Biochemical and histological changes in liver of *Nectomys squamipes* naturally infected by *Schistosoma mansoni*

Alterações na bioquímica e histologia do fígado de *Nectomys squamipes* naturalmente infectado por *Schistosoma mansoni*

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Abstract

The South American water rat *Nectomys squamipes* is a wild mammal reservoir of *Schistosoma mansoni* in Brazil. In the present study, wild rodents were collected in the field and categorized into two groups: infected and uninfected by *S. mansoni*. Blood was collected to analyze changes in the serum glucose level (mg/dL) and liver fragments were used to determine the hepatic glycogen content (mg of glucose/g tissue). The histological examination showed inflammatory granulomatous lesions in different phases of development in the liver of rodents naturally infected with *S. mansoni*, in some cases with total or partial occlusion of the vascular lumen. Early lesions were characterized by the presence of inflammatory infiltrate around morphologically intact recently deposited eggs. Despite the significance of these histological lesions, the biochemical changes differed in extent. *N. squamipes* naturally infected by *S. mansoni* showed no variation in hepatic glycogen reserves. These findings were accompanied by a significant increase in plasma glucose contents, probably as a consequence of amino acids deamination, which are degraded, resulting in the formation of intermediates used as precursors for the glucose formation, without compromising the reserves of liver glycogen. In the wild, naturally infected *N. squamipes* can maintain *S. mansoni* infections without undergoing alterations in its carbohydrate metabolism, which minimizes the deleterious effects of *S. mansoni*.

Keywords: Biochemical alterations, rodents, wild reservoirs, alternative model.

Resumo

Nectomys squamipes é um mamífero silvestre reservatório de *Schistosoma mansoni* no Brasil. No presente estudo, os roedores silvestres, colhidos no campo, foram classificados em dois grupos: infectado e não infectado por *S. mansoni*. O sangue foi colhido para análise da alteração no nível de glicose sérico (mg/dL) e fragmentos de fígado foram usados para determinar o conteúdo de glicogênio hepático (mg de glicose/g tecido). A análise histológica demonstrou lesões granulomatosas em diferentes fases de desenvolvimento no tecido hepático dos roedores naturalmente infectados com *S. mansoni*, localizados principalmente na região periportal, com total ou parcial oclusão do lúmen vascular. As lesões foram caracterizadas por presença de infiltrado inflamatório ao redor de ovos morfologicamente intactos recentemente depositados. Apesar da grande significância das lesões histológicas, as alterações bioquímicas não diferiram no mesmo

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grau. *N. squamipes* naturalmente por *S. mansoni* não apresentaram variação na reserva de glicogênio hepático. Esses achados foram acompanhados pelo aumento significativo nos conteúdos de glicose plasmática, provavelmente como consequência ao processo desaminativo de aminoácidos, que passam a ser degradados notadamente para a formação de glucose, sem contudo comprometer a reserva de glicogênio hepático. Em condições naturais a infecção de *S. mansoni* pode ser mantida usando *N. squamipes* como hospedeiro definitivo, sem alterações significativas nos conteúdos de glicogênio hepático, minimizando os efeitos deletérios causados por *S. mansoni* nos roedores *N. squamipes* naturalmente infectados.

Palavras-chaves: Alterações bioquímicas, roedores, reservatórios silvestres, modelo alternativo.

Introduction

Schistosomiasis is an expanding chronic parasitosis caused by *Schistosoma* sp. that affects some 200 million people worldwide (WHO, 2009) and about 2,3 million in Brazil (BRASIL, 2013). *Schistosoma mansoni* has been found in many endemic areas in Brazil naturally infecting wild animals (MODENA et al., 2008; MALDONADO et al., 2006). *Nectomys squamipes*, which is the main non-human definitive host of *S. mansoni* in Brazil (D'ANDREA et al., 2000), is characterized by its semi-aquatic habits and wide geographic distribution (BONVICINO et al., 2008; MALDONADO et al., 1994; RODRIGUES-SILVA et al., 1992; REY, 1993). Therefore, the occurrence of this semi-aquatic rodent in endemic areas is a factor that makes it difficult to control this zoonosis (SOUZA et al., 1992; D'ANDREA et al., 2000; GENTILE et al., 2006).

Cheever et al. (1998) reported the formation of hepatic granulomas in mice infected by *S. mansoni*. Likewise, Costa-Silva et al. (2002), in a comparative study of natural and experimental infection by *S. mansoni* in *N. squamipes*, detected the presence of peri-ovular hepatic lesions in pre-granulomatous phases under natural conditions, while experimentally infected rodents showed a limited pattern of granulomas and a strong initial or peri-ovular inflammatory reaction 52 days after being infected. Despite advances in the body of knowledge about pathology gained in studies of experimental (LENZI et al., 1995) and natural infections by *S. mansoni* (SOUZA et al., 1992), little is known about the biochemical alterations resulting from natural infection by *S. mansoni*.

In enzymatic studies, Bueding (1950) found that schistosome survival and reproduction depend directly on the absorption and utilization of the vertebrate host's carbohydrates as a of source energy, in a quantity of glucose equal to 20% of their weight per hour. The parasite metabolizes glucose by the Embden-Meyerhoff pathway, mainly under anaerobic conditions (MANSOUR; BUEDING, 1953, 1954; BUEDING; MacKINNON, 1955). Therefore, plasma glucose is expected to decrease, inducing physiological compensation by the host. Rodrigues et al. (1968) demonstrated that the biochemical mechanism in mice exposed to *S. mansoni* cercariae differed statistically from control animals as a result of protein biosynthesis and increased oxygen consumption by isolated mitochondria.

Studies to determine the level of serum glucose have revealed significant changes in the glycogen stocks of animals infected by *S. mansoni*. Wu et al. (2010) observed accentuated metabolic changes in hamsters co-infected by *Schistosoma japonicum* and *Necator americanus* under experimental conditions, with significant decreases in the levels of glucose, succinate, citrate and amino acids in the plasma of the co-infected animals compared to the control groups.

The most important event in schistosomiasis is the hepatic changes that occur in the definitive hosts (AMARAL et al., 2002), which are related to fibrosis (TAO et al., 2003). Other studies have shown that hepatic function only changes in more severe forms of the disease, with organomegalies and ascites, or in association with uncompensated hepatitis or cirrhosis (FAHIM et al., 2000; EL-SHAZLY et al., 2001). Therefore, the objectives of this study were to evaluate, for the first time, the carbohydrate profile and hepatic histological changes resulting from natural infection by *S. mansoni* in *N. squamipes* in comparison to uninfected wild specimens.

Materials and Methods

Study area

Adult specimens of *N. squamipes* were captured at three sites in the municipality of Sumidouro, RJ, Brazil, an area endemic for *S. mansoni* in the mountainous region of the state of Rio de Janeiro: Encanto (20° 1'07" S-43° 8'01" W), Pamparrão (20° 2' S-43° 8' W) and Soledade 3 (22° 03' S-42° 35' W). Five capture transects were established along streams, which are the natural habitat of *N. squamipes* (ERNEST; MARES, 1986; GENTILE; FERNANDEZ, 1999). The rodents were captured using a Tomahawk® trap in two 3-night periods, one in March and the other in May 2009, when the average temperature was 20 °C and relative humidity was 49%. All the procedures were carried out with the approval of the Animal Ethics Committee of the Oswaldo Cruz Foundation (CEUA Protocol Nº L-049-08), and with the permission of the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) (under Permit Nº 13373-1). Two females were infected by *S. mansoni* and four were uninfected, while six male rodents were infected and four were not.

Collection of the samples

All the captured animals were euthanized in a CO_2 chamber on the morning after the capture. Samples of approximately 5 mL of blood were collected by cardiac puncture and placed in sterile plastic tubes. After 30 minutes the blood samples were centrifuged

at 2700 xg (Eppendorf Minispin® centrifuge) for 10 minutes to separate the serum from the plasma content. The serum was then stored at –20 °C for further use in serological analyses.

Subsequently, perfusion of the portal-hepatic system was performed according to Smithers and Terry (1965) to identify adult helminths in the mesenteric veins, and the positive results were confirmed by the presence of helminths and eggs in coprological examinations (HOFFMAN et al., 1934). Based on these results, the animals were divided into two groups: *S. mansoni*-infected and uninfected.

The liver of each rodent was excised, weighed, and a 1 g fragment was removed from the right lobule. Each fragment was placed in a sterile plastic tube and frozen at –20 ° C for subsequent determination of the hepatic glycogen content. The liver samples for histological analyses were taken from the same lobule, divided into equal fragments (1 cm³), and fixed in 4% formalin for 24 hours. The fixed tissues were placed in plastic flasks and allowed to rest for 24 hours, after which they were transferred to flasks containing 70% ethanol until they were processed.

Eight uninfected and six *S. mansoni*-infected animals were used in the biochemical assays and eight animals of each group were used in the histological examinations.

Biochemical analysis of blood glucose

The serum glucose concentration of the wild *N. squamipes* specimens naturally infected and uninfected by *S. mansoni* was determined by adding 10 μL of serum to a medium containing a solution of 0.05M of sodium phosphate buffer, pH 7.45, 0.03 mM of aminoantipyrine, 15 mM of sodium p-hydroxybenzoate, and at least 12 KU of glucose oxidase and 0.8 KU of peroxidase (E.C. 1.11.1.1) per liter. The absorbance was read at 510 nm against a blank reaction and utilizing a standard of 100 mg/dL of D-glucose (Doles® Reagentes). Spectrophotometric readings were performed with three repetitions and the results were expressed in mg/dL.

Biochemical analysis of hepatic glycogen

Glycogen was extracted from the liver samples in a cold acid medium, according to Pinheiro and Gomes (1994), and its concentration was determined by 3.5-dinitrosalicylic acid reaction (3.5DNS) (SUMNER, 1924) and expressed in mg of glucose/g of tissue (fresh weight).

Histopathological analysis of liver

The liver samples were processed according to routine histology techniques and were embedded in paraffin blocks to obtain sections (5 μm thickness). The sections were stained with hematoxylin and eosin (HUMASON, 1979), observed under an Olympus BX51 microscope MRc5 equipped with an AxioCam digital camera, and processed with the AxioVision program.

Statistical analysis

Statistical comparisons were made of the biochemical parameters of the two groups: wild animals naturally infected with *S. mansoni* and uninfected animals. The results of the biochemical measurements were expressed as mean ± standard deviation and were compared using the unpaired *t*-test (α = 5%).

Results

Biochemical results

The hepatic glycogen concentration in *S. mansoni*-infected *N. squamipes* specimens (4.47 ± 5.68 mg of glucose/g of tissue, fresh weight) and uninfected animals (4.47 ± 3.76 mg of glucose/g of tissue, fresh weight) did not vary significantly (Table 1).

An alteration in the plasma glucose levels was observed in infected groupin both groups. The naturally infected rodents showed a significantly higher level of serum glucose (136.5 ± 90.44) than the uninfected animals, corresponding to an increase of 25.27% in serum glucose level in infected animals (Table 1).

Histopathological results

The histological examination revealed lesions in different developmental phases in the liver of *N. squamipes* naturally infected by *S. mansoni*, located mainly in the periportal region, in some cases with total or partial occlusion of the vascular lumen (Figure 1a). The lesions in the initial phase were characterized by inflammatory infiltrate composed of lymphocytes, plasmocytes, neutrophils, and eosinophils around recently deposited eggs, which were morphologically intact, with clearly observable structures, including the miracidium (Figure 1b). Some areas contained microscopic neutrophilic abscesses typical of the granulomatous exudative phase (Figure 1b). These were absent from the uninfected groups.

The exudative-productive stage was characterized by the presence of a small to moderate quantity of macrophages and giant cells (Figure 1c). Some granulomas showed degeneration and even complete disintegration of the eggs, and fibroblast proliferation and collagen deposition was more prominent in the portal spaces (Figure 1c). Additionally, yellowish brown pigment was found in the macrophages and Kupffer cells (Figure 1b). We also observed granulomas in the involution phase, with mineralized centers, variable degrees of fibrosis and a small number of inflammatory cells (Figure 1d). No lesions of any type were found in the uninfected wild animals (Figures 1e and f).

Table 1. Concentration of hepatic glycogen, expressed in mg of glucose/g of tissue, fresh weight, and plasma glucose, expressed in mg/dL, in *Nectomys squamipes* naturally infected by *Schistosoma mansoni*.

Groups	Glycogen (mg of glucose/g tisue, fresh weight) X ± SD	Glucose (mg/dL) X ± SD
Uninfected	4.47 ± 3.76[a]	102.0 ± 31.39[a]
Infected	4.47 ± 5.68[a]	136.5 ± 90.44[b]

X ± SD = mean ± standard deviation. [a,b] = Means followed by different letters differ significantly from each other (5%).
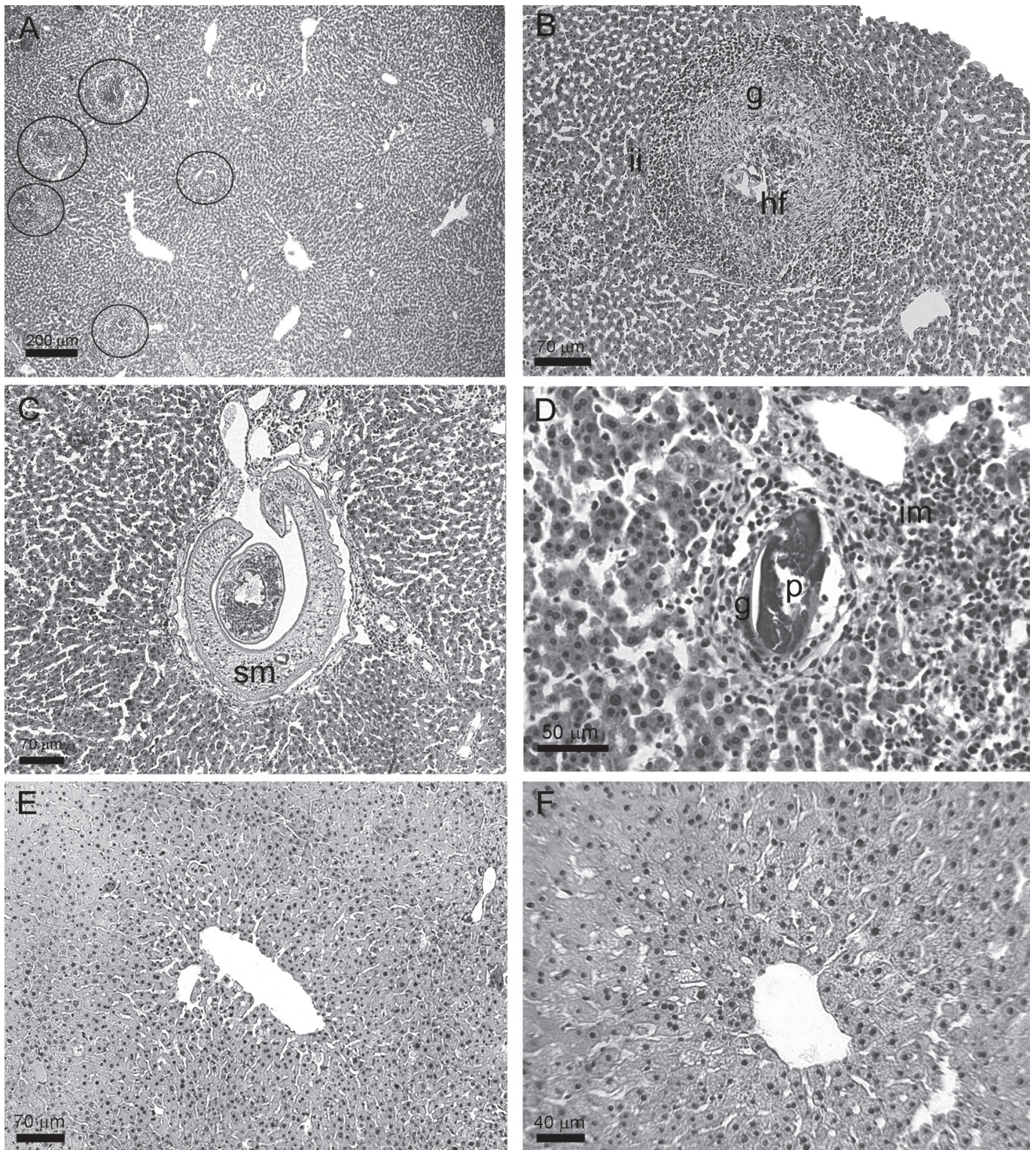
Figure 1. Liver fragments from *Nectomys squamipes* naturally infected with *Schistosoma mansoni*. a – multifocal lesions (black circles) in different development phases in the periportal region. b - Granuloma in the exsudative-productive phase, with the presence of helminth fragments (hf), inflammatory infiltrate (ii) with numerous eosinophils, fibroblasts, plasmocytes, neutrophils, lymphocytes and macrophages having brownish-yellow pigmentation. c - An adult *Schistosoma mansoni* (sm) couple inside the portal-hepatic space, blocking the light from the branch of the portal vein. d - Granuloma (g) in the involutive phase, with mineralized vestiges of the parasite (p) inside the portal space, with the presence of mononuclear inflammatory infiltrate (im). e - No *Schistosoma mansoni* parasitic granulomas were found in the laboratory control group. f - Absence of parasitic granulomas in the uninfected wild group. (All sections were stained with hematoxylin and eosin).

Discussion

One of the most important aspects of this study was to assess, for the first time, the influence of natural infection of *N. squamipes* by *S. mansoni* from a biochemical standpoint. *Schistosoma mansoni* can occur in nature in biological cycles independent of the presence of humans, with *N. squamipes* acting as one of the main non-human hosts of this parasite.

Ahmed and Gad (1995) studied mice experimentally infected with *S. mansoni* and observed an increase in the activity of enzymes involved in carbohydrate metabolism, mainly of pyruvate kinase (E.C. 2.7.1.40) and phosphofructokinase (E.C. 2.7.1.11), starting in the fifth week after infection. As a direct consequence, the formation of pyruvate from glucose accelerated, causing a higher concentration of this substrate in the plasma.

The wider range of variations in the liver glycogen levels of wild rodents naturally infected by *S. mansoni* may result from irregular glucose consumption in response to stress caused by the infection. In addition, the animals' general metabolic conditions are altered in response to infection, weakening them and inhibiting their foraging behavior. Couto et al. (2008) observed the same energy imbalance in an experimental study of metabolic changes in undernourished mice infected by *S. mansoni.* In a study of *Holochilus brasiliensis nanus* from a pre-Amazon region naturally infected with *S. mansoni*, Bastos et al. (1985) demonstrated that the animals infected at 30 days of age suffered reduced glycemic levels as the infection evolved, while those infected at 40 days of age showed no significant difference in plasma glucose levels during eight weeks of infection when compared to animals uninfected by *S. mansoni*. In the present study, *S. mansoni* infection in rodents caused loss of glycemic homeostasis, resulting in an increase of free plasma glucose as a typical response observed under conditions of physiological stress.

No significant differences were observed in the glycogen content of the naturally infected and uninfected groups. This may indicate a physiological adaptation to minimize the deleterious effects of *S. mansoni* in naturally infected *N. squamipes*.

Carvalho (1982) and Silva and Andrade (1989), who studied the histopathological alterations in *N. squamipes* naturally infected by *S. mansoni*, did not observe significant pathological alterations in hepatic morphology . The authors of both studies stated that hepatic lesions resulting from the infection were discrete, suggesting the occurrence of good compatibility in the parasite-host relationship. In the present study, naturally infected animals showed an immune response characterized by the strong presence of macrophages in the formation of different developmental stages of granulomas. The same finding was reported by Costa-Silva et al. (2002), who studied *N. squamipes* naturally infected by *S. mansoni*. According to those authors, histopathological changes – both qualitative and quantitative – were less marked in naturally infected animals than in the experimental groups, suggesting that the intensity of the response to infection depends on the host species, the *S. mansoni* strain, and the number of cercariae. Additionally, the nutritional state of vertebrate hosts used as study models, as well as the handling protocol, influence the response to infection by *S. mansoni*. The results presented here deserve consideration about the real effects of *S. mansoni* infection on the physiology of its wild vertebrate host under natural conditions.

In conclusion, although histological changes were observed, they were not sufficient to induce significant alterations in the liver glycogen contents the glycemic profile of animals naturally infected by *S. mansoni*, which displayed the same metabolic variations as those of uninfected wild specimens. These findings suggest a natural physiological adaptation of the water rat *N. squamipes* to the *S. mansoni* parasite, and emphasize the importance of this rodent as a reservoir of *S. mansoni*, enabling its transmission, as well as the possibility of using these hosts as biological indicators of infection in eco-epidemiological control programs.

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