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Effects of experimental *Angiostrongylus cantonensis* infection on the reproductive biology of *Biomphalaria straminea* and *Biomphalaria tenagophila*

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ABSTRACT

Eosinophilic meningoencephalitis is an endemic zoonosis in Southeast Asia and the Pacific Islands, but in recent years, new cases have been reported in various countries outside these regions, including Brazil, where it is considered an emerging disease. In this study, the effect of infection by the nematode *Angiostrongylus cantonensis*, one of the main etiologic agent of this disease, on the reproductive biology of the planorbid snails *Biomphalaria straminea* and *B. tenagophila* was investigated during the pre-patent period. Alterations in the reproductive biology of *B. straminea* and *B. tenagophila* were analyzed in laboratory-reared specimens infected by *A. cantonensis* during 21 days; the number of eggs, number of egg masses, number of eggs/mass, number of eggs/snail, viable eggs/snail, survival and galactogen content in the albumen gland were measured. The results indicated the occurrence of initial compensation in reproductive effort in both snail species, but at different moments in the pre-patent period. More specifically, a reduction of 46.53% in the eggs/egg mass ratio in infected *B. straminea* was observed, a reflection of a 50% decline in the concentration of galactogen contained in the albumen gland. Changes in this parameter were also noted in *B. tenagophila,* but only at the end of the study period, with a reduction of 15.49%. Histological analyses indicate that changes observed can be explained by the tissue damages caused by the migration and development of the larvae. These results shed more light on the host-parasite relationship and indicate the importance of studying reproductive aspects for efforts to control infected snails. Considering that terrestrial snails can also transmit eosinophilic meningitis (in addition to aquatic mollusks), the data obtained expand knowledge of this host-parasite relationship and provide support for programs to control this zoonosis.

1. Introduction

The nematode *Angiostrongylus cantonensis* (Chen, 1935) is a zoonotic parasite that can accidentally infect humans, causing multiple neurological symptoms, with the disease often being referred to as neural angiostrongyliasis. The disease occurs because of the neurotropism of the third-stage larvae (L_3), which can infect vertebrates. These larvae, once ingested, migrate to the host's brain where they cause a series of clinical alterations, the most common being eosinophilic meningoencephalitis (Martins et al., 2015). Epidemiological studies on the distribution of this metazoonosis have confirmed its presence in more than 30 countries. It is considered endemic in Southeast Asia and the Pacific Islands, with isolated cases or small outbreaks having also been reported in the Caribbean, southeastern United States, Australia, Egypt, Nigeria, Ivory Coast and South America (Brazil and Ecuador) (Caldeira et al., 2007; Morassutti et al., 2014; New et al., 1995; Thiengo et al., 2013; Wang et al., 2008). According to Wang et al. (2008), up to the time their paper was written over 2,800 cases had been confirmed

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worldwide. Morassutti et al. (2014) reported the occurrence of neural angiostrongyliasis in 34 patients in Brazil. This scenario summarizes the rapid geographic dispersal of this nematode, and its importance in posing an increasing health risk in many regions of the world.

According to Yong and Eamsobhana, (2013), different species of murid rodents are used as definitive hosts of the rat lungworm *A. cantonensis* under natural conditions, as they form the black rat *Rattus rattus* species complex and form genus *Maxomys*. Besides this, land and freshwater snails are obligatory intermediate hosts to this parasite. Thus, understanding the transmission foci of the parasite is dependent on the presence of these mollusks which assure completion of larval development, and provide potential for infection of new definitive hosts (Maldonado et al., 2010; Simões et al., 2012). In Brazil, malacological investigations in recent years have confirmed the participation of numerous gastropod species as natural intermediate hosts of *A. cantonensis*, with involvement of both terrestrial (Caldeira et al., 2007; Maldonado et al., 2010; Thiengo et al., 2010) and aquatic species. Among the aquatic gastropod hosts is the genus *Biomphalaria* (Ibrahim, 2007; Thiengo et al. 2010). In addition, Kim et al. (2014) reported that many gastropod species from 34 additional families could also act as hosts.

During the intramolluscan development, helminth larvae settle in a specific ecological niche, formed by the host's hemolymph and tissues. The parasites compete directly for nutrients, besides releasing excretion/secretion products that can interfere with the snail's metabolism (Pinheiro et al., 2009; Tunholi-Alves et al., 2014, 2015; Lima et al., 2016) and reproductive biology (Tunholi et al., 2013). Among the reproductive changes recorded in the literature, specifically in reference to *A. cantonensis/Biomphalaria* interface, parasitic castration is most commonly found. Sullivan et al. (1985) defined this phenomenon as "the total or partial interruption of the host's reproductive performance". According to Baudoin (1975), this phenomenon can be explained by two mechanisms: a direct process, related to tissue damage caused by the parasites, which destroys important structures related to the host's reproductive system such as the albumen gland and ovotestis, (Tunholi-Alves et al., 2011); or an indirect process associated with depletion of energy reserves driven to reproductive processes, especially galactogen, can also explain the parasitic castration (Faro et al., 2013). The availability of galactogen has a direct effect on the viability of the developing embryos, since this polysaccharide is a constituent of the perivitellinic fluid, and acts as an energy source for embryo development and newly hatched snails (Goudsmit, 1973; Gomot et al., 1989). This reserve, found only in the albumen gland, and the initial step of its synthesis is the interconversion of glucose to galactose by the enzyme UDP-glucose-4- epimerase (E.C. 5.1.3.2) (van Elk and Joosse, 1981).

The effects of helminths' infections have been observed in various host-parasite models, mainly those involving larval trematodes, in which the intensity varies according to the parasite and host species and strain (Etges and Gresso, 1965; Hodasi, 1972; Wilson and Denison 1980; Faro et al., 2013). Although in recent years the clinical, pathological and epidemiological aspects of neural angiostrongyliasis have been extensively studied (Kim et al., 2002; Diaz, 2010; Murphy and Johnson, 2013; Cowie et al., 2013), data on the metabolic and reproductive profile of snails infected by *A. cantonensis* are still scarce. This is cause for concern, because recommendations from the World Health Organization (WHO, 2017) and the Ministério da Saúde in Brazil (Brasil, 2014) for control of diseases transmitted by snails pointed out the importance of monitoring and controlling the populations of these invertebrate hosts.

Given that the presence of the snail host is an essential requirement for the parasite to complete its life cycle, this stage of life history could be a focus point for controlling the disease. Therefore, the enrichment of the knowledge about reproductive changes in snails caused by *A. cantonensis* is an essential point for developing effective methods to prevent the spread of neural angiostrongyliasis. In the present study, we analyzed alterations in the reproductive biology of *B. straminea* and *B. tenagophila* infected by *A. cantonensis* during the pre-patent period (3 weeks or 21 days post infection - DPI), using as parameters the number of eggs, number of egg masses, number of eggs/mass, number of eggs/snail, embryonated eggs/snail, hatched snail/snail, survival, and the galactogen content in the albumen gland. Additionally, histopathological aspects of the ovotestis were analyzed. The results contribute to a better understanding of the snail-parasite relationship in the species studied.

2. Material and methods

2.1. Maintenance of the snails

The snails, *B. straminea* (strain Jaguarari, Rio Grande do Norte, Brazil) and *B. tenagohila* (strain Andorinhas, Espírito Santo, Brazil), born and reared in the laboratory, free of infection, were kept in aquariums containing 3 L of dechlorinated water and 0.5 g of $CaCO_{3,}$ per aquarium, in the National Schistosomiasis-Malacology Reference Laboratory of the Instituto Oswaldo Cruz (Fiocruz), in the city of Rio de Janeiro. The aquariums were cleaned at least twice a week and the snails were fed with lettuce leaves (*Lactuca sativa* L.) *ad libitum*, renewed on alternate days. Polystyrene plates measuring $\sim 2\ cm^2$ were placed in each aquarium to serve as substrates for egg laying.

2.2. Infection of the snails and biological replications

The specimens of *A. cantonensis* used in this study were obtained from naturally infected *A. fulica* snails collected in 2011 in São Gonçalo municipality, Rio de Janeiro state (22°48'26.7"S, 43°00'49.1"W). The life cycle of this parasite had been maintained in the Laboratory for Biology and Parasitology of Wild Mammal Reservoirs (LBPMR) of Fiocruz, in Rio de Janeiro, using *Rattus norvegicus* as the definitive host and *B. glabrata* as an intermediate host. The experiment was approved by the Fiocruz committee on ethical animal use (Permit 47/14).

Fecal samples of *R. norvegicus* were collected from the boxes were the animals were maintained. The feces were processed (1 g) by the Baermann technique, as modified by Willcox and Coura (1989). First-stage larvae (L_1) were obtained by collecting and processing the feces of experimentally infected rodents.

Specimens of *B. straminea* (6–8 mm shell diameter) and *B. tenagophila* (8–12 mm shell diameter) with an average age of 90 days-old, were individually exposed to approximately 1200 L_1 larvae, in 24-hole cell culture plates. To the formation of an uninfected control group, the snails were put in the wells just filled with water. *Biomphalaria tenagophila* and *B. straminea* have distinct growth patterns and adult sizes, being *B. straminea* smaller than *B. tenagophila*. Hence, the snails were grouped by mean age and size to allow a better comparison of the results. Subsequently, after 24 h exposed to the larvae, the snails were removed from each well, and the wells were observed (using Lugol reagent) to verify the presence of larvae. Then after exposure period (24 h), the snails of each group (30 specimens) were transferred to aquarium, containing 1500 ml of dechlorinated water, the aquariums were kept in a room with controlled temperature of 25 °C and a 12 h dark/light cycle throughout the experiment, and the experimental groups were formed.

A previous study on the experimental infection of *B. glabrata* with *A. cantonensis* showed that the pre-patent period lasts about 21 days (Harris and Cheng, 1975). Therefore, we used three periods of infection in our analysis: 6 days post exposure (beginning of infection), 11 days post exposure (middle of infection), and 21 days post exposure (end of pre-patent period). Each period of infection analyzed was composed by one group of uninfected snails (30 mollusks) + one group of *A. cantonensis*-infected snails (30 mollusks); all groups were formed with triplicates (n = 180 snails per period; n = 540 snails total).

After 6th, 11st and 21st DPI, respectively, the snails were dissected