



Unveiling the oxidative metabolism of *Achatina fulica* (Mollusca: Gastropoda) experimentally infected to *Angiostrongylus cantonensis* (Nematoda: Metastrongylidae)

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Abstract

For the first time, alterations in the oxidative metabolism of *Achatina fulica* experimentally infected with different parasite loads of *Angiostrongylus cantonensis* were determined. For this, the hemolymph activities of lactate dehydrogenase (LDH) and hexokinase and the glucose concentrations in the hemolymph, as well as the polysaccharide reserves in the digestive gland and cephalopedal mass, were assessed. Additionally, the contents of some carboxylic acids in the hemolymph of infected and uninfected snails were determined by high-performance liquid chromatography (HPLC), permitting a better understanding of the alterations related to the host's oxidative metabolism. As the main results, activation of oxidative pathways, such as the glycolytic pathway, was demonstrated in response to the increase in the activity of hexokinase. This tendency was confirmed by the decrease in the contents of glucose in the hemolymph of parasitized snails, indicating that the infection by *A. cantonensis* alters the host's metabolism, and that these changes are strongly influenced by the parasite load. This metabolic scenario was accompanied by activation of the anaerobic fermentative metabolism, indicated not only by an increase in the activity of (LDH), but also by a reduction of the content of pyruvic acid and accumulation of lactic acid in the hemolymph of parasitized snails. In this circumstance, maintenance of the host's redox balance occurs through activation of the fermentative pathways, and LDH plays a central role in this process. Together, the results indicate that *A. cantonensis* infection induces activation of the anaerobic metabolism of *A. fulica*, characterized not only by the accumulation of lactic acid, but also by a reduction in the pyruvic acid and oxalic acid contents in the hemolymph of the infected snails.

Keywords Carboxylic acids · Host-parasite relationship · *Achatina fulica* · *Angiostrongylus cantonensis*

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Introduction

According to systematic studies, the genus *Angiostrongylus* is composed of 21 species, which are widely distributed in the world, posing a threat to the health of wild and domestic animals (Robles et al. 2016). Of these, two stand out, *A. costaricensis* and *A. cantonensis*, due to their potential involvement in infection and pathological alterations in humans. The first species is a causal agent of abdominal angiostrongyliasis in the Americas, including Brazil, while the second is the main cause of eosinophilic meningoencephalitis in humans, a parasite-borne disease that causes significant alterations in the central nervous system due to the neurotropic behavior of the infective larvae (Martins et al. 2015).

To date, epidemiological studies have confirmed more than 2800 human cases of neural angiostrongyliasis, reported in

about 30 countries, with the majority of cases having been notified in Southeast Asia and Pacific Islands (Cowie 2013; Wang et al. 2008). In the Americas, the first reports of infection were confirmed in the USA (New et al. 1995), followed by occurrences in other countries, such as Jamaica (Slom et al. 2002), Haiti (Raccurt et al. 2003), Cuba (Dorta-Contreras et al. 2011), and Brazil (Thiengo et al. 2010). The most recent data, presented by Morassutti et al. (2014), confirm the occurrence of 34 human cases of neural angiostrongyliasis in Brazil, where it is classified by the Ministry of Health as an emerging parasitic disease.

Biologically, *A. cantonensis* presents a heteroxenous life cycle, involving the sequential participation of two hosts, where terrestrial and aquatic snails act as intermediate hosts and rodents (mainly *Rattus rattus* and *Rattus norvegicus*) are the natural definitive hosts. According to Caldeira et al. (2007), the low specificity established between the nematode and its intermediate host is the main factor for the rapid dispersion of the parasite. Among the different species of mollusks that participate in this parasite's development, particular attention has been focused on *Achatina fulica*, which has often been found to have a high prevalence of natural infection (Thiengo et al. 2010). This situation was demonstrated by Oliveira et al. (2014). According to the authors, the prevalence rate found was greater than 50%, but without a pattern of variation during the months studied. This information is in line with the results published by Maldonado et al. (2010), who also observed high prevalence of the nematode in specimens of *A. fulica* collected in the South and Southeast regions of Brazil. These findings are important, since Brazil and other regions of the world are experiencing an explosive invasion of this gastropod, a condition that is propitious for the rapid dispersion of this zoonosis (Lima et al. 2009; Lv et al. 2009).

Therefore, because of the importance attributed to gastropods in the transmission of *A. cantonensis*, studies of the parasite-host interaction have been conducted as part of a strategy to develop measures to control the snail and consequently the pathogens transmitted by it (Tunholi-Alves et al. 2012, 2014, 2015). According to these studies, infection by *A. cantonensis* induces significant metabolic alterations in *Biomphalaria glabrata*, a potential intermediate host, characterized by depletion of polysaccharide reserves, activation of anaerobic fermentative pathways, increased deamination rate of amino acids, accumulation of nitrogen degradation compounds, and an intense lipolytic process. Despite these findings, little is known about the alterations that occur during the development of *A. cantonensis* in *A. fulica* and the influence of different parasite loads on the snail's metabolic status, an important parameter already studied in interaction models involving mollusks-trematodes (Bandstra et al. 2006; Tunholi et al. 2013, 2016). Therefore, this study was conducted to shed

more light on the *A. cantonensis*-*A. fulica* interface and determine the impacts of different parasite loads on the metabolic profile of infected snails.

Material and methods

Maintenance of the snails and formation of groups

The snails used in this study were obtained from a colony kept in the Laboratório de Biologia e Parasitologia de Mamíferos Silvestres e Reservatórios, Instituto Oswaldo Cruz (Fiocruz), located in the city of Rio de Janeiro, Brazil. Nine experimental groups were formed: one control group (uninfected) and eight groups individually exposed to different quantities of larval nematodes (500, 1000, 1500, 2000, 2500, 5000, 10,000, and 15,000 L₁ larvae). Each group contained ten snails, reared in the laboratory from hatching, to be certain of their age and that the snails were free of infection by other parasites. The entire experiment was conducted in duplicate, using a total of 180 snails, of which 20 snails constituted the control group and 160 snails the challenged groups. The terrariums were kept in a room with controlled temperature of 25 °C throughout the experiment. The snails were fed with fresh lettuce leaves (*Lactuca sativa* L.) ad libitum.

The terrariums were cleaned every other day, when the lettuce leaves were replaced to prevent their fermentation.

Parasites

The strain of *A. cantonensis* studied was isolated by collecting naturally infected *A. fulica* specimens in São Gonçalo (Rio de Janeiro state) (22° 49' 37" S; 43° 03' 14" W). The cycle was maintained in the Laboratório de Biologia e Parasitologia de Mamíferos Silvestres Reservatórios (IOC) FIOCRUZ—Rio de Janeiro by passages in *R. norvegicus* (Wistar) used as definitive host and *Biomphalaria glabrata* as intermediate host, with the permits for the use of animals obtained from Oswaldo Cruz Foundation (FIOCRUZ) Ethical Committee on Animal Use (permit number LW 24/10). The first-stage larvae (L₁) utilized in this study were obtained from this experimental cycle maintained in the Laboratory of Patologia do Instituto Oswaldo Cruz (Fiocruz).

Infection of the snails

The feces of parasitized *R. norvegicus* were collected and used to obtain the larvae by the technique of Baermann, employed to separate and decant the L₁ larvae (Willcox and Coura 1989). After processing the fecal samples, specimens of *A. fulica* with 90 days of age in average were exposed individually to different quantities mentioned above.

The determination of the different quantities of L₁ larvae was performed according to Bonfim et al. (2014). Briefly, after decantation and separation of L₁ larvae by the technique Baermann, a 100 µL aliquot was analyzed in magnifying glass to estimate the number of larvae. From this relationship, the parasite load was estimated.

The L₁ larvae of *A. cantonensis* were spread on pieces of fresh lettuce leaves, which were in turn placed in Petri dishes with moistened filter paper at the bottom. The snails were added over the lettuce leaves. The Petri dishes were closed, and the snails were maintained in contact with the larvae overnight. After this, they were transferred to terrariums and maintained as described above.

Dissection and collection of the hemolymph and tissues

At the end of the 3-week experimental period, the snails from control and infected groups were dissected and the hemolymph was collected by cardiac puncture and the tissues separated (DGG and CM), stored in Eppendorf tubes and maintained at –80 °C until the biochemical analyses. All the samples were kept in an ice bath during dissection. The choice of the study period (3 weeks) was based on Tunholi-Alves et al. (2011), period that corresponds to the prepatent development of *A. cantonensis*.

Determination of glucose concentration and LDH activity

The determination of the glucose was performed according to Tunholi et al. (2013). For this, 10 µL of sample was added to 1 mL of color reagent (0.05 M phosphate buffer solution, pH 7.45 ± 0.1; 0.03 mM aminoantipyrine and 15 mM of sodium p-hydroxybenzoate; 12 kU of glucose oxidase; and 0.8 kU peroxidase per liter). The product formed by oxidation of 4-aminoantipyrine was determined by spectrophotometry with maximum absorption at 510 nm, using a standard solution of glucose at a concentration of 100 mg/dL (Mello-Silva et al. 2010). The readings were expressed in milligram per deciliter.

The determination of LDH activity was conducted according Tunholi-Alves et al. (2014). The readings were taken in a spectrophotometer at 505 nm and the results were expressed in UI.

Determination of the glycogen concentration

The glycogen contents of the DGG and cephalopedal mass were determined according to the method 3.5 DNS (Sumner 1924; Pinheiro and Gomes 1994) and expressed as milligram glucose per gram tissue, wet weight. The samples used for biochemical analyses were obtained from ten snails/experimental group analyzed individually.

Chemicals

Standards of oxalic, succinic, pyruvic, and lactic acids were purchased from Sigma–Aldrich (Steinheim, Germany) in the highest purity grade available. Acetonitrile, sodium dihydrogen phosphate, and phosphoric acid were of analytical purity or for chromatographic use. Ultrapure water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Stock standard solutions were dissolved in mobile phase, phosphate buffer adjusted to pH 2.2 with phosphoric acid, and stored at 4 °C.

HPLC analysis

All HPLC experiments were carried out in a Shimadzu LC-20AT system equipped with photodiode array detector (PDA; SPD-M20A, Shimadzu, Japan) coupled to an LC Solution ChemStation data processing station. Separations were carried out with reversed phase column C18 (150 × 4.5 mm I.D., 5 µL, Allure® Organic Acids, Restek) in isocratic conditions. The mobile phase consisted of 1% acetonitrile in 20 mol L⁻¹ NaH₂PO₄ aqueous solution, adjusted to pH 2.2 with H₃PO₄. The temperature was set at 36 °C and the flow rate was 0.8 mL/min. The chromatograms were monitored at 210 nm and the injection volume was 20 µL. The identification of organic acids present in the samples was based on a comparison of UV spectra and retention times with those of the pure standard solutions. Quantification was performed on the basis of linear calibration plots of peak area against concentration. Calibration lines were constructed based on five concentration levels of standard solutions. The calibration graphs for oxalic, succinic, pyruvic, and lactic acids were linear ($r = 0.99$) in all cases. All experiments were performed in triplicate.

The hemolymph was vortexed and centrifuged for 10 min at 2520g. The supernatant was separated and undissolved particles were removed by filtration using 45-µm membrane filters. Aliquots of 20 µL were used for the chromatographic analysis.

Hexokinase activity

DGGs were homogenized in 1 mL extraction buffer containing 20 mM Tris–HCl, pH 7.5 and centrifuged at 10,000×g for 10 min. The supernatant was assayed for hexokinase (HK) activity in 20 mM Tris–HCl pH 7.5 containing 6 mM MgCl₂, 1 mM ATP, 0.5 mM NAD⁺, 10 mM NaF, and the reaction started with 2 mM glucose. The glucose 6-phosphate formed was measured by adding an equal volume of 20 mM Tris–HCl pH 7.5, 6 mM MgCl₂, 1 unit/mL glucose 6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, and 0.3 mM β-NAD⁺. The production of β-NADH was read at 340 nm using a molar extinction coefficient of 6.22 M⁻¹ as described by Galina and Da Silva (2000). The study was

performed using six snails/experimental group analyzed individually.

Histological analyses

After 3 weeks of infection, four specimens of each group were dissected and transferred to Duboscq-Brasil fixative (Fernandes 1949). The soft tissues were processed according to routine histological techniques (Humason 1979). The sections (5 μm) were stained using hematoxylin and eosin and observed under a Zeiss Axioplan light microscope; images were captured with an MRc5 AxioCam digital camera.

Infectivity analysis

The cephalopodal masses of ten snails from each group infected with *A. cantonensis*, obtained 21 days after infection, were individually fragmented and digested in a 0.7% HCl solution for 6 h and subsequently subjected to the method of Baermann for L_3 recovery. The larvae were counted with the aid of a stereoscopic microscope and a manual cell counter (Bonfim et al. 2014).

Statistical analyses

The results were expressed as mean \pm standard deviation and submitted to one-way ANOVA and then the Tukey–Kramer test ($P < 0.05\%$) to compare the means (InStat, GraphPad, v.4.00, Prism, GraphPad, v.3.02, Prism Inc.).

Results

The infection by *A. cantonensis* induced significant changes in the oxidative metabolism of *A. fulica*, with a load-dependent response. For the control group, due to the absence

of variation in the parameters analyzed, the numerical values, expressed as (mean \pm standard deviation), were grouped in a single value called zero load, to better reveal the variations between the parasitized groups.

As a main result, a significant reduction in the hemolymph glucose content was observed only in the groups exposed to a load greater than or equal to 5,000 L_1 larvae (0.22 ± 0.023 mg/dL), differing significantly from the control group. This represents a reduction of approximately 77.77% in relation to the uninfected snails (0.99 ± 0.061 mg/dL), where the lowest concentration was observed in the group submitted to a load of 15,000 L_1 larvae (0.17 ± 0.054 mg/dL) (Fig. 1a). That variation was accompanied by activation of allosteric centers related to regulation of the glycolytic pathway, including hexokinase (HK), an enzyme involved in the first stage of oxidation of glucose molecules. The activity of HK was significantly greater in the groups submitted to a load higher than 2,500 L_1 larvae (0.2 ± 0.015 U/mg of tissue), indicating the importance of the pathway to maintain the host's energy status (Fig. 1b).

This metabolic scenario was accompanied by activation of the fermentative anaerobic metabolism, characterized by an increase in the activity of lactate dehydrogenase (LDH). This increase was observed only in the experimental groups with the largest parasite loads, of 10,000 (4.65 ± 0.25 UI) and 15,000 (4.82 ± 0.35 UI) L_1 larvae, differing significantly in relation to the control group (3.57 ± 0.42 UI) (Fig. 2a).

To better understand the impact of the infection on the oxidative metabolism of *A. fulica*, we also measured the concentrations of some carboxylic acids in the hemolymph, as important bioindicators of transition from aerobic to anaerobic pathways. The main finding was a significant decrease in the contents of oxalic acid in the groups exposed to 2,500 (36.89 ± 5.25 mM) and 5,000 (32.76 ± 4.35 mM) L_1 of *A. cantonensis*, differing significantly from the average value of the control group (64.55 ± 5.6 mM) (Fig. 2b). This

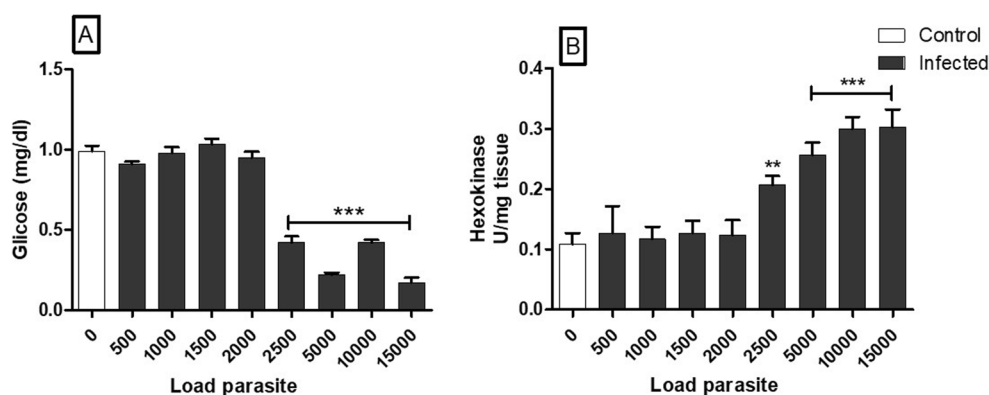
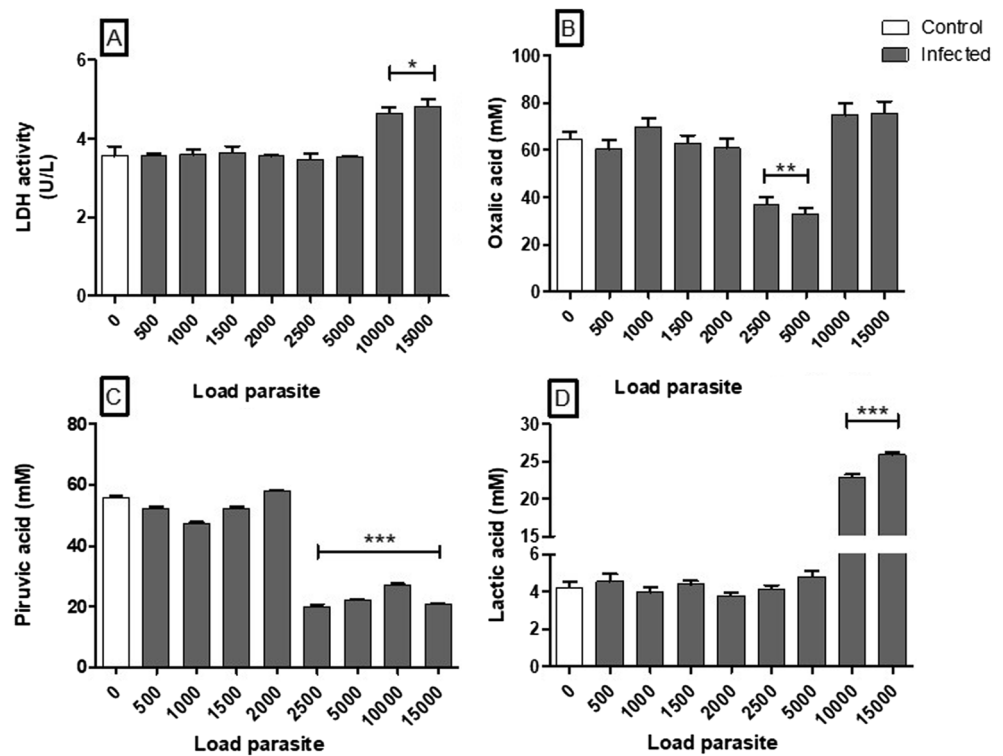


Fig. 1 Glucose concentration (mg/dL) (a) in the hemolymph and hexokinase activity (U/mg tissue) (b) in the digestive gland of *Achatina fulica* experimentally infected by different parasite loads (500, 1000, 1500, 2000, 2500, 5000, 10, 000, and 15, 000 L_1 larvae) of

Angiostrongylus cantonensis. Values are expressed as (mean \pm standard deviation). **/** indicate means differ significantly in relation to the control group, $P < 0.01$; $P < 0.05$, respectively

Fig. 2 Lactate dehydrogenase activity (UI) (a) and contents of organic acids (oxalic (b), pyruvic (c), and lactic acid (d)) (mM) in the hemolymph of *Achatina fulica* infected by different parasite loads (500, 1000, 1500, 2000, 2500, 5000, 10,000, and 15,000 L₁ larvae) of *Angiostrongylus cantonensis*. Values are expressed as (mean \pm standard deviation). */**/*** indicate means differ significantly in relation to the control group, $P < 0.05$, $P < 0.01$, and $P < 0.001$



tendency was also observed for the pyruvic acid contents, where the lowest concentrations occurred in the groups exposed to the highest parasite loads. The infection by 15,000 L₁ of *A. cantonensis* (20.78 ± 0.65 mM) resulted in a decline of approximately 62.79% in the pyruvic acid content, differing from the mean of the control group (55.85 ± 0.87 mM) (Fig. 2c). In contrast to these parameters, the exposure to infective larvae induced an accumulation of lactic acid in the hemolymph of *A. fulica*, where the highest values were found in the groups exposed to 10,000 (22.89 ± 0.72) and 15,000 larvae (25.9 ± 0.55 mM), increases of 422 and 513%, respectively, different from the average value of the uninfected snails (4.22 ± 0.55 mM) (Fig. 2d).

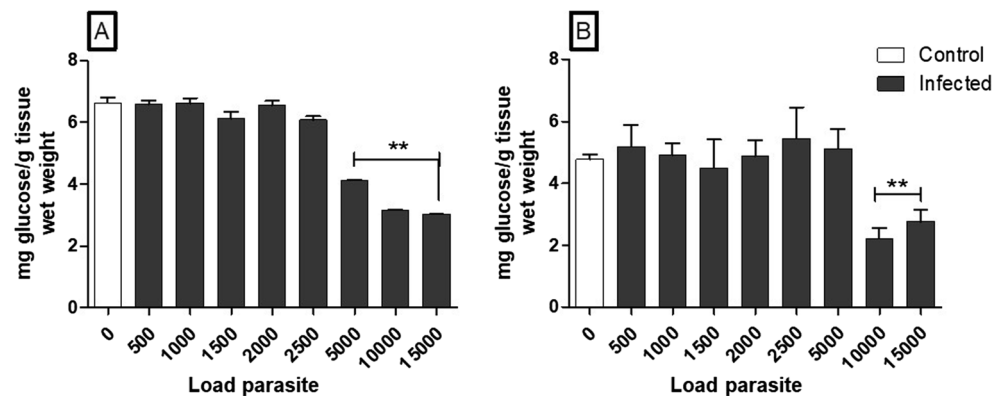
Variations in the level of polysaccharide reserves were found in both sites analyzed. There was a significant decrease

in the glycogen stored in the digestive gland only of the snails exposed to a parasite load greater than 5,000 L₁ larvae (4.13 ± 0.017 mg glucose/g tissue), representing a reduction of 37.70% in relation to the average of the control group (6.63 ± 0.31 mg glucose/g tissue) (Fig. 3a). The same tendency was observed for the level of glycogen stored in the cephalopedal mass, with the smallest values being in the snails exposed to the highest load, differing significantly from the uninfected mollusks (4.78 ± 0.28) (Fig. 3b).

Variations in the parasite load of the different experimental groups were observed, with the number of L₃ larvae recovered being proportional to the number of L₁ larvae to which the snails were exposed (Fig. 4).

Histopathological changes were also observed indicating the presence of granulomatous reactions in different tissues,

Fig. 3 Glycogen content in the digestive gland (a) and cephalopedal mass (b), expressed in milligram glucose per gram tissue, fresh weight, in *Achatina fulica* experimentally infected by different parasite loads (500, 1000, 1500, 2000, 2500, 5000, 10,000, and 15,000 L₁ larvae) of *Angiostrongylus cantonensis*. ** indicate means differ significantly in relation to the control group, $P < 0.01$



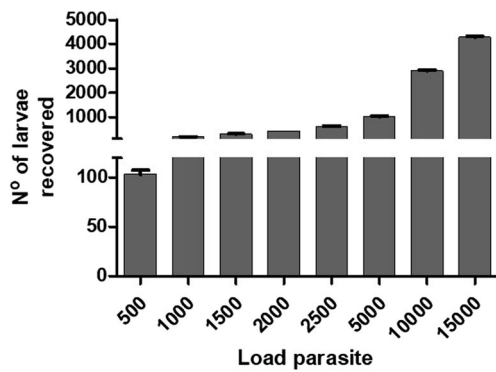


Fig. 4 Number of L₃ recovered at the end of 3 weeks of infection

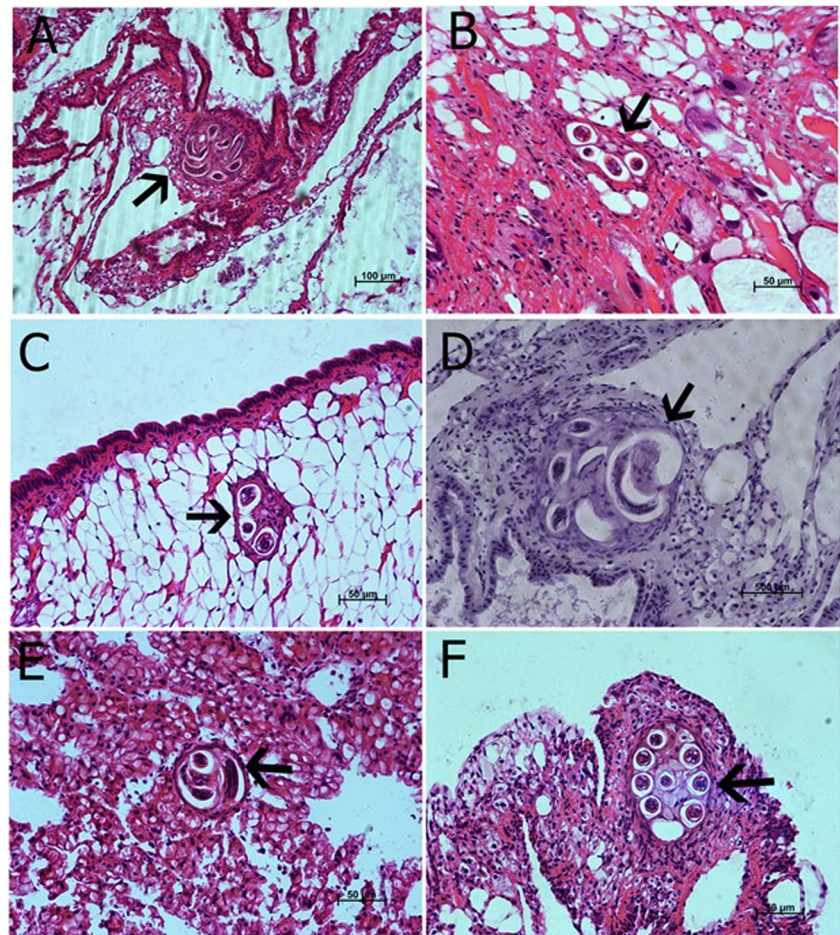
including mufla, kidneys, cephalopedal mass, and mantle, with increased cellularity, formation of vacuoles, and presence of degenerative processes (Fig. 5).

Discussion

Control of the energy metabolism is an essential life process (Fraga et al. 2013). In pulmonate gastropods, the oxidative

metabolism is principally maintained by metabolization of the carbohydrates that are stored in the form of glycogen in specific tissues, such as the digestive gland, cephalopedal mass, and mantle (Pinheiro et al. 2009). Under conditions of physiological stress, these reserves are drastically mobilized, allowing the formation of glucose units that will integrate the metabolic pathways in the cells, for production of adenosine triphosphate (ATP). This process occurs through activation of multiple reaction steps, where, besides glycogenolysis, the glycolytic pathway and Krebs cycle play central roles (Bezerra et al. 1997; Massa et al. 2007; Tunholi-Alves et al. 2014) (Fig. 1). This situation was demonstrated by El-ansary et al. (2000), in studying the impact of infection by *Schistosoma mansoni* on the carbohydrate metabolism of *B. alexandrina*. According to the authors, the reduction of the glucose level in the hemolymph occurred in parallel with a significant increase in the activity of hexokinase, pyruvate kinase, and glucose phosphate isomerase in snails after 2 weeks of infection in relation to the control group. That finding indicates that in infected snails, the increased demand for energy is supplied by acceleration of the glycolytic pathway, resulting in the formation of intermediate compounds that are essential to the host's survival and the complete

Fig. 5 A perilarval reaction (→) in the kidney (a), mufla (b), cephalopedal mass (c). Perilarval reaction between the kidney and the wall of the mantle cavity, with the carbohydrate concentrations evidenced by the presence of matrix elements, in the larvae (d). Presence of larvae in the digestive gland (e) and mufla (f)



development of the parasite. In the present study, we only observed a significant reduction in the hemolymph glucose levels in the groups exposed to a parasite load greater than 2.500 L₁ of *A. cantonensis*. According to Tunholi et al. (2013, 2016), the variations observed in the metabolism of infected mollusks result from the action of the developing larval stages of the parasite, characterized not only by tissue damages, but also due to the direct competition for nutrients and the secretion/excretion from the parasite's own metabolism, which alters the composition of the internal medium. Therefore, because previous studies have confirmed the presence of enzymatic centers related to the glycolytic pathway in the larval stages of *A. cantonensis* (Shih and Chen 1982), our results indicate that glucose is used as a source of energy by the parasite. This condition was demonstrated by Wang et al. (2013), who characterized the transcriptomic profile of larval stages of *A. cantonensis*. According to the authors, a significant proportion of amino acid sequences were associated with the activation of metabolic pathways of the nematode, including those related to the metabolism of carbohydrates and oxidative pathways involved in the production of energy. Together, these findings confirm the ability of the nematode to capture glycidic sources from the host organism, including glucose, as a plastic and energy substrate for its development, contributing to the establishment of a hypoglycemic state in *A. fulica*.

With respect to the reduction in the content of glucose in the hemolymph, the infection by *A. cantonensis* increased the activity of HK, one of the allosteric centers involved in regulating the glycolytic pathway. Moraes et al. (2007), studying the metabolism of carbohydrates during embryogenesis of the tick *Rhipicephalus Boophilus microplus*, observed a relative increase in the catalytic action of this enzyme, indicating that glucose is the main substrate related to the cellularization process of that arthropod. In this circumstance, since the energy demand rises abruptly, polysaccharide reserves are mobilized from specific regions of the snail to assure maintenance of energy homeostasis. Therefore, the authors attributed the increased activity of HK as a response to glycogenolysis, to assure the phosphorylation of glucose molecules, and consequently, the synthesis of molecules with high energy content, including ATP and NADH, which are essential to support important cell functions. In this study, since the infection induced physiological alterations similar to those described by Moraes et al. (2007), the increased catalytic action of HK in the groups exposed to a load higher than 2.500 L₁ larvae of *A. cantonensis* probably resulted from greater energy demand by the host during the parasitic development.

We also noted a significant depletion of polysaccharide reserves in the digestive gland and cephalopedal mass. The largest variations occurred in the group exposed to a parasite load of 15,000 L₁ larvae. This situation, besides indicating the existence of precisely regulated homeostatic mechanisms

among the tissues and hemolymph, indicates a load-dependent response pattern, corroborating the results previously published by Brockelman and Sithithavorn (1980). According to them, the infection by 2000 *A. cantonensis* L₁ in *A. fulica* did not result in a decrease in the levels of glycogen in the host's digestive gland, since the values fluctuated near those of the control group, similar to our results. Therefore, even though glycogenolysis exercises a role in re-establishment of normoglycemia in these organisms, we believe that the maintenance of basal levels of glucose initially occurs through redirecting metabolic pathways, especially from use of non-glycidic substrates like lipids (Alves et al. 2014; Tunholi-Alves et al. 2012, 2014) and proteins (Tunholi et al. 2011), where the mobilization of glycogen occurs belatedly, depending on what is needed for maintenance and the intensity of the stressing agent.

Studies of snail-parasite interactions have demonstrated that the alterations in the metabolism of carbohydrates are frequently accompanied by changes in the oxidative profile of these organisms (Abou Elseoud et al. 2010; Bezerra et al. 1997; Tunholi et al. 2013). Based on spectrometry and liquid chromatography, we demonstrated that the developing larval states induce the activation of fermentative pathways in the host, characterized by overlap of enzymatic centers, such as lactate dehydrogenase, at the same time, culminating in suppression of others, including cytochrome and oxidoreductase. This condition, reported both in models involving trematodes and nematodes (Tunholi-Alves et al. 2014), over the long-term results in an energy deficit of the host, since the quantity of ATP generated by the reduction of pyruvate into lactate is significantly lower compared to complete oxidation of the molecules in the Krebs cycle and phosphorylation chain. According to Tunholi et al. (2016), this process probably results from the inhibitory action of parasite antigens released in the snail's hemolymph, inhibiting the catalytic activity of enzymes that compose the Krebs cycle and hence the electron transport chain. In this circumstance, maintenance of the host's redox balance occurs anaerobically by the reduction of pyruvate to lactate via LDH, guaranteeing reoxidation of NADH⁺. This step is essential to the host's survival, by allowing new glucose molecules to enter the glycolytic pathway, generating intermediate substances that will integrate important pathways in the cells.

We only noted the transition from aerobic to anaerobic metabolism in the snails exposed to parasite loads greater than or equal to 10.000 L₁ larvae, indicating that the alterations observed in the oxidative metabolism of *A. fulica* is strongly influenced by the parasite load. The infection induced an increase in the activity of LDH, contributing to accumulation of lactate in the hemolymph and reduction of pyruvate, a product and substrate of the reaction. A contrary variation profile was found in the snails exposed to parasite loads higher than 2500 and lower than 10.000 L₁ larvae. A significant reduction in the

levels of oxalic acid in the hemolymph of *A. fulica* was observed, differing from the values of the control group. Two mechanisms can explain the occurrence of this metabolic pattern:

- (I) - Acceleration of the oxide reduction reactions that integrate the tricarboxylic acid cycle. As mentioned, both the Krebs cycle and electron transport chain play central roles in the oxidative metabolism of gastropods, by guaranteeing complete oxidation of glucose molecules (Tunholi-Alves et al. 2014). In this context, acetylated compounds such as acetyl-CoA play a key role in the cell metabolism, by acting as the main fuel to assure the functioning of these pathways. However, for its complete metabolization, a condensation step is first necessary. This process is catalyzed by specific enzymatic centers, with oxaloacetate molecules being responsible for the incorporation of these compounds. Therefore, the decrease in the levels of this acid found in the infected snails indicates greater consumption of acetylated compounds in response to the acceleration of the reactions that compose the Krebs cycle, guaranteeing production of the energy necessary for the host to survive and the nematodes to develop fully.
- (II) - Another possibility is the diversion of the oxaloacetate molecules to gluconeogenesis. This metabolic route assures the production of glucose from non-glycemic substrates, in an attempt by the host to reestablish its normoglycemia. That condition has been considered by several researchers, indicating the metabolic plasticity of the snail when faced with infection, since glucose is the principal substrate related to the snail's energy metabolism.

To characterize the *A. fulica*-*A. cantonensis* interface better, we also conducted a histopathological study, as a tool to better understand the physiological alterations found in the host's oxidative metabolism. The results demonstrated the existence of perilarval granulomatous reaction in different tissues, including mufla, kidneys, cephalopedal mass, and mantle, with increased cellularity, formation of vacuoles, and presence of degenerative processes. Additionally, the PAS staining revealed the presence of glycogen granules in the larval stages of the nematode. This result demonstrates the parasite's ability to capture glucose monomers from the hemolymph of the intermediate host, contributing to the establishment of hypoglycemia in *A. fulica*.

The study reported here was the first to investigate the physiological changes in *A. fulica* exposed to different loads of *A. cantonensis*. The variations found indicate a load-dependent response, with activation of the fermentative pathway being observed in the groups with highest parasite loads. Furthermore, the infection induced activation of the glycolytic pathway, a metabolic situation characterized by a decline in

the levels of glucose in the hemolymph and increase in the activity of HK. This physiological condition was accompanied by marked glycogenolysis due to the depletion of the reserves of polysaccharides in the digestive gland and cephalopedal mass, the main sites for parasite development.

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