

## A Comparative Study of the Organic Acid Content of the Hemolymph of *Schistosoma mansoni*-Resistant and Susceptible Strains of *Biomphalaria glabrata*

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*The freshwater snail Biomphalaria glabrata is an intermediate host of the trematode Schistosoma mansoni. However, some strains of B. glabrata are resistant to successful infection by S. mansoni larvae. The present work examines the profile of organic acids present in S. mansoni-resistant and -susceptible strains of B. glabrata, in order to determine whether the type of organic acid present is related to susceptibility. The organic acids were extracted from the hemolymph of two susceptible B. glabrata strains (PR, Puerto Rico and Ba, Jacobina-Bahia from Brazil), and from the resistant strains 13-16-R<sub>1</sub> and 10R<sub>2</sub>, using solid phase extraction procedures followed by high performance liquid chromatography. The organic acids obtained were analyzed and identified by comparison with known standards. Pyruvate, lactate, succinate, malate, fumarate, acetate, propionate,  $\beta$ -hydroxybutyrate and acetoacetate were detected in all hemolymph samples. Under standard conditions, the concentration of each of these substances varied among the strains tested and appeared to be specific for each strain. An interesting variation was the low concentration of pyruvate in the hemolymph of PR-snails. Only the concentration of fumarate was consistently different ( $p \leq 0.05$ ) between resistant and susceptible strains.*

Key words: *Biomphalaria glabrata* - host-parasite interaction - organic acids - resistance - *Schistosoma mansoni* - susceptibility

Mollusk-parasite relationships represent a vast field of research. Several studies have examined the influence of parasites on the host organism (Marshall et al. 1974, McManus & James 1975, Becker 1980), the mechanisms of host location (MacInnis et al. 1974, Haas 1985), and the mollusk's resistance to the parasite, i.e. incompatibility of the host (Wright 1974, Richards 1975, Bayne & Loker 1987, Zelck et al. 1995, Boehmler et al. 1996). In general, mollusks represent important intermediate hosts for the multiplication of trematode larvae. In case of *Schistosoma mansoni*, the larvae can multiply to reach a weight equivalent up to 50% of that of the *Biomphalaria glabrata* digestive gland (Schwanbek et al. 1986). *S. mansoni* larvae obtain their energy and growth substrates from the host, and in exchange release products of their intermediate metabolism into the host's body. For larval growth to occur, alterations

are required in the host's own metabolism (Becker 1980).

Many aspects of the host-parasite interaction remain to be clarified. Parasites such as *S. mansoni* relay on one specific host, in this case *B. glabrata*. The host itself may possess factors which determine whether an infection will be successful. Resistant *B. glabrata* possess a natural defense system which protects them against *S. mansoni* larvae, which generally die one to three days after penetrating the snails. Although susceptible snails are able to protect themselves against disease-transmitting agents, *S. mansoni* appears to be able to avoid the snail's defense system, thus allowing its own development in the host.

Organic acids are important components of intermediate metabolism and participate in both catabolic (eg. glycolysis) and anabolic (eg. gluconeogenesis) pathways. Pyruvate and lactate are indicators of glycolytic processes under aerobic conditions, while fumarate, succinate and malate are indicators of the tricarboxylic acid cycle. The presence of ketone bodies, such as  $\beta$ -hydroxybutyrate and acetoacetate, as well as of fatty acids, such as acetate and propionate, is indicative for lipid metabolism (Meyer et al. 1986, Bezerra & Becker 1993).

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The present work reports on the concentration of organic acids in the hemolymph of non-infected *B. glabrata* and examines whether there is any difference in the intermediate metabolism of resistant and susceptible snails. Since the development of the larva in the host depends on hemolymph components, we hypothesize a relationship between the profile of organic acids present and the snail's ability to resist the parasite.

## MATERIALS AND METHODS

*B. glabrata* were maintained in 30 liters aquaria under standard conditions, at a density of 30 snails per tank. Water temperature was maintained at  $26 \pm 2^\circ\text{C}$  and the animals were kept on a 12 hr light: dark cycle. The snails were fed *ad libitum* with a balanced diet (Becker & Lamprecht 1977). Only adult snails with a shell diameter of  $18 \pm 2$  mm were used in the study.

Two *S. mansoni*-susceptible strains (PR, Puerto Rico, and Ba, Jacobina-Bahia) and two *S. mansoni*-resistant strains (13-16-R<sub>1</sub> and 10R<sub>2</sub>) of *B. glabrata* were employed.

**Sample preparation - Hemolymph collection:** the snail's shell was first cleaned with a paper towel and the hemolymph then collected with a Pasteur pipette inserted through a tiny hole made in the pericardial region of the shell. The hemolymph from three snails was pooled and then centrifuged (120g, 5 min,  $2^\circ\text{C}$ ), in order to remove hemocytes and cellular debris. The resulting supernatant was used in the analyses described below. A total of ten samples from each strain was examined.

**Extraction and separation of the organic acids** - The organic acids were extracted immediately from the centrifuged hemolymph using Bond-Elut® columns (SAX-anion exchange-quaternary amine, manufactured by Analytichem International, Harbor City, USA) as described by Rumsby et al. (1987). Under vacuum, the columns were activated by consecutive washes with 1 ml of 0.5M HCl, 1 ml of methanol and 2 ml of HPLC-grade water. They were loaded with 200  $\mu\text{l}$  of hemolymph and 2 ml water. The columns were disconnected from the vacuum pump and 250  $\mu\text{l}$  of 0.5M sulphuric acid were applied to elute the organic acids retained on the matrix. The eluate was centrifuged at 1200 g for 5 min and the supernatant stored at  $-70^\circ\text{C}$  until analysis by high performance liquid chromatography.

The liquid chromatography (HPLC-system Milton Roy-Analyst 7800) was performed at room temperature using a BIORAD-Aminex ion exclusion HPX-87H column (300x7.8 mm) designed specifically for the separation of organic acids. The separation column was protected by a BIORAD-Aminex HPX-85 guard column. The mobile phase

was sulphuric acid (0.5mM) delivered at a flow rate of 0.8 ml/min. The elution profile was determined at 210nm. The injection volume of each sample was 100  $\mu\text{l}$ .

Statistical calculations were based on the non-parametric U-test of Wilcoxon, Mann and Whitney (Sachs 1986). The differences were considered to be significant when  $p \leq 0.05$ .

## RESULTS

The organic acids pyruvate, lactate, succinate, fumarate, malate, acetate, propionate, acetoacetate and  $\beta$ -hydroxybutyrate were detected in the hemolymph of all *B. glabrata* strains under normal conditions. Figure shows the level of each of these substances in the hemolymph samples and provides a statistical comparison of the results obtained. With the exception of fumarate, there was no consistent difference in the levels of these acids between *S. mansoni*-susceptible and resistant strains of *B. glabrata*.

## DISCUSSION

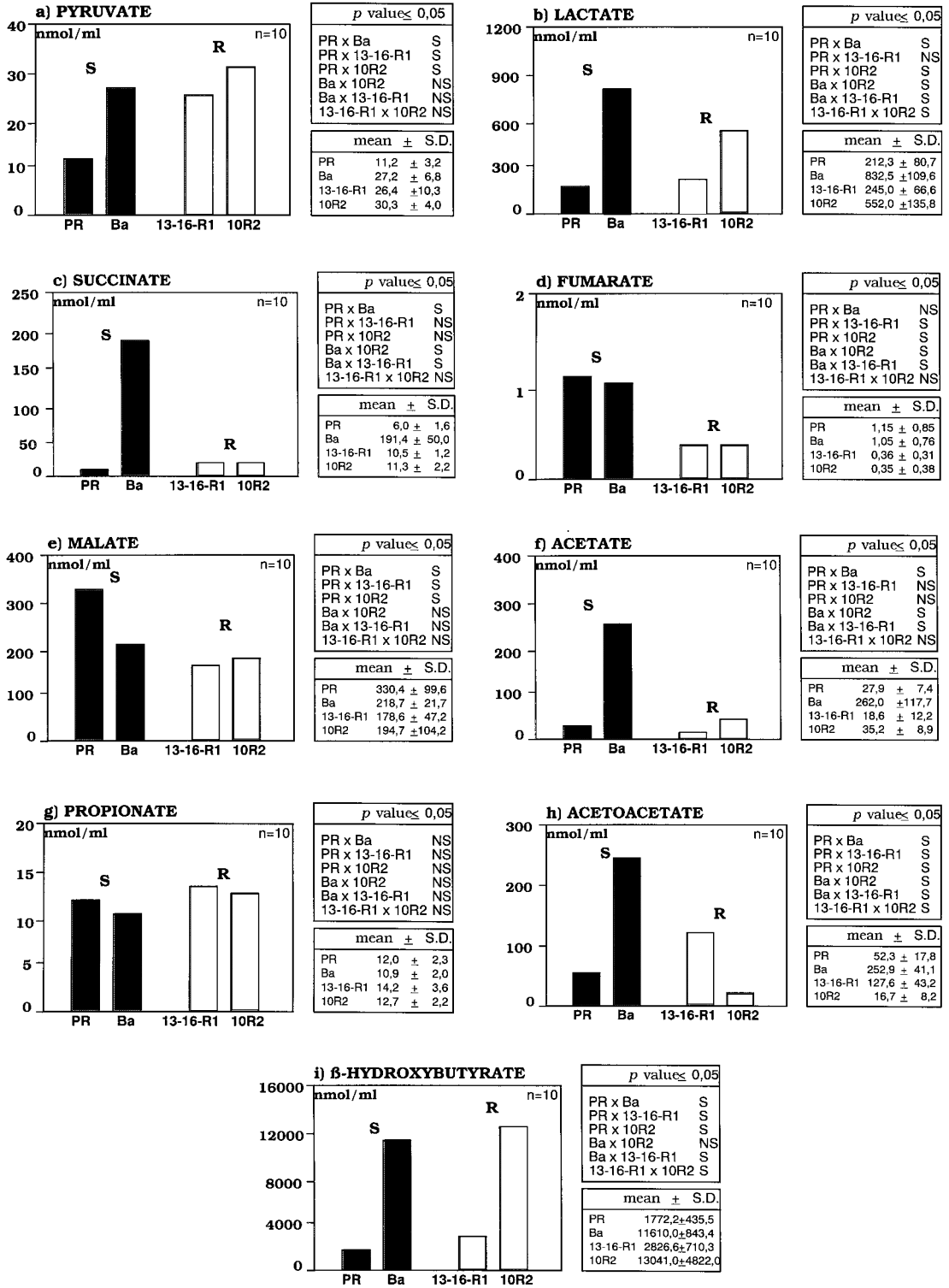
The various studies of host-parasite compatibility published so far are not always comparable because of the wide variety of methods and experimental designs employed (Frandsen 1979).

Our extraction method is commonly used in the medical field for urine and blood analyses and the evaluation of physiological disorders in man (Rehman et al. 1982). By adapting the methodology to invertebrates, it was possible to separate and quantify nine organic acids at the same time in one sample of *B. glabrata* hemolymph. These acids represent metabolites directly linked to energy production and facultative metabolic ways in invertebrates (Ishak et al. 1975, Patience et al. 1983, Hardewig et al. 1994).

Under the conditions employed, the four strains studied showed different concentrations of organic acids, the profiles of which appeared to be specific for each strain. Gilbertson et al. (1967) also reported variations in the amino acid concentrations of *B. glabrata* strains from different geographical locations.

An interesting finding in the present work was the low concentration of pyruvate in the susceptible strain PR. Pyruvate is the end product of the glycolytic process and serves as a substrate for the formation of acetyl-CoA, which will either enter the Krebs' cycle or form acetate or lactate. The low concentration of pyruvate in the PR strain may therefore be explained as a less intensive glycolytic rate than in resistant snails, or by its faster usage as a substrate and hence faster removal from the hemolymph of susceptible snails.

The success of both the parasite's penetration



The organic acids concentrations. Pyruvate (a), lactate (b), succinate (c), fumarate (d), malate (e), acetate (f) and propionate (g); and of the ketone bodies acetoacetate (h) and β-hydroxybutyrate (i) in *Schistosoma mansoni*-susceptible (S) and resistant (R) strains of *Biomphalaria glabrata*. PR, Puerto Rico. Ba, Bahia. The values shown are the mean ± S.D. Significant differences ( $p < 0.05$ ) are marked by S while non-significant differences are indicated by NS.

of the snail's skin (Loker & Bayne 1982, Bayne & Loker 1987) and the establishing of an infection (Frandsen 1979) are generally correlated with the hemolymph composition. In this context, it is possible that the parasite is able to absorb host substances which will provide either energy or structural substrates to enable the parasite to become established (Wright 1974). The presence of parasite-repelling substances may indicate either a host metabolic state not conducive to the parasite's settling or the presence of important concentration differences for certain substrates.

The concentration of lactate showed no distinctive profile between susceptible and resistant snails. The strains Ba and 10R<sub>2</sub> both had a high level of this acid. Flechter and LoVerde (1981) suggested a correlation between *S. mansoni* lactate dehydrogenase values and the parasite's infectivity in snails. However, the results shown here indicate no difference in the hemolymph lactate levels of susceptible and resistant snails.

Only for fumarate we detected a consistent significant difference between resistant and susceptible strains. It is interesting to note that the fumarate-reductase system is gaining increasing attention in invertebrate biochemistry. Particularly the presence of this system has a valuable physiological meaning in the adaptation of invertebrates with facultative anaerobic metabolism under adverse ecological conditions and is very important in the studies of helminth parasites metabolism to elucidate the characteristics of the fumarate-reductase system in relation to energy generation and to the effects of anthelmintics on the energy metabolisms (Takamiya et al. 1984, 1993, Tielens 1994, Saz 1990, Kita 1992). In conclusion, the results of this work show a variable concentration of organic acids in the hemolymph of *S. mansoni*-resistant and -susceptible strains of *B. glabrata*. These differences cannot be correlated with the snail's ability to eliminate the parasite.

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