LUMEN
ET VIRTUS

# Alterations in the metabolism of nitrogenous products of *Bulimulus tenuissimus* (mollusca) in response to experimental infection with larvae of *Angiostrongylus cantonensis* (nematoda)

https://doi.org/10.56238/levv15n38-037

**Florence Gonçalves Martins[1], Juberlan Silva Garcia[2] and Jairo Pinheiro[3]**

**RESUMO**

Mollusks are an essential requirement for the completion of the life cycle by the metaastrogilid nematode *Angiostrongylus cantonensis*, the causative agent of infections in domestic and wild animals, mainly rodents, and also of neural angiostrongyliasis or eosinophilic meningitis in humans. The terrestrial gastropod *Bulimulus tenuissimus* is widely distributed in the Brazilian territory and there are other species of the same genus that occur in Brazil and other countries, overlapping with the regions where there are reports on the occurrence of *A. cantonensis* and angiostrongyliasis. Despite this, the records in the literature about this species as an intermediate host for *A. cantonensis* are recent. The present study aimed to experimentally infect the mollusk *B. tenuissimus*, using L1 larvae of *A. cantonensis*, under laboratory conditions, and to monitor the physiological changes that occurred in the metabolism of nitrogen products. The concentration of total proteins increased in the first week (19.56%), returning to values close to those observed for the control group, which seemed to be related to tissue lysis, since the same pattern of variation was observed for AST and ALT activities. Associated with this, an inversion of the excretory pattern, becoming predominantly uricotelic, was observed in the second week after exposure, returning to the ureotelic pattern at the end of the pre-patent period. The results reveal that *B. tenuissimus* is an experimental host that presents a physiological response pattern to infection with *A. cantonensis* robust, being able to maintain its basic physiological processes, in addition to meeting the demand of the developing parasite.

**Palavras-chave:** Bulimulidae, Metastrogylidae, Angiostrongyliasis, Neural, Mollusk host.

[1] Postgraduate Course in Veterinary Sciences, Institute of Veterinary Sciences, UFRRJ, BR465, km7, Seropédica, RJ. BRAZIL. CEP 23.897-000. Postdoctoral Intern, Department of Physiological Sciences, Institute of Biological and Health Sciences. UFRRJ. BR465, km7, Seropédica, RJ. BRAZIL. CEP 23.897-000.
[2] LBPMR, FIOCRUZ. Avenida Brasil, 4365, Manguinhos, Rio de Janeiro, RJ. BRAZIL. CEP 21.040-360.
[3] Postgraduate Course in Veterinary Sciences, Institute of Veterinary Sciences, UFRRJ, BR465, km7, Seropédica, RJ. BRAZIL. CEP 23.897-000. Postdoctoral Intern, Department of Physiological Sciences, Institute of Biological and Health Sciences. UFRRJ. BR465, km7, Seropédica, RJ. BRAZIL. CEP 23.897-000.
Department of Physiological Sciences, Institute of Biological and Health Sciences. UFRRJ. BR465, km7, Seropédica, RJ. BRAZIL. CEP 23.897-000.

## INTRODUCTION

*Angiostrongylus cantonensis* (Chen, 1935) is a parasitic nematode, known as rat lungworm, because of its location during adulthood in its definitive hosts, the rodents. It was first described as *Pulmonema cantonensis* Chen, 1935, in Canton, China, during parasitological research in rats. The parasite can also infect other animals, wild and domestic, including ruminants, horses, dogs and birds, as well as humans, and is therefore considered a zoonosis (LINDO et al., 2002).

Infection in humans occurs through the ingestion of raw food containing the infecting larvae or by ingestion of intermediate or paratenic hosts. It is one of the main causes of eosinophilic meningoencephalitis, a disease that can be lethal. Human infection was first described in Taiwan in 1945, and since then several outbreaks with thousands of cases have been described (WANG et al., 2012). In Brazil, cases of neural angiostrongyliasis in humans have been described, the first record being presented at a congress by Graziela Maria Zanini and Carlos Graeff-Teixeira in two patients, in January 2007, in the State of Espírito Santo, Brazil (CALDEIRA et al., 2007).

The life cycle of *A. cantonensis* requires more than one host. Rodents, usually of the genus *Rattus* Fisher Waldhein, 1803 are definitive hosts, in which the adults of the parasite reproduce and the females release their eggs inside the pulmonary arterioles. In eggs, the first-stage larvae (L1) develop and hatch, migrating into the alveoli and from there to the pharynx where they are swallowed and through the gastrointestinal tract, they will be eliminated in the feces. Terrestrial and aquatic gastropod mollusks are the intermediate hosts and become infected through ingestion and/or penetration of these first-stage larvae. In the mollusk, the larvae go through two molts, and become infectious (L3) around 15 days. Mollusks are ingested bythe definitive hosts or by paratenic hosts, which can be planarians, frogs, crustaceans and lizards (WANG et al., 2008; MALDONADO et al., 2012).

The parasite has little specificity for the intermediate host and a great diversity of terrestrial and aquatic mollusk species has been described as host. The studies have been directed to the identification of the species involved in each region where *A. cantonensis* is detected in rodents and especially where human cases occur. The isolation and identification of *A. cantonensis* under laboratory conditions were recorded, for the first time in Brazil, from mollusks collected in the State of Espírito Santo, after the notification of two cases of angiostrongyliasis in humans. *Sarasinula marginata* (Semper, 1885) (Veronicellidae), *Subulina octona* (Bruguiëre, 1789) (Subulinidae), *Achatina fulica* (Bowdich, 1822) (Achatinidae) and *Bradybaena similaris* (Fèrussac, 1821) (Bradybaenidae) were collected, all containing larvae of *A. cantonensis* (CALDEIRA et al., 2007).

The family Bulimulidae Tryon, 1896, is mainly South American and includes medium to large mollusks (10 to 80 mm), with *Bulimulus tenuissimus* (d'Orbigny, 1935) being widely distributed throughout Brazil (MORRETES, 1949; OLIVEIRA & ALMEIDA 1999; SIMONE 2006).

They have an oval shell, measuring 12 to 20 mm in length, with five to six turns. Under laboratory conditions, the animals can reach up to 27 mm in length (SILVA et al. 2008).

In this family, it is the species that has parasitological importance, as it acts as an intermediate host of several parasites of domestic animals. In Brazil, Carvalho et al. (2012), in a collection of mollusks in an endemic area for *A. cantonensis* , examined some specimens belonging to the Bulimulidae family, generically identified as *Tomigerus* sp., *Pseudoxychona* sp., *Rhinus* sp., *Bulimulus* sp., which did not present infection by this parasite.

Oliveira et al. (2015) in a collection in the municipality of São Gonçaço, RJ collected together with other mollusks, *B. tenuissimus*, but this species did not present positivity for this parasite.

In this context, bulimulids have also been collected, in Brazil and in other countries, without, however, any record of the presence of A. *cantonensis* larvae (KIM et al., 2014; CARVALHO et al., 2012). However, recently, in a Master's Dissertation, defended in July 2017, at the Federal University of Sergipe Foundation, Jucicleide Ramos de Souza, collected 978 specimens of terrestrial mollusks, identified in 11 species and two genera: *A. fulica* (399 specimens), *Cyclodontina fasciata* (225), *Subulina octona* (135), *Bulimulus tenuissimus* (104), *Leptinaria unilamellata* (56), *Sarasinula linguaeformis* (30), *Streptartemon cookeanus* (7), *S. quixadensis* (2), *Omalonyx* sp. (7), *Alopeas gracille* (6), *Tamayoa banghaasi* (3), *Latipes erinaceus* (2), *Helicina* sp. (2). After obtaining nematode larvae by chemical digestion of soft tissues, the author performed the artificial infection in *Rattus norvegicus* (Berkenhout, 1769), of the Wistar lineage, and the nematodes (larvae and adults) recovered from mollusks and rodents had their DNA extracted, amplified towards the COI region (mitochondrial gene derived from Cytochrome Oxidase I) and sequenced for the specific identification of nematodes. Infection by *A. cantonensis* was recorded in mollusks from all municipalities investigated, involving three host species, and of the B. *tenuissimus* mollusks collected in Barra dos Coqueiros, SE, 4.3% were naturally infected with *A. cantonensis* (RAMOS-DE-SOUZA, 2017).

As a consequence of the alterations that have arisen in the metabolism of carbohydrates, caused by parasitism, the mollusks start to make use of other, alternative substrates to obtain energy, thus maintaining their basal physiological state and ensuring the development of the parasite.

The effect of infection by *A. cantonensis* on the activity of the enzymes alanine aminotransferase (ALT – E.C. 2.6.1.2) and aspartate aminotransferase (AST – E.C. 2.6.1.1) and the concentration of total proteins, uric acid and urea in the hemolymph of *B. glabrata* were investigated by Tunholi-Alves et al. (2012). Infection with *A. cantonensis* induced severe changes in the metabolism of the host mollusk, triggering physiological mechanisms to minimize the deleterious effects caused by the larvae. There was a significant decrease, which occurred gradually as the

infection progressed, in the concentration of total proteins in the infected moluccas. This change was accompanied by an increase in urea concentrations and a decrease in uric acid levels in the hemolymph, suggesting that in this model the infection induces proteolysis and inversion of the excretion pattern of infected hosts, revealing a change in the excretory pattern from uricotelic to ureothelic in these conditions. In addition, variations in aminotransferase activities were observed, with significantly higher levels in the infected groups than in the control group.

Lima et al. (2016), in their Doctoral Thesis work, observed that, in *B. straminea,* A strong positive relationship between the post-infection period and the hemolymphatic protein concentration ($R^2$=0.90) occurred, with an increase in the concentration of circulating proteins 11 days after infection in relation to the uninfected mollusks. However, in *B. tenagophilaHappened* a decrease throughout the observed period, especially in the period in which there is development of the first-stage larva (L1). A significant increase was observed in the activities of aminotransferases, AST and ALT, in *B. straminea*, confirming a direct relationship between the period of infection and enzyme activity. Similarly, the infection of *B. tenagophila* presented high values for the enzymatic activity of ALT, with its peak markedly visible in the third week of infection, when we already have the presence of third stage larvae (L3) completely formed inside the body of the host mollusk.

Infection by *A. cantonensis* caused alterations in the excretion metabolism of nitrogen products of both mollusk species. In *B. straminea,* 6 days after infection, there was a transition in the excretion pattern, with a decrease in uric acid concentration and an increase in circulating urea concentration in the hemolymph.

On the other hand, urea concentrations show an increase of 570.17% for mollusks, but at the next point of intramollusk larval development analyzed by this author, an inversion of this profile is observed, with an increase in uric acid after 11 days of infection and establishment of this behavior until the end of the observed period. Concomitantly, urea concentrations fall and remain low until 21 days post-infection.

Similar results were presented by the author for *B. tenagophila* with an inversion in excretion patterns, with a reduction in the hemolymphatic concentration of uric acid in the first days of infection with its lowest value in the first week of infection in contrast to the control group, with a reduction corresponding to 58.54%. The decrease in uric acid concentration was accompanied by an increase in the levels of urea available in the hemolymph in the same period, with its maximum value in the first week of infection. As in *B. straminea* heading towards the second week of infection, a new alternation is seen, now with an increase in uric acid levels and a drop in urea levels. Thus, in both models used by Lima et al. (2016), we observed the change in the excretory pattern from uricotelic to ureotelic as a result of the pre-patent development of intramolluscus larvae.

The mollusks *B. tenuissimus* were described as an intermediate host of *A. cantonensis* for the first time by Martins et al. (2018), under experimental conditions, and by Ramos-de-Souza et al. (2018), under natural conditions. Therefore, little is known about physiological aspects of the mollusk-nematode larval interaction at this interface. In view of the state of the art regarding the study of the interaction between *B. tenuissimus-A. cantonensis*, the present work aimed to carry out experimental infections of this mollusk with L1 larvae of *A. cantonensis*, seeking to analyze the changes in the metabolism of host nitrogen products over the pre-patent period of infection.

## MATERIAL AND METHODS

### COLLECTING AND MAINTAINING MOLLUSCS

The mollusks *B. tenuissimus* were collected manually from a vegetable garden in the municipality of Seropédica at Km 46 of the old Rio-São Paulo road (Latitude: 22º 44' 38" S; Longitude:43º 42' 27" W; Altitude: 26m) and taken to the Biophysics Laboratory at the Department of Physiological Sciences (ICBS-UFRRJ) where they were kept in plastic terrariums (31 x 21 x 10 cm) containing 3 cm of sterilized vegetable soil (120oC for 1 hour) at the bottom. Each terrarium thus prepared housed 60 mollusks that were fed fresh lettuce leaves (*Lactuca sativa*), carrot slices (*Daucus carota*), chayote (*Sechium edule*) and cucumber (*Cucumis sativus*) and crushed and sifted poultry feed enriched with calcium carbonate in a ratio of 3:1 (SILVA et al., 2008). The diet was offered in plastic containers of 3 cm in diameter and 0.5 cm in height. The maintenance of the terrariums was done three times a week, when they were sprinkled with water, if necessary, and the food was changed; The earth was also turned over to locate the eggs and the dead animals were removed.

The postures thus collected gave rise to the mollusks used in this experiment. The counted eggs were separated in petri dishes on damp paper towels until the hatching of the chicks. These were kept in plastic containers (11cm x 7cm x 4cm) with a diet of lettuce, carrot, chayote and cucumber on moistened paper towels for 15 days. The food was only offered after this period, in 1 cm diameter containers with 0.2 cm height, to reduce the mortality of the neonates (the puppies entered the containers containing the food, but could not get out) (Fig. 4).

These mollusks, thus raised in the laboratory, were used only when they reached sexual maturity, with the beginning of laying, which occurred when the animals reached 90-120 days of age.

### OBTAINING THE PARASITES

The parasites used in this study were obtained from an experimental cycle maintained at the Laboratory of Biology and Parasitology of Wild Mammals Reservoirs (LABPMR) of the Oswaldo Cruz Foundation, RJ, Brazil. This cycle began from third-stage larvae of *A. cantonensis* obtained

from mollusks of the species *Achatina fulica* collected in the municipality of São Gonçalo, RJ (22º 48' 26.7"S, 43º00'49.1"W). The cycle is maintained using *Rattus norvegicus* as the definitive host and *Biomphalaria glabrata as* an intermediate host and has the experimental protocol approved by the Ethics Committee for the Use of Animals (CEUA LW 47-14)

## EXPERIMENTAL INFECTION

The feces of *R. norvegicus* were collected and processed according to the Baermann technique (WILLCOX & COURA, 1989) to collect the L1 larvae of *A. cantonensis.*

After fasting for 24 hours, B. *tenuissimus* mollusks were individually placed in 24-well plates where 1200 L1 larvae of *A. cantonensis* were placed on small pieces of cucumber, being kept for 24 h, then being removed from the wells and transferred to prepared terrariums as previously described.

## FORMATION OF THE GROUPS

The infection was followed for three weeks. The mollusks were divided into infected and control groups. In all, 18 groups were formed with ten mollusks each, nine of which were infected (three groups per week) and nine more control groups, uninfected (three groups per week). Each group of ten mollusks was kept in a 12cm x 8 cm plastic pot with 3cm of soil and were followed as described above.

The population density in the containers (10 mollusks/terrarium) was maintained throughout the study through groups of mollusks that were subjected to the same conditions, called the replacement group. As the mollusks died, new mollusks from the replacement group were transferred to the terrariums, in order to maintain population density.

## DISSECTION AND MATERIAL COLLECTION

Each week after infection, the shells of the mollusks were washed and dried on paper towels. The hemolymph of the mollusks was collected by breaking the apex of the shell while the cephalopedius mass was gently pushed into the shell. The hemolymph leaked and was collected in microtubes kept in an ice bath at 10ºC during collection and later stored at -20ºC until the hemolymph was used for biochemical analysis (Fig. 5).

The shells were removed, dried in an environment and stored wrapped in paper towels for later calcium quantification.

After the shells were removed, the mollusks were dissected on ice and the tissues of the cephalopedia mass, digestive gland and albumen gland were kept in an ice bath at 10ºC during collection, being weighed afterwards and then stored at -20ºC until its use.

After the pre-patent period of infection with *A. cantonensis*, specimens of *B. tenuissimus* from each infected group were subjected to chemical digestion with pepsin-HCl for recovery of L3 larvae, according to Thiengo et al. (2010).

## DETERMINATION OF CONCENTRATIONS OF GLUCOSE, TOTAL PROTEINS, UREA, URIC ACID AND ACTIVITY OF LACTATE DEHYDROGENASE, ASPARTATE AMINOTRANSFERASE AND ALANINE AMINOTRANSFERASE ENZYMES

For the analysis of the parameters concentration of glucose, total proteins, urea and uric acid, and enzymatic activity of ALT, AST and LDH, the automated biochemical analyzer A15 (Biosystems®) was used, and a dilution of the centrifuged matrix sample, without cells, in a proportion of 1:1 with Milli-Q water.

For the determination of total protein concentration, 4 µL of dilute sample was added to 300 µL of color reagent (copper acetate 6 mmol. $^{L-1}$, potassium iodide 12 mmol. $^{L-1}$, sodium hydroxide 1.15 mol. L-1). The standard used was bovine serum albumin, at a concentration of 50 g.L-1. The readings were taken in a spectrophotometer at 540 nm, and the results were expressed in g.L-1 (GORNALL et al., 1949).

To determine the urea content, 10µL of hemolymph were added to 1.5 µL of the working solution containing (Tris 100 mmol/L, 2-oxoglutarate 5.6 mmol/L, urease >140 U/mL, glutamate dehydrogenase > 140 U/mL, ethylene glycol 220g/L, sodium azide 0.95g/L, pH 8 + NADH 1.5 mml/L, sodium azide 9.5 g/L). The formed product was determined by spectrophotometry at a wavelength of 340 nm, using a standard urea solution at 50 mg/dL, being expressed in mg/dL.

To determine the uric acid content, 1 mL of working reagent (155 mM buffer, 0.1 mM 4-aminoantipyrine ≥, peroxidase ≥ 1,000 U/L, 0.02% sodium azide and surfactants + 155 mM buffer, 3.5-dichloro-2-hydroxybenzene sulfonate (DHBS) acid (DHBS) ≥ 2.5 mM, uricase ≥ 300 U/L, sodium azide 0.02% and surfactant). The absorbances were read by spectrophotometry, at 505 nm, against reaction blank and using a standard solution of uric acid 6 mg/dL, expressed in mg/dL.

For the evaluation of the enzymatic activity of ALT, 25 µL of diluted sample was added to 300 µL of reagent (Tris 150 mmol. $^{L-1}$, L-alanine 750 mmol. L-1, lactate dehydrogenase > 1350 U.L-1, pH 7.3, NADH 1.3 mmol. $^{L-1}$, 2-oxoglutarate 75 mmol. $^{L-1}$, sodium hydroxide 148 mmol. L-1, sodium azide 9.5 g.L-1). The product formed was determined by spectrophotometry at a wavelength of 340 nm, expressed in U/L1 (GELLA et al., 1985).

For the evaluation of the enzymatic activity of AST, 25 µL of diluted sample was added to 300 µL of reagent (Tris 121 mmol. $^{L-1}$, L-aspartate 362 mmol. L-1, malic dehydrogenase> 460 U.L-1, lactate dehydrogenase > 660 U.L-1, pH 7.8, NADH 1.3 mmol. $^{L-1}$, 2- Oxoglutarate 75 mmol. $^{L-1}$,

sodium hydroxide 255 mmol. L $^{-1}$, sodium azide 9.5 g.L-1). The results, expressed in U.L-1, were read in a spectrophotometer at 340 nm (GELLA et al., 1985).

### STATISTICAL ANALYSES

The results were expressed as mean ± mean standard error, submitted to ANOVA *Two-way* with Tukey-Kramer post-test (α=5%) to compare the means and polynomial regression analysis to evaluate the relationship between the time of infection and the physiological parameters analyzed (Prism, GraphPad, v. 5.00, Prism Inc.).

## RESULTS

The concentration of total proteins in the hemolymph of *B. tenuissimus* infected with *A. cantonensis* is shown in Table 6, where we observe that the proteinemia increased significantly only in the first week after exposure, when the total protein content reached a value 19.56% higher than that of the uninfected mollusks (15.30 ± 0.33 g/L), progressively returning to values close to those observed for the control group (0). When submitted to polynomial regression analysis, a positive but weak relationship was observed between the time of infection and the total protein content in the hemolymph (Fig. 1).

The urea content in the hemolymph of *B. tenuissimus* of the infected mollusks remained without significant variations until the second week after exposure to the larvae of *A. cantonensis* (Table 1), but in the third week of infection, we observed an increase of 308.32% in the concentration of this nitrogen excretion product in the hemolymph, in relation to the value observed for the group of uninfected mollusks (4.00 ± 1.53 mg/dL), which is the only significant variation observed in this parameter. Due to this variation, a strong positive relationship was observed between the urea content in the hemolymph of *B. tenuissimus* and the time of infection with *A. cantonensis* (Fig. 2).

In the first week after exposure of *B. tenuissimus* to L1 larvae of *A. cantonensis*, we observed a 100% increase in uric acid content in the mollusk's hemolymph (Table 5) compared to the control group (0.38 ± 0.12 mg/dL). With the evolution of the infection, we observed a new significant increase in uric acid concentration in the third week after exposure (0.39 mg/dL), reaching a value practically equal to that of uninfected mollusks. However, the comparison of means made by the Tukey-Kramer test revealed that there was no significant difference between the uric acid contents observed in the different pre-patent periods analyzed and that of the uninfected mollusks (0). The regression analysis revealed a weak positive relationship between the analyzed parameters (Fig. 3).

In the present study, the urea/uric acid ratio observed was 10.53 in the group of uninfected mollusks, being reduced in the second and third weeks after exposure to the larvae of *A. cantonensis*, second week = 2.63; third week = 2.56.

However, at the end of the analysis period, the third week, there was a significant increase to 27.22 in this ratio.

Table 1. Variation in the contents of total proteins (g/L), urea and uric acid, expressed in mg/dL, in *Bulimulus tenuissimus* hemolymph exposed to L1 larvae of *Angiostrongylus cantonensis*, experimentally, for three weeks. X ± SD = mean ± standard deviation. N = minimum number of coherent spectrophotometric determinations. a,b=means followed by different letters, differ from each other at the level of 5% of significance.

| Time of infection (weeks) | Total protein concentration (g/L) | Urea concentration (mg/dL) | Uric acid concentration (mg/dL) | N |
|---|---|---|---|---|
| 0 | 15,33 ± 0,33a | 4.00 ± 1.53a | 0.38 ± 0.12a | 3 |
| 1 | 18,33 ± 2,33b | 2.00 ± 0.58a | 0.76 ± 0.27b | 3 |
| 2 | 13,00 ± 1,00a | 1.00 ± 0.58A | 0.39 ± 0.07a | 3 |
| 3 | 14,33 ± 0,88a | 16,33 ± 1,67b | 0.60 ± 0.08b | 3 |

Figure 1. Relationship between the time of infection, in weeks, of *Bulimulus tenuissimus* with larvae of *Angiostrongylus cantonensis*, and the total protein content in the hemolymph of the mollusk over the pre-patent period of three weeks.

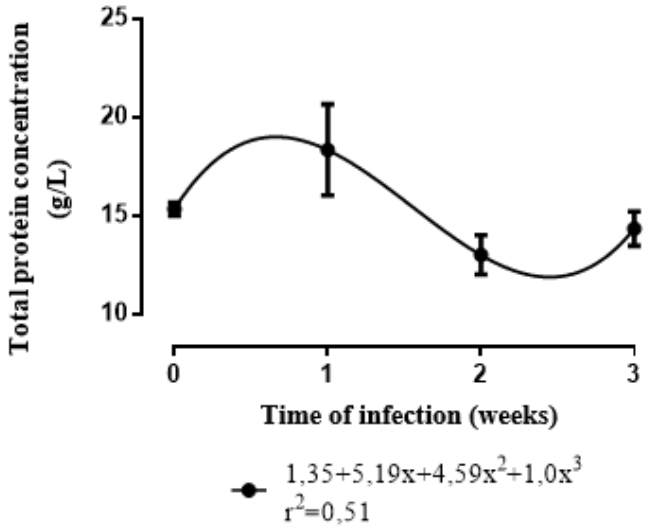


Figure 2. Relationship between the time of infection, in weeks, of *Bulimulus tenuissimus* with larvae of *Angiostrongylus cantonensis*, and the urea content in the hemolymph of the mollusk over the pre-patent period of three weeks.

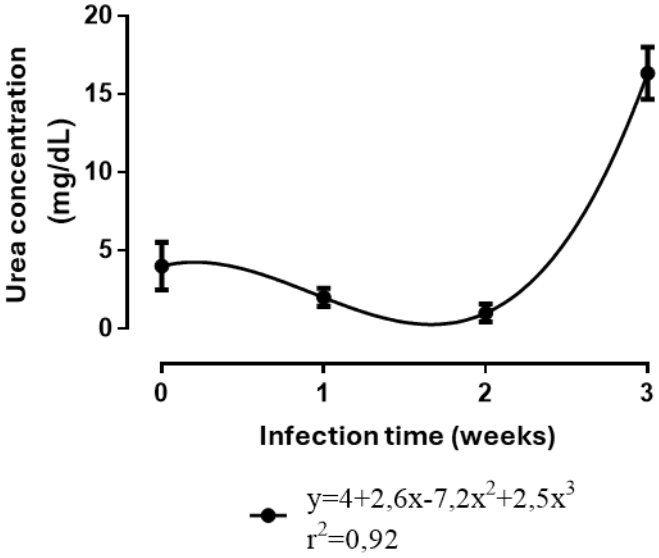
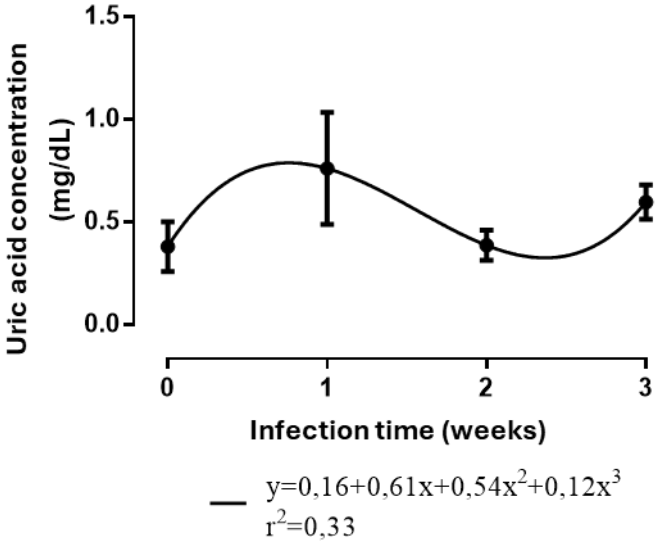



Figure 3. Relationship between the time of infection, in weeks, of *Bulimulus tenuissimus* with larvae of *Angiostrongylus cantonensis*, and the uric acid content in the hemolymph of the mollusk over the pre-patent period of three weeks.




The activity of aminotransferases, AST and ALT, was analyzed over the three weeks after exposure of *B. tenuissimus* to L1 larvae of *A. cantonensis*. AST activity increased in the first week after exposure, reaching values 96.50% higher than those observed for the control group (190.33 ± 4.98 U/L), which was significantly different from all the others observed in this experiment. From this period of infection, there was a progressive reduction in the activity of this enzyme in the second and third weeks of infection. Even with this reduction, AST activity remained different from that observed for uninfected mollusks (Table 2). Third-order polynomial regression analysis revealed a

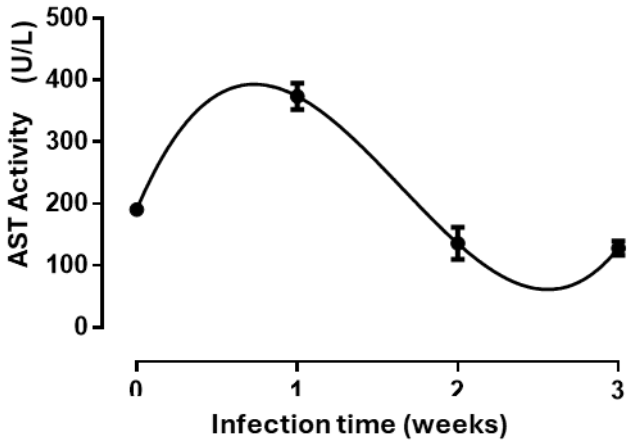
strong positive relationship between infection times and AST activity, indicating a trend toward an oscillating pattern of variation in AST activity (Fig. 4a).

ALT activity did not vary significantly in the first week of infection of *B. tenuissimus* with *A. cantonensis* (Table 6), although we obtained a higher value for the activity of this enzyme in this period of infection than in the control group (7.67 ± 0.67 U/L). From the second week of infection, ALT activity showed sequential reductions, remaining significantly lower than the activity of this enzyme in the hemolymph of uninfected mollusks. However, the relationship between ALT activity in hemolymph *B. tenuissimus* showed a weak positive relationship with the time of infection with *A. cantonensis* (Fig. 4b).

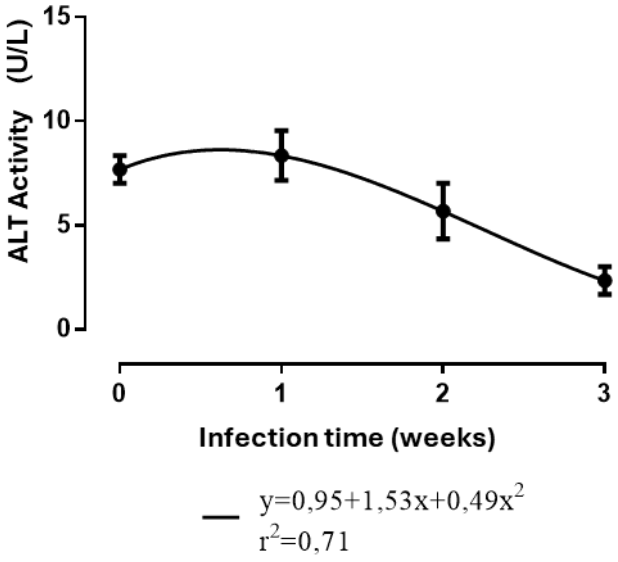
Table 2. Variation in the activity of AST (aspartate aminotransferase) and ALT (alanine aminotransferase) aminotrasphereases in *Bulimulus tenuissimus* hemolymph exposed to L1 larvae of *Angiostrongylus cantonensis*, experimentally, for three weeks. X ± SD = mean ± standard deviation. N = minimum number of coherent spectrophotometric determinations. a,b,c=averages followed by different letters, differ from each other at the level of 5% of significance.

| **Time of infection (weeks)** | **Aminotransferase activity** | | **N** |
|---|---|---|---|
| | **AST (U/L)** | **ALT (U/L)** | |
| 0 | 190,33 ± 4,98a | 7.67 ± 0.67a | 3 |
| 1 | 374,00 ±21,52b | 8.33 ± 1.20a | 3 |
| 2 | 135,67 ± 26,33c | 5.67 ± 1.33b | 3 |
| 3 | 128,00 ±11,53c | 2.33 ± 0.67b | 3 |

Figure 4. Relationship between the activity of aminotransferases in *Bulimulus tenuissimus* hemolymph and the time after exposure, in weeks, to L1 larvae of *Angiostrongylus cantonensis*, experimentally, for three weeks. A. Aspartate aminotransferase (AST) activity, expressed in U/L. B. Alanine aminotransferase (ALT) activity, expressed in U/L.




A




B

## DISCUSSION

In a broad review, Becker (1980) evidenced the metabolic alterations that arise in *B. glabrata* due to infection with *Schistosoma mansoni*, reporting the reduction that occurs in the glycemia of the infected mollusk, leading to consequent alterations in the contents of amino acids and proteins in the hemolymph of these intermediate hosts. Such alterations have also been evidenced in other host-parasite systems, involving both trematodes (Tunholi et al., 2011) and nematodes (Tunholi-Alves et al., 2012).

The knowledge accumulated by the various results obtained in our Research Group (GP Physiology of Mollusks of Medical-Veterinary Interest – Directory of Research Groups - CNPq), has shown that, although Livingstone and Zwaan (1983) point out that the energy metabolism of mollusks is based mainly on carbohydrates as substrates to obtain the energy necessary for their growth, development and maintenance of basal metabolism, we have observed that there are divergences in this aspect with regard to aquatic (freshwater) and terrestrial mollusks. In terrestrial mollusks, carbohydrate deposits are more significant in quantity than in aquatic mollusks, which indicates that in the latter, other substrates are used to a greater extent than in terrestrial mollusks under conditions of metabolic and physiological normality (Becker & Schmale, 1975).

In the present study, the physiological stress situation leads to an increase in the mollusk's glycemia in the first week of infection and a consequent reduction in glycogen deposits in the tissues of the cephalopediosus mass and digestive gland, evidencing the increased mobilization of the mollusk's glycidic energy reserves to compensate for the increase in demand that is established due to the development of intramollusk larvals (MARTINS et al., 2018).

Brockelman (1980) observed similar changes in *glucose content in A. fulica* infected with *A. cantonensis*. In 1978, Brockelman observed in this same intermediate host parasite-mollusk system, a reduction in total protein content from 1.77 mg% to 0.6 mg%, a 66% reduction in this value.

The intense migration and growth of L1 larvae until they complete their pre-patent development (from L1 to L3) causes a series of tissue damages, causing the extravasation of cellular proteins, and this physiological change may be what leads the mollusk to recruit a greater amount of proteins, probably from the injured tissues, which is evident with the increase in the activity of aminotransferases, AST and ALT in the hemolymph of *B. tenuissimus* in the first week after exposure to L1 of *A. cantonensis*.

Tunholi-Alves et al. (2012) observed an increase in AST activity in *B. glabrata* infected with *A. cantonensis*, with changes in the activity of both enzymes, with AST activity increased by up to 49.62% and ALT by 98.13% at the end of the three-week period of intramollusk larval development.

As a result, we observed a reduction in the concentration of urea and an increase in the concentration of uric acid in the hemolymph of *B. tenuissimus* in the first week after exposure to the larvae of *A. cantonensis*. With the continuation of intramollusk larval development, we observed a loss in the metabolic capacity to maintain the homeostasis of the metabolism of nitrogen products, because even with the decrease in the concentration of total proteins in the hemolymph to values close to those observed in the control group, there is a very significant increase in the concentration of urea in the hemolymph, and the content of uric acid, Although it was reduced in the third week after exposure, it still remained high in relation to the control group. Similar results were obtained by Souza et al. (2000) when analyzing and comparing the effects of infection by *E. coelomaticum* on the metabolism of nitrogenous products in *B. similaris*, with a reduction in urea content and an increase in uric acid content in mollusks at 30 days of infection with this trematode.

In this way, the hyperproteinemia that is established leads the mollusk to metabolize these proteins in higher than normal concentrations, forming a high amount of nitrogenous products, such as ammonia, which need to be detoxified by the excretory route. Terrestrial mollusks, due to their greater difficulty in maintaining their water balance, being exposed to excessive water loss and, therefore, to desiccation, seek physiological mechanisms that prevent excessive water loss. Thus, under normal conditions, these mollusks predominantly excrete urea as nitrogenous degradation/excretion products, exhibiting a predominant ureotelic excretory pattern. However, the excretion of urea leads to consequent water losses in significant quantities.

For this reason, when there is an increased production of urea by mollusks, such as in situations of starvation and infection by helminth larvae, in terrestrial molluscs, nitrogenous products tend to be predominantly excreted through the elimination of uric acid, which can be excreted in a

highly concentrated form and with little water loss to the mollusk, leading the mollusk to change its excretion pattern to uricotelic.

Becker & Schmale (1975) observed in *B. glabrata* maintained in severe fasting the change in urea concentration from 0.160 mg% to 5.12 mg%. According to Pinheiro (1996), in his study with *B. similars*, 30 days of severe fasting are similar to 104 days of infection with *E. coelomaticum* in terms of metabolic alterations observed. Thus, we can extrapolate the studies of these authors to comparisons with what should happen with *B. glabrata* infected with *S. mansoni*.

Tunholi-Alves et al. (2015), in a study with *A. fulica* experimentally infected with *A. cantonensis*, observed a reduction in the preotheinemia of infected mollusks, and this change was accompanied by significant increases in the concentration of urea and uric acid, suggesting the occurrence of an intense deamination of amino acids in the hemolymph of these hosts as a result of the infection. These authors also suggested that the increased activity of AST and ALT could be related to the use of proteins to obtain amino acids, objectifying their direction to the gluconeogenesis pathway to obtain de novo glucose, thus constituting an alternative way of obtaining energy by the mollusks due to the increased energy demand resulting from the continuous withdrawal of nutrients by the developing parasites and the need for maintenance of the basal metabolism of the hosts.

## ACKNOWLEDGMENT

Funding: This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001. This work was supported by the Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ – Process number E26-203.004/2016) and Conselho Nacional para o Desenvolvimento Científico e Tecnológico (CNPq – Process number 447211/2014-5).

## REFERENCES


1. Becker, W. (1980). Metabolic interrelationship of parasitic trematodes and molluscs, especially Schistosoma mansoni in Biomphalaria glabrata. *Zeitschrift fur Parasitenkunde, 63*, 101–111.

2. Becker, W., & Schmale, H. (1975). The nitrogenous products of degradation ammonia, urea and uric acid in the hemolymph of the snail Biomphalaria glabrata. *Zeitschrift fur Parasitenkunde, 51A*, 407–411.

3. Brockelman, C. R., Chusatayano, W., & Baidikul, V. (1976). Growth and localization of Angiostrongylus cantonensis in the molluscan host, Achatina fulica. *The Southeast Asian Journal of Tropical Medicine and Public Health*, 1, 30-37.

4. Brockelman, C. R. (1978). Effects of parasitism and stress on hemolymph protein of the African giant snail, Achatina fulica. *Parasitology Research, 57*, 137–144.

5. Caldeira, R. L., Mendonça, C. L. F., Goveia, C. O., Lenzi, H. L., Graeff-Teixeira, C., Lima, W. S., Mota, E. M., Pecora, I. L., Medeiros, A. M. Z., & Carvalho, O. S. (2007). First record of molluscs infected with Angiostrongylus cantonensis (Chen, 1935) (Nematoda: Metastrongylidae) in Brazil. *Memórias do Instituto Oswaldo Cruz, 102*, 887-889.

6. Carvalho, O. S., Scholte, R. G., Mendonça, C. L. F., Jannotti-Passos, L. K., & Caldeira, R. L. (2012). Angiostrongylus cantonensis (Chen, 1935) (Nematoda: Metastrongylidae) in molluscs from harbour areas in Brazil. *Memórias do Instituto Oswaldo Cruz, 107*, 740-746.

7. Gornall, A. G., Bordawill, C. S., & David, M. M. (1949). The determination of protein by the biuret reaction. *Journal Biology Chemistry, 177*, 751-780.

8. Kim, J. R., Hayes, K. A., Yeung, N. W., & Cowie, R. H. (2014). Diverse gastropod hosts of Angiostrongylus cantonensis, the rat lungworm, globally and with a focus on the Hawaiian Islands. *PLOS ONE, 9*, e94969.

9. Lima, M. G., Tunholi-Alves, V. M., Gaudêncio, F. N., Martins, F. G., Castro, R. N., Thiengo, S. C., Garcia, J. B., Maldonado, A., & Pinheiro, J. (2016). The influence of Angiostrongylus cantonensis (Nematoda, Metastrongylidae) infection on the aerobic metabolism of Biomphalaria straminea and Biomphalaria tenagophila (Mollusca, Gastropoda). *Experimental Parasitology, 171*, 1-9.

10. Lindo, J. F., Waugh, C., Hall, J., Cunningham-Myrie, C., Ashley, D., Eberhard, M. L., Sullivan, J. J., Bishop, H. S., Robinson, D. G., Holtz, T., & Robinson, R. D. (2002). Enzootic Angiostrongylus cantonensis in rats and snails after an outbreak of human eosinophilic meningitis, Jamaica. *Emergent Infectious Diseases, 8*, 324-326.

11. Livingstone, D. R., & De Zwaan, A. (1983). Carbohydrate metabolism of gastropods. In P. W. Hochachka (Ed.), *The Mollusca Metabolic Biochemistry and Molecular Biomechanics* (pp. 178-203). Academic Press.

12. Maldonado, A., Simões, R. O., & Thiengo, S. C. (2012). Angiostrongiliasis in the Americas. In J. Lorenzo-Morales (Ed.), *Zoonosis* (pp. 303-320). Rijeka, Croatia: In Tech.

13. Martins, F. G., Lima, M. G., Castro, R. N., Sant'Anna, L. D. O., Dos Santos, M. A. J., Garcia, J. S., & Pinheiro, J. (2018). Bulimulus tenuissimus (Mollusca) as a new potential host of

Angiostrogylus cantonensis (Nematoda), a histological and metabolic study. *Journal of Invertebrate Pathology, 154*, 65-73.

14. Morretes, F. L. (1949). Contribuição ao estudo da fauna brasileira de moluscos. *Papéis Avulsos do Departamento de Zoologia, 3*(7), 111-126.

15. Oliveira, A. P. M., Gentile, R., Maldonado Jr., A., Torres, E. J. L. T., & Thiengo, S. C. (2015). Angiostrongylus cantonensis infection in molluscs in the municipality of São Gonçalo, a metropolitan area of Rio de Janeiro, Brazil: role of the Achatina fulica in parasite transmission dynamics. *Memórias do Instituto Oswaldo Cruz, 110*(6), 739-744.

16. Oliveira, M. P., & Almeida, M. N. (1999). *Conchas dos caramujos terrestres do Brasil*. Juiz de Fora: Editar Editora Associada.

17. Ramos-de-Souza, J., Thiengo, S. C., Fernandez, M. A., Gomes, S. R., Corrêa-Antônio, J., Clímaco, M. D. C., Garcia, J. S., Maldonado-Junior, A., Barbosa, L., & Dolabella, S. S. (2018). First records of molluscs naturally infected with Angiostrongylus cantonensis (Nematoda: Metastrongyloidea) in Sergipe State, Northeastern Brazil, including new global records of natural intermediate hosts. *Revista do Instituto de Medicina Tropical de São Paulo, 60*, e51.

18. Ramos-de-Souza, J. R. (2017). *Pesquisa e Identificação de Larvas de Nematódeos Recuperadas de Achatina fulica Bowdich, 1822 e outros Moluscos Terrestres da Região Metropolitana de Aracaju, Sergipe* (Dissertação de Mestrado, Universidade Federal de Sergipe).

19. Silva, L. C., Meireles, L. M. O., Junqueira, F. O., & Bessa, E. C. A. (2008). Development and reproduction in Bulimulus tenuissimus (Mollusca: Bulimulidae) in laboratory. *Revista Brasileira de Zoologia, 25*, 220-223.

20. Simone, L. R. L. (2006). *Land and freshwater molluscs of Brazil*. São Paulo: Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP.

21. Souza, R. M. D., Gomes, E. M., Chagas, G. M., & Pinheiro, J. (2000). The influence of starvation and Eurytrema coelomaticum infection on the nitrogenous products of degradation in the hemolymph of Bradybaena similaris. *Brazilian Archives of Biology and Technology, 43*, 365-371.

22. Thiengo, S. C., Maldonado, A., Mota, E. M., Torres, E. J. L., Caldeira, R., Carvalho, O. S., Oliveira, A. P. M., Simões, R. O., Fernandez, M. A., & Lanfredi, R. M. (2010). The giant African snail Achatina fulica as natural intermediate host of Angiostrongylus cantonensis in Pernambuco, Northeast Brazil. *Acta Tropica, 115*, 194-199.

23. Tunholi, V. M., Lustrino, D., Tunholi-Alves, V. M., Mello-Silva, C. C. C., Maldonado, A. Jr., Pinheiro, J., & Rodrigues, M. L. A. (2011). Biochemical profile of Biomphalaria glabrata (Mollusca: Gastropoda) after infection by Echinostoma paraensei (Trematoda: Echinostomatidae). *Parasitology Research, 109*, 885-891.

24. Tunholi-Alves, V. M., Tunholi, V. M., Amaral, L. S., Mota, E. M., Junior, A. M., Pinheiro, J., & Garcia, J. (2015). Biochemical profile of Achatina fulica (Mollusca: Gastropoda) after infection by different parasitic loads of Angiostrongylus cantonensis (Nematoda, Metastrongylidae). *Journal of Invertebrate Pathology, 124*, 1-5.

25. Tunholi-Alves, V. M., Tunholi, V. M., Pinheiro, J., & Thiengo, S. C. (2012). Effects of infection by larvae of Angiostrongylus cantonensis (Nematoda, Metastrongylidae) on the metabolism of the

experimental intermediate host Biomphalaria glabrata. *Experimental Parasitology, 131*, 143-147.

26. Wang, Q. P., Lai, D. H., Zhu, X. Q., Chen, X. G., & Lun, Z. R. (2008). Human angiostrongyliasis. *The Lancet Infective Diseases, 8*, 621-630.

27. Wang, Q. P., Wu, Z. D., Wei, J., Owen, R. L., & Lun, Z. R. (2012). Human Angiostrongylus cantonensis: an update. *European Journal of Clinical Microbiology and Infectious Diseases, 31*, 389-395.

28. Willcox, H. P., & Coura, J. R. (1989). Nova concepção para o método de Baermann–Moraes – Coutinho na pesquisa de larvas de nematódeos. *Memórias do Instituto Oswaldo Cruz, 84*, 539-565.