. The reduction of certain carboxylic acids in the DGG of *Helisoma trivolvis* (Pa) suggests that estivation stimulates a decreased production of these acids or increased utilization by the snail tissue. Differences in the concentrations of certain acids between *H. trivolvis* (Pa) and *H. trivolvis* (Co) probably reflect strain differences.

INTRODUCTION

Estivation (also written as aestivation) of pulmonate snails in the family Planorbidae relates to a dormancy of these snails under conditions of drying out. Estivation allows the snails to survive long periods of drought. Morevoer, snails infected with larval trematodes may retain their infections during estivation and transfer of the larval parasites to new hosts may occur when the snails emerge from estivation following submersion in fresh water. In the field, such estivation occurs when snails are subjected to drying conditions in lakes or ponds during short term or extended periods of drought. In the laboratory, estivation can be induced by subjecting the planorbids to a high relative humidity (circa 98%) and temperatures of about 22 to 24°C in a moist closed chamber as described by White et al. (2007). During estivation, snail metabolism is reduced and the organisms do not feed on exogenous food stuff. Information at the cellular and molecular level of the effects of estivation on planorbids is sparse. In our laboratory, we have begun estivation studies on planorbids in the genera *Biomphalaria* and *Helisoma*. Snails in these genera are important vectors of larval trematodes and nematodes and are associated with the transmission of numerous helminthic diseases to humans and wildlife; planorbid snails in these genera also serve as models for various research studies in the biomedical sciences. Our studies to date on the topic have examined the effects of experimentally-induced estivation on various analytes in both uninfected and infected planorbids in the genera *Biomphalaria* and *Helisoma*. In general, our snail estivation studies have demonstrated a reduction of most of the analytes we examined and in most cases parasitism by larval trematodes has exacerbated the effects of estivation on diminution of certain metabolites in the snails. The studies reported in this paper are a continuation of the effects of estivation on analytes in two strains of planorbid snails in the genus *Helisoma*. More information on the rational for the present work is given in the paragraphs below.

We maintain two strains of *Helisoma trivolvis* (Say, 1816) in our laboratory, one of which is *H. trivolvis* (Co) and the other *H. trivolvis* (Pa). The Co strain lacks melanin, is refractory to infection with miracidia of all trematodes tested to date, and is easy to culture in the laboratory (Schneck & Fried, 2005). It is also used as a model in invertebrate neurobiology (Kater, 1974). The Pa strain is ubiquitous in lakes and ponds in North America, is pigmented with melanin, and is infected with various species of larval trematodes (Schmidt & Fried, 1997).

Recent studies in our laboratory have examined the effects of estivation on various analytes in *Biomphalaria glabrata* (Say, 1816). Studies on lipids (White et al., 2006), carbohydrates (Jarusiewicz et al., 2006) and lipophilic pigments (Arthur et al., 2006) in the DGGs

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of these snails showed a decrease in these analytes as a function of snail estivation. One study on carboxylic acids in *B. glabrata* has determined changes in certain acids as a function of infection with *Schistosoma mansoni* larvae (Massa et al., 2007). One study on *B. glabrata* showed alterations in the concentrations of certain carboxylic acids as a function of estivation (Bezerra et al., 1999). Detailed studies on effects of estivation on the carboxylic acid content of any strain of *H. trivolvis* are not available. Therefore, the purpose of this study was to determine the effects of estivation on certain carboxylic acids in both the Pa and Co strains of *H. trivolvis*.

MATERIALS AND METHODS

Snail Maintenance

H. trivolvis (Co) has been maintained in our laboratory in continuous culture since the mid 1980s. This strain is maintained in Mason jar cultures, 15 to 20 snails per jar, in 800 mL of aerated artificial spring water (ASW). For further details, including the formulation of the ASW, see Schneck & Fried (2005). *H. trivolvis* (Pa) is available from April through November from local farm ponds and lakes in Northampton County, Pa (see Schmidt & Fried, 1997 for details). *H. trivolvis* (Pa) can be collected in the wild, brought into the laboratory, and maintained there for several months using the same cultivation procedure described for *H. trivolvis* (Co). Because some of these snails may be naturally infected with larval trematodes, they were examined by routine snail isolation procedures for larval trematodes; infected snails were removed from cultures and discarded.

Estivation

Usually 5 to 15 *H. trivolvis* (Pa) snails of each strain were estivated for 2, 3, or 7 days and a similar number of *H. trivolvis* (Co) for 7 days in a moist chamber at 24°C and a relative humidity of 98%. Details of the estivation chamber design were given in White et al. (2006). Preliminary studies showed that *H. trivolvis* (PA) did not survive the effects of estivation as well as *H. trivolvis* (Co), and therefore we used shorter estivation times for the studies on *H. trivolvis* (Pa).

At the end of each estivation period, snails were tested for survival by immersing them in ASW. Live snails became activated within 0.5 hr in ASW as shown by the extension of the head foot through the aperture. Snails that did not extend the head foot through the aperture were examined by mechanical probing with a needle after the shells were removed. Those that were not responsive were considered dead. The number of snails that survived estivation was recorded. Controls (unestivated snails) were maintained in Mason jar cultures and fed leaf lettuce as described above for the same times as those that estivated.

Sample Preparation

Each snail was removed from the ASW and placed in a Petri dish. The shell was cracked gently and removed from the snail body. The DGG was dissected from the body and homogenized with 6 mL of 50% Locke's solution with a glass homogenizer. The homogenizer was washed with 2–3 mL of solution, which was then added to the homogenate. The DGG homogenate was centrifuged at 2500 g for 10 min at 25°C. The carboxylic acids were recovered from the supernatant by solid-phase extraction (SPE) as described below. Each sample represented the supernatant of one DGG and had a final volume of 8 ± 1 mL.

Carboxylic Acid Extraction

SPE was performed as outlined by Massa et al. (2007a). The acids were extracted from the DGG homogenate using Varian strong anion exchange columns (quaternary amine; 100 mg; 3 mL, Varian Inc., Palo Alto, Ca, USA). Under vacuum, the columns were cleaned and activated with 1 mL of 0.5 M HCL, 1 mL of methanol, and 2 mL of deionized (DI) water. The DGG homogenate supernatant was then passed through a column under vacuum. The column was cleaned again with 2 mL of DI water. The carboxylic acids were eluted from the columns using 1 mL of 0.5 M sulfuric acid.

HPLC Analysis

Acetic, fumaric, lactic, malic, pyruvic, and succinic acid salts were purchased from Sigma (St. Louis, Mo, USA). Stock solutions of each organic acid were prepared at a concentration of 1.00×10^3 ppm in 0.5 M H_2SO_4 and the stock solutions were diluted to 10, 25, 50, and 100 ppm.

High performance liquid chromatography (HPLC) was performed at 30°C using an Agilent Technologies (Wilmington, DE, USA) 1100 Series HPLC Instrument with an autosampler amd ultraviolent (UV) detection at 210 nm. A Bio-Rad Laboratories (Hercules, CA, USA) Aminex ion exclusion HPX-87H column (300 × 7.8 mm) was used. 0.5 mM sulfuric acid was used as the mobile phase with an injection volume of 100 µL.

Linear calibration curves were generated using Microsoft Excel relating standard concentrations to their peak areas. The interpolated amounts of each organic acid quantified by HPLC were calculated using the following equation:

Table 1

Carboxylic acid concentrations in the digestive gland-gonad complex (DGG) of unestivated and estivated *H. trivolvis* (Pa).

	Unestivated DGG		Estivated DGG (2 Days)	
Acid	Sample Size	ppm (μg/g ± Standard Error)	Sample Size	ppm (μg/g ± Standard Error)
Fumaric	18	100 ± 8.0	11	96 ± 8.5
Malic	9	450 ± 85	5	290 ± 53
Pyruvic	8	20 ± 5.7	4	19 ± 2.9
Succinic	10	350 ± 66	6	540 ± 180
	Unestivated DGG		Estivated DGG (3 Days)	
Acid	Sample Size	ppm (μg/g ± Standard Error)	Sample Size	ppm (μg/g ± Standard Error)
Fumaric	16	130 ± 18	7	140 ± 13
Malic	9	270 ± 35	7	<2.0*
Pyruvic	10	23 ± 7.4	7	<2.0*
Succinic	9	280 ± 36	7	<2.0*
	Unestivated DGG		Estivated DGG (7 Days)	
Acid	Sample Size	ppm (μg/g ± Standard Error)	Sample Size	ppm (μg/g ± Standard Error)
Fumaric	8	120 ± 8.8	4	98 ± 16
Malic	7	350 ± 54	4	<2.0*
Pyruvic	6	34 ± 10	4	<2.0*
Succinic	6	550 ± 150	4	<2.0*

* A significant reduction in the concentration of carboxylic acids in the estivated DGG relative to the controls (Student's *t*-test, $P \leq 0.05$).

$$\text{Organic Acid (ppm)} = \frac{(I)(V)}{(M)}$$

where I = instrument solution concentration (ppm) interpolated from the standard calibration curve, V = sample volume (mL), and M = snail DGG mass (g).

RESULTS

For *H. trivolvis* (Pa), a total of 22 of 60 snails survived estivation for 2, 3, or 7 days. Of these, 11 of 20 survived 2 days, 7 of 25 survived 3 days, and 4 of 15 survived 7 days. The organic acid content of these snails and of 42 unestivated snails was determined.

For *H. trivolvis* (Co), 7 of 12 estivated snails survived 7 days. As with the Pa strain, the organic acid content for the 7 estivated and the 8 unestivated Co strain samples was determined on the last day of estivation. *H. trivolvis* (Co) was more capable of surviving long-term estivation (7 days) than was *H. trivolvis* (Pa).

The DGG samples of both Pa and Co strains of *H. trivolvis* showed peaks with similar retention times to the standards. Typical retention times of the standards in minutes were as follows: pyruvic, 9.2; malic, 9.7; succinic, 12.2; lactic, 12.4; acetic, 14.9; and fumaric, 16.2.

The carboxylic acids and their concentrations in ppm detected in DGG samples of unestivated and estivated snails of *H. trivolvis* (Pa) are listed in Table 1. Similar information on DGG samples of unestivated and estivated snails of *H. trivolvis* (Co) snails are listed in Table 2. Of all the acids tested (acetic, fumaric, lactic, malic, pyruvic, and succinic acids), all but acetic and lactic were consistently detected in DGG samples of unestivated snails of both strains.

For *H. trivolvis* (Pa) estivated for 2 days, fumaric, malic, pyruvic, and succinic acids were detected and quantified (Table 1). The Student's *t*-test ($P \leq 0.05$) showed no significant difference in the concentrations of these acids between the unestivated and estivated samples. For *H. trivolvis* (Pa) estivated for 3 and 7 days, concentrations of fumaric acid were at the levels of those in the unestvated cohorts, whereas the concentrations of malic, pyruvic, and succinic acids in these snails were below the detection of 2 ppm of the HPLC analysis determined by extrapolation from the standard calibration curves. At 3 and 7 days, the concentrations of malic, pyruvic, and succinic acids in the DGGs of estivated *H. trivolvis* were significantly lower than those in the unestivated cohorts.

For *H. trivolvis* (Co) maintained for 7 days, fumaric, malic, pyruvic, and succinic acids were detected in both estivated and unestivated snails (Table 2). There was no significant difference ($P \leq 0.05$) in the concentrations of fumaric, malic, and pyruvic acids in unestivated versus estivated snails (Table 2). There was a

Table 2

Carboxylic acid concentrations in the digestive gland-gonad complex (DGG) of unestivated and estivated *H. trivolvis* (Co).

	Unestivated DGG		Estivated DGG (7 Days)	
Acid	Sample Size	ppm (μg/g ± Standard Error)	Sample Size	ppm (μg/g ± Standard Error)
Fumaric	8	90 ± 9.3	7	82 ± 7.1
Malic	8	340 ± 23	7	500 ± 100
Pyruvic	8	16 ± 1.8	7	30 ± 6.5
Succinic	8	400 ± 56	7	1300 ± 280*

* A significant increase in the concentration of carboxylic acids in the estivated DGG relative to the controls (Student's *t*-test, $P \leq 0.05$).

significant increase ($P \leq 0.05$) in the concentration of succinic acid in estivated versus unestivated *H. trivolvis* snails at 7 days.

DISCUSSION

Bezerra et al. (1999) stated that estivation of *B. glabrata* resulted in decreased snail metabolic activity. They suggested that the process of snail estivation could be better understood by studying the effects of estivation on various snail analytes. Earlier studies have been done to determine how estivation affects certain metabolites in *Biomphalaria* snails. Perhaps the most noteworthy of these studies was that of Von Brand et al. (1957), who showed that lipids, polysaccharides, lactic acid and certain volatile organic acids were depleted in estivated *B. glabrata* compared with the non-estivated controls. Bezerra et al. (1999) noted various changes in the organic content of estivated versus non-estivated B. glabrata. White et al. (2006) found a significant decrease in depot fats (triacylglycerols) in estivated versus non-estivated *B. glabrata*. Jarusiewicz et al. (1996) found a decrease in maltose and glucose in estivated versus non-estivated *B. glabrata*. The above-mentioned studies all documented significant changes in certain key analytes associated with metabolism in estivated planorbids compared with the non-estivated cohorts. These changes reflect the decreased metabolic activity that occurs during the period of dormancy that we associate with estivation.

Certain carboxylic acids decrease with estivation in planorbid snails. Bezerra et al. (1999) showed that for *B. glabrata* estivated for 7 days, the concentration of pyruvic acid in the digestive gland decreased but that of lactic, succinic, malic, and acetic increased compared to the unestivated controls. They used the term digestive gland in their study, but presumably they were examining the DGG. The gonad is at the most distal part of the DGG and Bezerra et al. (1999) made no mention of removing the gonad from the digestive gland in their study. Our findings on *H. trivolvis* (Pa) estivated for 3 and 7 days also showed a decrease in pyruvic acid in accord with the findings of Bezerra et al. (1999) on *B. glabrata*. In contrast to the findings of an increase in succinic and malic acids in estivated *B. glabrata*, we found a decrease in these two acids in *H. trivolvis* (Pa) estivated for 3 and 7 days. Our finding of an increase in succinic acid in the DGG of *H. trivolvis* (Co) estivated for 7 days is in accord with the increase noted by Bezerra et al. (1999) for *B. glabrata* for the same time. The significant changes in the concentrations of carboxylic acids in the DGGs of estivated planorbids is difficult to determine until more information is available on the metabolism of these snails during estivation.

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