

## Interaction between primary and secondary sporocysts of *Schistosoma mansoni* and the internal defence system of *Biomphalaria* resistant and susceptible to the parasite

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*The outcome of the interaction between Biomphalaria and Schistosoma mansoni depends on the response of the host internal defence system (IDS) and the escape mechanisms of the parasite. The aim of this study was to evaluate the responsiveness of the IDS (haemocytes and soluble haemolymph factors) of resistant and susceptible Biomphalaria tenagophila lineages and Biomphalaria glabrata lineages in the presence of in vitro-transformed primary sporocysts and secondary sporocysts obtained from infected B. glabrata. To do this, we assayed the cellular adhesion index (CAI), analysed viability/mortality, used fluorescent markers to evaluate the tegumental damage and transplanted secondary sporocysts. B. tenagophila Taim was more effective against primary and secondary sporocysts than the susceptible lineage and B. glabrata. Compared with secondary sporocysts exposed to B. tenagophila, primary sporocysts showed a higher CAI, a greater percentage of dead sporocysts and were labelled by lectin from Glycine max and Alexa-Fluor 488 fluorescent probes at a higher rate than the secondary sporocysts. However, the two B. tenagophila lineages showed no cercarial shedding after inoculation with secondary sporocysts. Our hypothesis that secondary sporocysts can escape the B. tenagophila IDS cannot be confirmed by the transplantation experiments. These data suggest that there are additional mechanisms involved in the lower susceptibility of B. tenagophila to S. mansoni infection.*

Key words: *Schistosoma mansoni* - sporocysts - *Biomphalaria tenagophila* - internal defence system - escape mechanisms

Planorbidae of the genus *Biomphalaria* are the intermediate hosts of the parasite *Schistosoma mansoni*. A high degree of specificity is an important characteristic of the mollusc-digenea interaction. According to the literature, a restricted number of *Biomphalaria* species and lineages are susceptible to a specific strain of *S. mansoni* (Basch 1976). *Biomphalaria glabrata*, *Biomphalaria tenagophila* and *Biomphalaria straminea* are the three species responsible for the transmission of schistosomiasis in Brazil (Paraense 1975). Many authors (Corrêa et al. 1979, Santos et al. 1979, Bezerra et al. 1997, 2003, Martins-Souza et al. 2003, Rosa et al. 2004, 2005, 2006, Barbosa et al. 2006, Coelho et al. 2008, Pereira et al. 2008) have described the existence of a geographic lineage of *B. tenagophila* (provided from Ecological Station of Taim, state of Rio Grande do Sul, Brazil) resistant to *S. mansoni*. This lineage represents an important population in which to study the defence mechanisms of these invertebrate hosts to *S. mansoni* infection. The compatibility between *S. mansoni* and *Biomphalaria* is established by genetic factors of the parasite and of the

intermediate host (Adema & Loker 1997). *Biomphalaria* has an internal defence system (IDS) composed of cells (haemocytes) and soluble haemolymph factors that are stimulated in the presence of parasites (van der Knaap & Loker 1990, Gliński & Jarosz 1997, Hahn et al. 2000, 2001). Parasite escape mechanisms that respond to the host IDS can assure the survival and adaptability of the parasite (Yoshino & Bayne 1983, Adema & Loker 1997). Previous studies have demonstrated that success of the infection depends on the parasite interfering with the mollusc IDS (Fryer & Bayne 1990). The parasite can disturb the IDS by producing proteases (Yoshino et al. 1993) and by the action of enzymes that neutralise reactive oxygen species (Connors & Yoshino 1990). In addition, it has been demonstrated that sporocysts release glycoproteins (excreted/secreted products) that bind to carbohydrate-binding receptors on the haemocyte cell surface to attenuate the initial cellular recognition of the parasite (Johnston & Yoshino 2001). These molecules can inhibit IDS mechanisms (Adema & Loker 1997) by suppressing the synthesis of many haemocyte molecules (Lodes et al. 1991) or by reducing haemocyte motility and phagocytic activity (Fryer & Bayne 1990, Lodes & Yoshino 1990). Other important parasite escape mechanisms include the use of molecular mimicry, likely through the production of sporocyst surface membrane molecules similar to those present on the host, and the ability of the parasite to absorb host antigens (Yoshino & Bayne 1983, Damian 1987, Salzet et al. 2000, Lehr et al. 2008, 2010, Peterson et al. 2009, van Die & Cummings

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2010). According to Salzet et al. (2000), the latter mechanism can prevent the recognition of the parasite by the host IDS. Furthermore, van Die and Cummings (2010) and Lehr et al. (2010) have suggested that glycans play a role in the parasite molecular mimicry process. Although the evolutionary advantages of this adaptive process for the parasite are well understood, it is not known how this process interferes with schistosomiasis (Salzet et al. 2000) or whether this mechanism could interfere with the snail's resistance mechanisms. To this end, the aim of this study was to evaluate the mechanisms of *B. tenagophila* Taim (resistant) and Cabo Frio (susceptible) IDS systems in the presence of primary sporocysts transformed in vitro and secondary sporocysts derived from infected snails. To accomplish this, we measured cellular adhesion, assayed sporocysts viability and mortality, evaluated the tegumental damage using fluorescent markers and transplanted secondary sporocysts.

## MATERIALS AND METHODS

*Snails and parasites* - Snails *B. tenagophila* (Taim and Cabo Frio lineages) and *B. glabrata* (12-14 mm of diameter) and the *S. mansoni* LE strain were used in this work. The snails had been maintained for more than 30 years in the Mollusc Room of the René Rachou Research Center - Oswaldo Cruz Foundation.

*Production of primary and secondary sporocysts* - Primary sporocysts were obtained from miracidia that were cultivated and transformed in vitro according to the technique described by Mattos et al. (2006) and Bahia et al. (2006). Briefly, livers of mice 50 days after infection with *S. mansoni* were macerated, sedimented and transferred to a glass flask containing 500 mL of water without chlorine. This flask was exposed to artificial light, and the miracidia were collected. The miracidia were transformed in Roswell Park Memorial Institute-1640 culture medium containing 5% foetal bovine serum (FBS) (Gibco Limited, Paisley, Scotland, UK) and 100 µg/mL penicillin/streptomycin antibiotics (Sigma) in biochemical oxygen demand (BOD) incubators at 26°C for 24 h. The primary sporocysts were then washed with Chernin Balanced Saline Solution (CBSS) (pH 7.4; 100 mOsm).

The secondary sporocysts were obtained from *B. glabrata* infected with *S. mansoni* (Pereira et al. 1984). Briefly, the cephalopodal region from snails infected with 50 miracidia was dissected after 13 days of infection. This tissue, with the secondary sporocysts, was ground and fragments were transferred to a nylon mesh (200 µm) and maintained in contact with CBSS (pH 7.4) for 2 h in a water bath at 28°C. Next, CBSS from below the mesh was centrifuged at 1,000 rpm for 2 min and the secondary sporocysts were counted under a microscope.

*Haemolymph collection and counting of haemocytes* - Whole haemolymph was collected from *B. tenagophila* (Taim and Cabo Frio lineages) and *B. glabrata*. Each snail shell was cleaned with 70% alcohol and maintained overnight in antibiotic solution (100 µg/mL penicillin/streptomycin and 4 mg amphotericin B). Total haemolymph (TH fraction) was collected by cardiac puncture using a 21G needle (Zelck & Becker 1990, Bezerra et al.

1997) and centrifuged at 1,000 rpm for 10 min at 4°C. The supernatant was transferred to another tube [(S) soluble fraction only] and the pelleted haemocytes were resuspended in CBSS supplemented with 2% essential aminoacids (Sigma), 5% FBS and 2% antibiotics penicillin/streptomycin; haemocytes were resuspended in a CBSS volume equal to the haemolymph that was initially collected [(H) fraction of haemocytes only]. The haemocytes were then diluted 1/10 in CBSS solution containing 0.4% Trypan Blue and counted in a Neubauer's chamber (Martins-Souza et al. 2009). In the experiments, haemolymph from 15 snails was pooled and the post-separation fractions were called TH, H or S.

*Evaluation of the cellular adhesion index (CAI) and the viability/mortality of primary and secondary sporocysts after contact with B. tenagophila (Taim and Cabo Frio lineages) and B. glabrata IDS components* - The experiments were performed in 24-well culture plates. Thirty microliters of S and TH fractions and  $2 \times 10^5$  H fraction from *B. tenagophila* (Taim and Cabo Frio lineages) and *B. glabrata* were separately exposed to 20 primary or secondary sporocysts. CBSS was then added for a final volume of 300 µL/well. For measuring the CAI and for viability/mortality experiments, 10 wells were prepared for each lineage and each fraction; wells with only sporocysts were used as controls. The culture plates were maintained in BOD incubators at 26°C. After 2 h incubation, 100 sporocysts from each lineage were analysed to determine their CAI using the protocol described by Castillo and Yoshino (2002). Sporocyst CAIs were scored as arbitrary values ranging from 1-4 according to the following parameters: CAI 1: no adherent haemocytes on the sporocyst surface; CAI 2: up to 10 adherent haemocytes; CAI 3: between 11-50 haemocytes; CAI 4: more than 50 adherent haemocytes. The formula to calculate CAI was:

$$\text{CAI} = \frac{\text{Total binding value}}{\text{Number of sporocysts scored}}$$

The total binding value was obtained by summing the individual values (1-4) of all the sporocysts scored from each lineage and haemolymph fraction. The following statistical analyses were used: an unpaired, two-tailed Student *t* test/Mann-Whitney test (2-tailed) or the One-Way Analysis of Variance/Kruskal-Wallis and Dunn's Multiple Comparison test. Analyses were carried out using GraphPad Prism 4 software. Differences were considered significant if  $p \leq 0.05$ .

Sporocyst viability was assessed 6 h post-incubation using an inverted microscope. The proportion of dead sporocysts was identified by 0.4% Trypan Blue staining. All the sporocysts present in each well were counted. The Chi-square test with Fisher's exact test (when necessary) was used to analyse the data. These tests were conducted using the Minitab 14 software.

*Evaluation of primary and secondary sporocyst tegumental damage after contact with B. tenagophila (Taim and Cabo Frio lineages) IDS components* - Thirty microliters of fractions S, TH and  $2 \times 10^5$  H fraction from the *B. tenagophila* Taim and Cabo Frio lineages were separately

incubated with 20 primary or secondary sporocysts in 24-well culture plates. The volume of each well was topped up to 300  $\mu$ L with CBSS after the distribution of IDS and sporocysts. The culture plates were maintained in BOD incubators at 26°C. Wells containing only sporocysts were used as a control. Six experimental wells were used for each lineage and each haemolymph fraction. After 3 h and 5 h of incubation, the sporocysts were labelled for 30 min with fluorescent probes Hoechst 33258 (a hydrophilic DNA-specific probe that binds with DNA only after damage to the cell membrane), lectin from *Glycine max* (soybean, specific for N-acetylgalactosamine) and Alexa-Fluor 488 phalloidin (specific for actin filaments). The parasites were then washed three times with CBSS and analysed with a fluorescence microscope (K-Zeiss). The filters used were as follows: red for lectin from *Glycine max* TRITC (excitation/maximal emission 543/571 nm), green for Alexa-Fluor 488 (excitation/maximal emission 499/520 nm) and blue for Hoechst 33258 (excitation/maximal emission 352/455 nm). Photographs were taken with a Canon EOS Digital Rebel XT camera. The colour histogram tool of the ImageJ 1.43 software was used to calculate mean fluorescence intensity/area for each image obtained in the experiments. Statistical analyses were performed using the Mann Whitney test (2-tailed) in the GraphPad Prism 4 software. Differences were considered significant if  $p \leq 0.05$ .

*Evaluating the development of secondary sporocysts from B. glabrata infected after sporocyst inoculations in B. tenagophila (Taim and Cabo Frio) and B. glabrata* - To determine whether the IDS from *B. tenagophila* Taim can recognise the secondary sporocysts obtained from *B. glabrata*, we inoculated *B. tenagophila* with parasites originally recovered from *B. glabrata*.

Thirty secondary sporocysts obtained from infected *B. glabrata* were inoculated into 65 *B. tenagophila* Taim and *B. glabrata* and into 35 *B. tenagophila* de Cabo Frio using a modified protocol described by Jourdan and Theron (1980). Briefly, snails were anaesthetised with sodium pentobarbital solution (0.4 mg/mL) for at least 6 h (Martins-Souza et al. 2001). A sample of secondary sporocysts was stained with Trypan Blue and the unstained parasites were counted. Next, the secondary sporocysts were inoculated (maximum 30  $\mu$ L) into the cephalopodal area using a syringe and 30G needle. After inoculation for 14, 21, 27 and 35 days, the snails were exposed individually to artificial light for 1 h to verify the shedding of cercariae.

## RESULTS

*CAI of primary and secondary sporocysts* - The CAI values are shown in Table I. The haemocytes from all *Biomphalaria* species were able to adhere to the surface of primary and secondary sporocysts. However, IDS of *B. glabrata* (mean CAI - TH:  $1.89 \pm 0.31$ , H:  $1.87 \pm 0.35$ ) showed significantly lower CAI values compared to IDS of *B. tenagophila* Taim (mean CAI - TH:  $2.13 \pm 0.25$ , H:  $2.02 \pm 0.22$ ) and Cabo Frio (mean CAI - TH:  $2.32 \pm 0.3$ , H:  $1.98 \pm 0.31$ ) ( $p < 0.03$ ). Similarly, *B. tenagophila* Taim cell binding to secondary sporocysts (mean CAI -

TABLE I

Mean cellular adhesion index (CAI) values of *Biomphalaria tenagophila* (Taim and Cabo Frio) and *Biomphalaria glabrata* cells binding of primary and secondary sporocysts of *Schistosoma mansoni*

Groups of IDS		Primary sporocysts	Secondary sporocysts
		CAI $\pm$ SD	CAI $\pm$ SD
<i>B. tenagophila</i> Taim	TH	$2.13 \pm 0.25^b$	$2.03 \pm 0.31^b$
	H	$2.02 \pm 0.22^b$	$2.27 \pm 0.30^{a,b}$
<i>B. tenagophila</i> Cabo Frio	TH	$2.32 \pm 0.3^{b,c}$	$2.03 \pm 0.23^b$
	H	$1.98 \pm 0.31$	$1.99 \pm 0.12^b$
<i>B. glabrata</i>	TH	$1.89 \pm 0.31$	$1.73 \pm 0.41$
	H	$1.87 \pm 0.35^c$	$1.41 \pm 0.2$

*a*: statistical difference between CAI of primary or secondary sporocysts exposed to *B. tenagophila* Taim and Cabo Frio internal defence system (IDS) [total hemolymph (TH) or fraction of hemocytes only (H)] and CAI of primary or secondary sporocysts exposed to Cabo Frio IDS (TH or H)  $p < 0.03$ ; *b*: statistical difference between CAI of primary or secondary sporocysts exposed to Taim IDS (TH or H) and CAI of primary or secondary sporocysts exposed to *B. glabrata* IDS (TH or H)  $p < 0.03$ ; *c*: statistical difference between CAI of primary and secondary sporocysts exposed to IDS from the same species or lineage ( $p < 0.02$ ).

H:  $2.27 \pm 0.30$ ) was significantly higher ( $p < 0.03$ ) than to the secondary sporocysts exposed to Cabo Frio (mean CAI - H:  $1.99 \pm 0.12$ ) and *B. glabrata* (mean CAI - H:  $1.41 \pm 0.2$ ). Moreover, there were significantly ( $p < 0.02$ ) more cells adhering to the surface of primary sporocysts than of secondary sporocysts when they were exposed to *B. tenagophila* Cabo Frio (mean CAI - TH:  $2.32 \pm 0.3$ ) and *B. glabrata* (mean CAI - H:  $1.87 \pm 0.35$ ). There were no statistically significant differences between the different fractions of IDS (TH and H) used in the experiments.

*Viability/mortality of primary and secondary sporocysts* - The results of the viability experiments are summarised in Table II. *B. tenagophila* Taim IDS resulted in significantly higher primary sporocysts mortality (TH = 47.5%, H = 58%, S = 56%) compared to IDS from *B. tenagophila* Cabo Frio (TH = 23%, H = 32%, S = 24.5%), *B. glabrata* (TH = 5%, H = 18%, S = 6%) and sporocysts control (20%) ( $p < 0.03$ ). However, the proportion of dead secondary sporocysts exposed to *B. tenagophila* Taim (TH = 7%, H = 8.5%, S = 6.5%) was significantly higher ( $p < 0.03$ ) only when compared to *B. glabrata* (TH = 3%, H = 3%, S = 2%). On the other hand, we observed that the secondary sporocysts died at a significantly lower rate ( $p < 0.01$ ) than the primary sporocysts when exposed to *B. tenagophila* Taim and Cabo Frio but not to *B. glabrata*.

*Presence of tegumental damage in primary and secondary sporocysts after contact with mollusc IDS (HT, H and S fractions)* - No differences were observed in the labelling of sporocysts analysed 3 h and 5 h after incubation with different fractions of haemolymph TH

from each lineage. Tegumental damage was shown by Hoechst 33258 staining to be similar for all experimental conditions. However, primary sporocysts exposed to *B. tenagophila* Cabo Frio (mean fluorescence intensity/area:  $2.8 \pm 1.6$ ) were significantly more labelled ( $p = 0.03$ ) than secondary sporocysts (mean fluorescence intensity/area:  $0.21 \pm 0.05$ ) (Fig. 1). Alexa-Fluor 488 was better in indicating the differences in the extent of tegument damage between the various experiments. The primary sporocysts exposed to Taim (mean fluorescence intensity/area:  $2.5 \pm 0.8$ ) presented significantly more ( $p = 0.04$ ) tegumental damage than both the sporocysts exposed to Cabo Frio (mean fluorescence intensity/area:  $1.1 \pm 0.84$ ) and control primary sporocysts (mean fluorescence intensity/area:  $1.1 \pm 0.9$ ). Moreover, the primary sporocysts exposed to Taim were labelled at a significantly higher rate ( $p = 0.04$ ) than the secondary sporocysts (mean fluorescence intensity/area:  $0.38 \pm 0.2$ ) (Fig. 2). A similar result was observed using lectin from Glycine max. Primary sporocysts exposed to Taim (mean fluorescence intensity/area:  $2.76 \pm 0.8$ ) showed significantly higher ( $p \leq 0.03$ ) labelling than the sporocysts exposed to Cabo Frio (mean fluorescence intensity/area:  $0.47 \pm$

0.43) and sporocysts control (mean fluorescence intensity/area:  $0.7 \pm 0.6$ ) and higher labelling than secondary sporocysts exposed Taim (mean fluorescence intensity/area:  $0.12 \pm 0.06$ ) (Fig. 3). The labelling intensities for all experimental conditions are shown in the Figs 4-6.

*Development of secondary sporocysts isolated from B. glabrata and inoculated into B. tenagophila (Taim and Cabo Frio) and B. glabrata - Sixty-five B. tenagophila Taim were inoculated with secondary sporocysts provided from infected B. glabrata. Thirty days after inoculation, only seven snails were alive (11%) and none were shedding cercariae. Of the 35 B. tenagophila Cabo Frio similarly inoculated with B. glabrata-derived secondary sporocysts, 11 snails were alive at the end of the*

TABLE II

Proportion of dead primary and secondary sporocysts of *Schistosoma mansoni* after exposure of parasite to the internal defence systems (IDS) of *Biomphalaria tenagophila* Taim (resistant), Cabo Frio (susceptible) and *Biomphalaria glabrata*

Groups of IDS		Dead primary sporocysts (%)	Dead secondary sporocysts (%)
<i>B. tenagophila</i> Taim	TH	47.5 <sup>a,b,c,d</sup>	7 <sup>b</sup>
	H	58 <sup>a,b,c,d</sup>	8.5 <sup>b</sup>
	S	56 <sup>a,b,c,d</sup>	6.5 <sup>b</sup>
<i>B. tenagophila</i> Cabo Frio	TH	23 <sup>b,d</sup>	9 <sup>c</sup>
	H	32 <sup>d</sup>	5
	S	24.5 <sup>b,d</sup>	5.5
<i>B. glabrata</i>	TH	5 <sup>c</sup>	3
	H	18 <sup>d</sup>	3
	S	6 <sup>c</sup>	2
Control	-	20	6

a: statistical difference between proportion of dead primary or secondary sporocysts exposed to *B. tenagophila* Taim and Cabo Frio IDS and proportion of dead primary or secondary sporocysts exposed to Cabo Frio IDS ( $p < 0.03$ ); b: statistical difference between proportion of dead primary or secondary sporocysts exposed to Taim IDS and proportion of dead primary or secondary sporocysts exposed to *B. glabrata* IDS ( $p < 0.03$ ); c: statistical difference between proportion of dead primary or secondary sporocysts exposed to Taim IDS and proportion of dead primary or secondary control sporocysts ( $p < 0.03$ ); d: statistical difference between proportion of primary and secondary sporocysts exposed to IDS from the same species or lineage ( $p < 0.01$ ); H: fraction of hemocytes only; S: only soluble fraction; TH: total hemolymph.

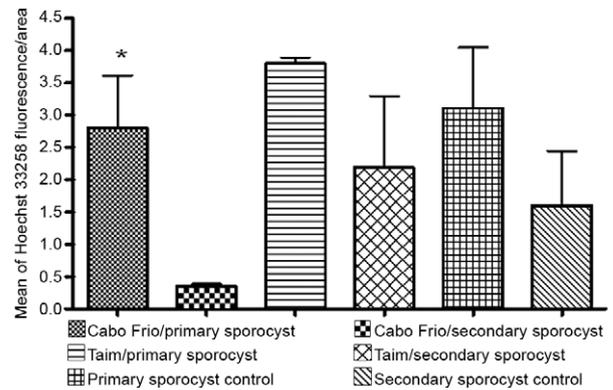


Fig. 1: mean Hoechst 33258 fluorescence/area in primary and secondary sporocysts exposed to internal defence systems of *Biomphalaria tenagophila* from Taim or Cabo Frio lineage and sporocysts control. Asterisk means difference between primary and secondary sporocysts exposed to Cabo Frio ( $p = 0.03$ ).

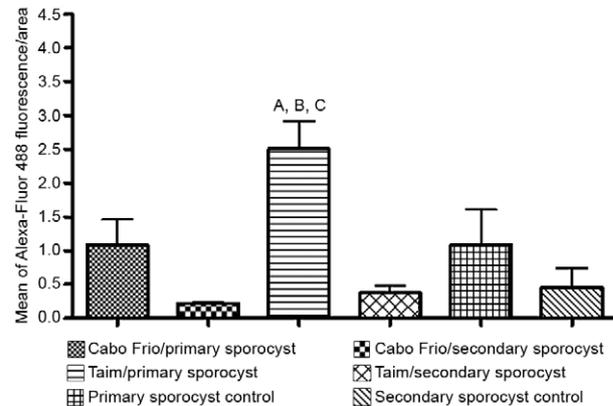


Fig. 2: mean Alexa-Fluor 488 fluorescence/area in primary and secondary sporocysts exposed to internal defence systems of *Biomphalaria tenagophila* from Taim or Cabo Frio lineage and sporocysts control. A: difference between primary sporocysts exposed to Cabo Frio and primary sporocysts exposed to Taim ( $p = 0.04$ ); B: difference between primary and secondary sporocysts exposed to Taim ( $p = 0.03$ ); C: difference between primary sporocysts exposed to Taim and primary sporocysts control ( $p = 0.04$ ).

experiments (31%). The survival of *B. tenagophila* Cabo Frio was high, but all the snails were negative for cercariae. Sixty-five *B. glabrata* were inoculated with secondary sporocysts provided from snails of the same species. At the end of the experiments, 21 snails were alive (32%) and 11 (52%) were shedding cercariae (Table III).

### DISCUSSION

*Biomphalaria tenagophila* from Taim is resistant to *S. mansoni*. However, the mechanisms used to eliminate the parasite are not completely understood (Coelho et al. 2008). In this study, we sought to demonstrate the efficacy of the IDS from *B. tenagophila* (resistant and susceptible strains) and *B. glabrata*. We used primary sporocysts produced in vitro and secondary sporocysts derived from *B. glabrata*. We also used a transplantation technique to assess the survival of secondary sporocysts derived from *B. glabrata*. Our hypothesis was that secondary sporocysts (from *B. glabrata*) would be less recognised or not recognised at all by the *B. tenagophila* Taim IDS because of the presence of host molecules on the sporocysts' surface. This concept of molecular mimicry was introduced by Yoshino and Bayne (1983) when they found haemolymph-like antigens on the surface tegument of sporocysts. Similar mimicry has been suggested in schistosomula by Guillou et al. (2007) and Horta and Ramalho-Pinto (1991) by demonstrating that decay-accelerating factor from human erythrocytes could be transferred to *S. mansoni* and protect it against complement-mediated haemolysis. We observed that the secondary sporocysts were recognised at a lower rate than the primary sporocysts in the in vitro experiments. This can likely be explained by the sporocysts acquiring or producing protein during their previous contact with the snail host, rendering them resistant to haemolytic

attack. However, no infection occurred when *B. tenagophila* were challenged with secondary sporocysts. We therefore speculated that perhaps *B. tenagophila* might recognise the molecules from *B. glabrata* on the sporocyst surface as "non-self" because the snails are different species. Another possible hypothesis is that *B. tenagophila* were able to destroy the secondary sporocysts in the same way as primary sporocysts in vitro. Thus, mimicry can be a valid strategy for IDS escape, but it is likely assisted by another mechanism. We hypothesised that additional mechanisms might be revealed during the transplantation experiment.

We used different haemolymph fractions (TH, H and S) to investigate whether the presence of soluble factors could increase the activity of haemocytes. We found no statistical difference between the fractions, indicating that the soluble factors did not increase haemocyte activity. Perhaps the amount of soluble factors used here (10% of total volume) was not sufficient to achieve a demonstrable effect on the haemocytes. Pereira et al. (2008) observed that when the fraction of soluble factors was 20% of the total volume, this produced an enhanced effect on haemocytes. Our analyses of the CAI showed

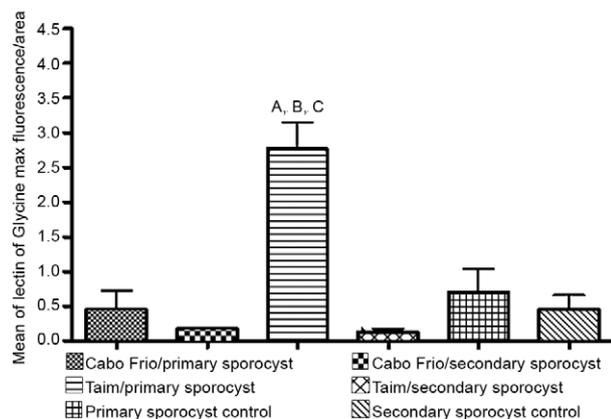


Fig. 3: mean lectin of Glycine max fluorescence/area in primary and secondary sporocysts exposed to internal defence systems of *Biomphalaria tenagophila* from Taim or Cabo Frio lineage and sporocysts control. A: difference between primary sporocysts exposed to Cabo Frio and primary sporocysts exposed to Taim ( $p = 0.007$ ); B: difference between primary and secondary sporocysts exposed to Taim ( $p = 0.03$ ); C: difference between primary sporocysts exposed to Taim and primary sporocysts controls ( $p = 0.03$ ).

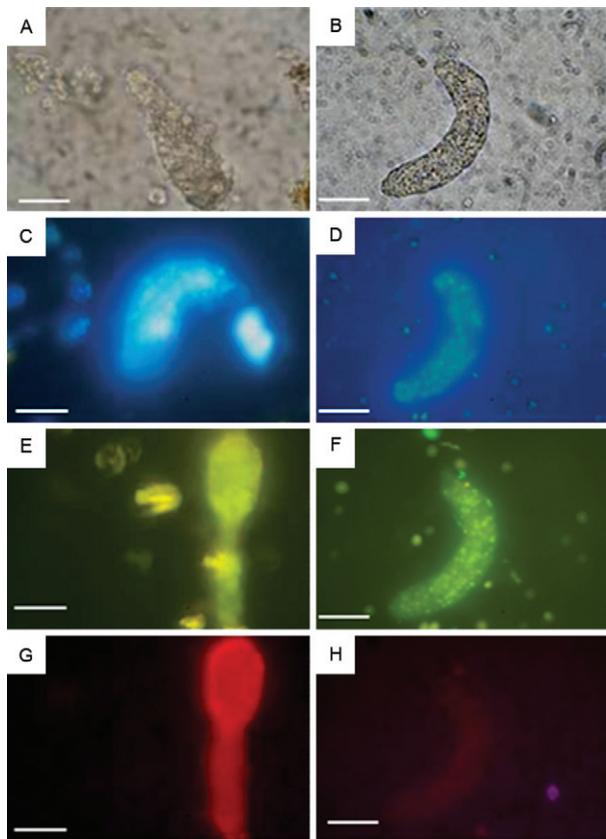


Fig. 4: action of internal defence systems of *Biomphalaria tenagophila* from Taim on the membrane of primary (A, C, E, G) and secondary (B, D, F, H) sporocysts. A, B: optical microscopy; C, D: labeled with Hoechst 33258 (fluorescence microscopy); E, F: labeled with Alexa-Fluor 488 (fluorescence microscopy); G, H: labeled with lectin of Glycine max (fluorescence microscopy). Bars = 25  $\mu\text{m}$ .

that in contact with the IDS of *B. tenagophila* Cabo Frio and *B. glabrata*, primary sporocysts presented higher CAI values than the secondary sporocysts, suggesting a better recognition of this intramollusc primary stage. On the other hand, haemocytes from Taim (which is resistant to *S. mansoni* infection) were able to adhere equally well to the primary and secondary sporocysts. In addition, the primary and secondary sporocysts exposed to *B. tenagophila* Taim presented the highest CAI value of all snails tested. In the viability/mortality experiments, the greatest percentage of dead primary and secondary sporocysts were observed upon exposure to *B. tenagophila* Taim and *B. tenagophila* Cabo Frio. Furthermore, both *B. tenagophila* Taim and Cabo Frio were able to kill more primary than secondary sporocysts. These results suggest that the *Biomphalaria* are more effective in recognising primary sporocysts than secondary sporocysts. We observed a higher mortality in the control sporocysts than the sporocysts exposed to *B. glabrata*. This could be because *B. glabrata*, which is most susceptible to *S. mansoni* infection, may produce molecules that can increase the sporocyst survival. The results using fluorescent probes corroborate to those previously described. *B. tenagophila* Taim causes more damage to the

tegumental surface of primary sporocysts. The primary sporocysts exposed to *B. tenagophila* Taim were labelled with Alexa-Fluor 488 and lectin from Glycine max at a greater rate than those inoculated into *B. tenagophila* Cabo Frio and the control sporocysts, and the primary sporocysts exposed to *B. tenagophila* Taim were labelled at a greater rate with Alexa-Fluor 488 and lectin from Glycine max than those sporocysts exposed to *B. tenagophila* Cabo Frio and the control sporocysts, and the primary sporocysts exposed to *B. tenagophila* Taim showed higher Alexa-Fluor 488 and lectin from Glycine max labelling intensities than secondary sporocysts. The probe Hoechst 33258 is used to assay membrane integrity and is thus a very sensitive probe for labelling tegument damage; even small amounts of tegument damage can be detected by this probe (Mattos et al. 2006, Oliveira et al. 2006). A similar sensitivity is not observed for the lectin from Glycine max (Mattos et al. 2006 in sporocyst) or Alexa-Fluor 488 (Oliveira et al. 2006 in adult worms). Consistent with these earlier results, only the sporocysts with intense damage were labelled with Alexa-Fluor 488 and lectin from Glycine max. These results demonstrate that damage by Taim IDS causes more N-acetylgalactosamine to be exposed on the parasite

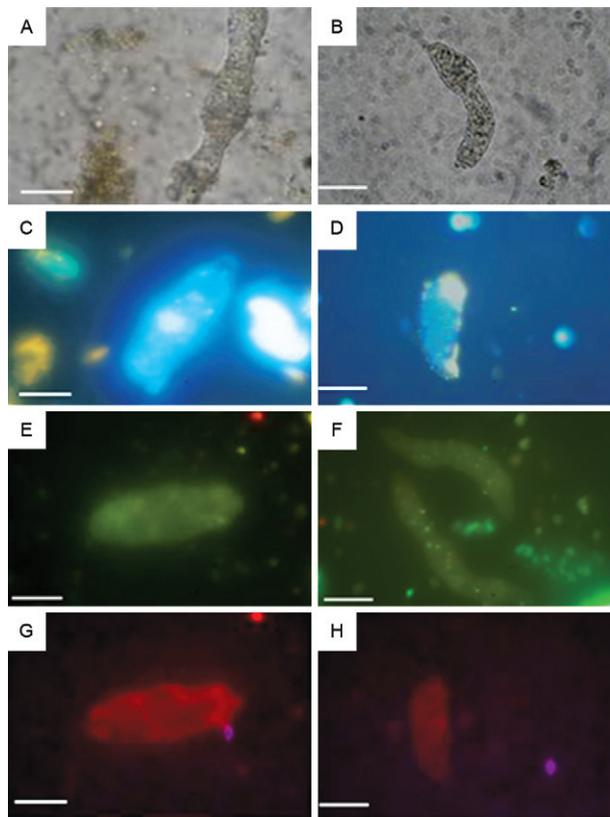


Fig. 5: action of internal defence systems of *Biomphalaria tenagophila* from Cabo Frio on the membrane of primary (A, C, E, G) and secondary (B, D, F, H) sporocysts. A, B: optical microscopy; C, D: labeled with Hoechst 33258 (fluorescence microscopy); E, F: labeled with Alexa-Fluor 488 (fluorescence microscopy); G, H: labeled with lectin of Glycine max (fluorescence microscopy). Bars = 25  $\mu$ m.

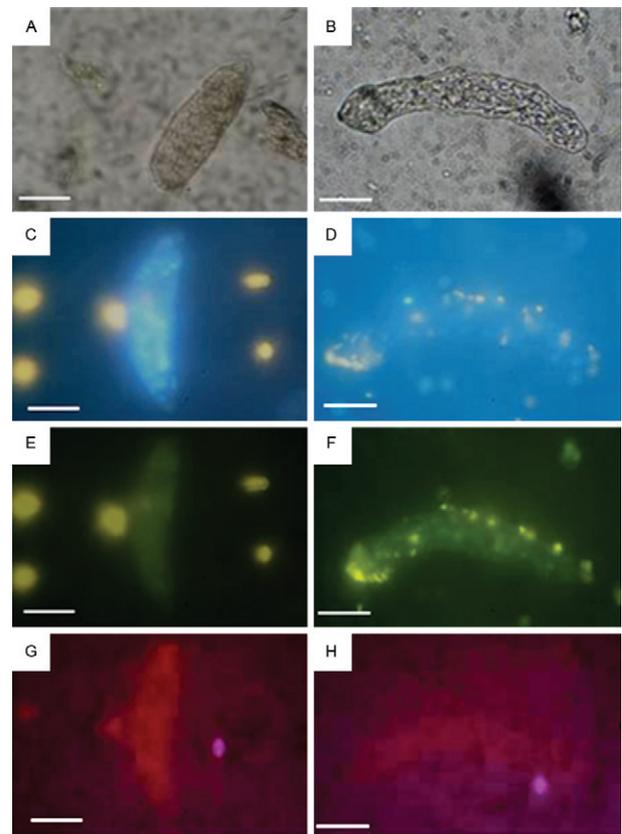


Fig. 6: primary (A, C, E, G) and secondary (B, D, F, H) sporocysts control. A, B: optical microscopy; C, D: labeled with Hoechst 33258 (fluorescence microscopy); E, F: labeled with Alexa-Fluor 488 (fluorescence microscopy); G, H: labeled with lectin of Glycine max (fluorescence microscopy). Bars = 25  $\mu$ m.

TABLE III

Numbers and percentage of alive and infected snails 30 days after inoculation with secondary sporocysts of *Schistosoma mansoni*

Species/lineages	Inoculated snails	Alive snails (%)	Infected snails (%)
<i>Biomphalaria tenagophila</i> Taim	65	7 (11)	0
<i>B. tenagophila</i> Cabo Frio	35	11 (31)	0
<i>Biomphalaria glabrata</i>	65	21 (32)	11 (52)

surface. Likewise, the Alexa-probe 488 labels the actin under the sporocyst surface. We attempted to infect *B. tenagophila* Taim and Cabo Frio with secondary sporocysts derived from *B. glabrata*; however, at the end of the experiments, only *B. glabrata* molluscs were infected. These data suggest that *B. tenagophila* Taim and Cabo Frio may recognise and destroy parasites derived from another *Biomphalaria* species. In addition, the mortality of *B. tenagophila* Taim was higher than that of the other snails. It has been well established that *B. tenagophila* Taim is completely resistant to *S. mansoni* (Corrêa et al. 1979, Santos et al. 1979, Bezerra et al. 1997, 2003, Martins-Souza et al. 2003, Rosa et al. 2004, 2005, 2006, Barbosa et al. 2006, Coelho et al. 2008, Pereira et al. 2008). Table II shows that *B. tenagophila* Taim kills sporocysts more effectively than the Cabo Frio strain or *B. glabrata*. Because the control values in Table II are high, it is not clear whether sporocysts are killed by *B. glabrata* or by the Cabo Frio strain.

The tegument of *S. mansoni* sporocysts is an important interface for molecular communication between the parasite and *Biomphalaria* (Johnston & Yoshino 1996). According to Loker (1994), the sporocyst's tegument is the main target for the snail's IDS. The haemocytes from resistant mollusc can recognise, encapsulate and destroy the sporocysts soon after *S. mansoni* invasion. However, the ability of parasites to avoid or disrupt the host's immune response is fundamental to the establishment of parasite-host compatibility (Yoshino & Bayne 1983). Similar molecules have been found in *S. mansoni* and in *Biomphalaria*, suggesting an evolutionary convergence of molecular expression between schistosomes and their snail hosts (Basch 1976, Yoshino & Bayne 1983, Peterson et al. 2009, van Die & Cummings 2010, Lehr et al. 2010). One may hypothesise that the similarity of these molecules is important for the escape process of the parasite; this hypothesis is known as molecular mimicry (Lehr et al. 2008, 2010). In these experiments, we used primary sporocysts transformed in vitro from miracidia and secondary sporocysts isolated from infected *B. glabrata*; these different intramollusc stages may present distinct surface molecules. The carbohydrates of glycoproteins and glycolipids represent the major chemical components of the schistosome intramollusc stage (Zelck & Becker 1990, Uchikawa & Loker 1991). These carbohydrates can be recognised by lectins present in the snail's IDS and it has been demonstrated that lectins play an important role in the parasite/mollusc

interaction (Uchikawa & Loker 1991, Johnston & Yoshino 1996, Martins-Souza et al. 2006). Lectins (carbohydrate-binding proteins) are also important receptors for the non-self recognition capabilities of snails and others invertebrates (Renwanz 1986).

These results indicate that the techniques used here can be performed to test whether carbohydrates on the parasite surface represent a form of molecular mimicry. Future work will use the Taim IDS to detect changes in carbohydrates on the sporocyst surface (induced by glycosidases, for example) and further comparisons between sporocysts isolated from *B. glabrata* and *B. tenagophila* may reveal those molecules recognised by the Taim IDS in transplantation experiments.

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