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## Effect of amphotericin B on the infection success of *Schistosoma mansoni* in *Biomphalaria glabrata*.

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1 **Effect of amphotericin B on the infection success of *Schistosoma mansoni* in *Biomphalaria***  
2 ***glabrata*.**

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22

23 ABSTRACT

24 In the present study, we examined the effect of amphotericin B on larval stages (miracidia and  
25 primary sporocyst) of the helminth *Schistosoma mansoni*, the causative agent of human  
26 schistosomiasis. Amphotericin B (AmB) is a polyene macrolide that disturbs the function of the cell  
27 membrane; it is widely used as prophylactic antimycotic agent in *in vitro* culture. We show for the  
28 first time that *S. mansoni* miracidia infectivity is considerably reduced after AmB treatment.  
29 Moreover we demonstrate that AmB does not affect the development, growth, viability, and  
30 behavior of miracidia and primary sporocysts. Our data indicate that AmB effects on *S. mansoni*  
31 sporocyst prevalence are linked to the oxidative properties of AmB. These may alter the capacity of  
32 sporocysts to respond to the oxidative stress generated by the snail immune defence system.

33

34 Keywords: Amphotericin B, *Schistosoma mansoni*, miracidium, *Biomphalaria glabrata*, reactive  
35 oxygen species (ROS).

36

37 INTRODUCTION

38

39 Schistosomiasis or bilharzia is a tropical parasitic disease affecting 200 million humans in 74  
40 countries, causing 200,000 deaths annually. It is the second most important tropical disease in terms  
41 of morbidity after malaria. Schistosomiasis is caused by flatworms of the genus *Schistosoma*, with  
42 the most common causative species being *S. haematobium*, *S. japonicum*, and *S. mansoni* (Chitsulo,  
43 et al., 2004, Gryseels, et al., 2006).

44 The cycle of the parasite is complex. It requires humans as definitive host and certain freshwater  
45 snail species as intermediate hosts. Infection occurs in water by the free-living larval stages  
46 (cercaria for the definitive host and miracidia for the intermediate host). Significant attention has  
47 been paid to freshwater snails over the past because of their both medical and epidemiological  
48 importance as intermediate hosts for schistosome parasites. Moreover, the interaction between  
49 *Schistosoma mansoni* and the snail *Biomphalaria glabrata* provides a model of choice in  
50 evolutionary biology to investigate the host-parasite coevolutionary dynamics (Beltran and Boissier,  
51 2008, Beltran, et al., 2008, Bouchut, et al., 2008, Roger, et al., 2008, Steinauer, 2009). In this  
52 context, the *in vitro* maintenance of schistosome intramolluscan stages is a valuable tool to  
53 investigate host-parasite interaction, particularly at the molecular level (Coustau and Yoshino,  
54 2000). Thus, *in vitro* culture is used often in this model to obtain pure parasites and/or secretion  
55 products to identify the molecules involved in host-parasite interplay (Bender, et al., 2002, Guillou,  
56 et al., 2007, Roger, et al., 2008, Roger, et al., 2008), to perform pharmacological investigations  
57 (Mattos, et al., 2006), or to study parasite development (Azzi, et al., 2009).

58 To avoid the risk of contamination in those *in vitro* culture, antimicrobial substances were widely  
59 used, mainly streptomycin, penicillin (as antibiotics) and amphotericin B (as antimycotic) (Smyth,  
60 1990). It is known that antimicrobials can have side effects and consequently may affect the  
61 cultivated organisms (Kuhlmann, 1995). Moreover, recent work has demonstrated activity of AmB  
62 against helminth parasites (Olds, et al., 1981, Reuter, et al., 2003).

63 In this paper we describe the effect of an antimicrobial mix on the miracidium infectivity. This  
64 antimicrobial substance is a mixture of two antibiotics, penicillin (PnG) and streptomycin (StrS)  
65 and one antimycotic: amphotericin B (AmB). Herein we show that AmB decreases the infectivity of  
66 the miracidium, the snail infective larval stage of schistosome parasite. To understand this anti-  
67 parasitic effect better, the mode of action of AmB was examined.

68

## 69 MATERIALS AND METHODS

70

### 71 *Biological material*

72 The host-parasite association *Biomphalaria glabrata* – *Schistosoma mansoni* originated from  
73 Brazil, and has been maintained in the laboratory for several years. Miracidia from *S. mansoni* were  
74 hatched from eggs axenically recovered from infected hamster (*Mesocricetus auratus*) livers after  
75 50 days according as described procedures (Roger, et al., 2008). Briefly, livers were collected and  
76 kept in sterile 0.85% saline, containing a 1X antibiotic/antimycotic mixture (100 units/ml penicillin  
77 G, 0.1mg/ml streptomycin sulfate, 0.25µg/ml amphotericin B). The livers were homogenized and  
78 the eggs were filtered, washed and transferred to natural mineral water (Volvic) to allow miracidia  
79 to hatch under illumination.

80

### 81 *Effect of antimicrobial substances on S. mansoni miracidia infectivity*

82 Different antimicrobial substances were added directly into the dish containing the miracidia,  
83 and they were incubated for 3 hours before snail infection. Five groups of miracidia were used: a  
84 control group (untreated miracidia), an antibiotic/antimycotic (AM) mixture 1X treated group, a  
85 penicillin G (PnG) treated group (100 units/ml), a streptomycin sulfate (StrS) treated group (0.1  
86 mg/ml), a PnG (100 units/ml)/StrS (0.1 mg/ml) treated group, and an amphotericin B (AmB) treated  
87 group (0.25 µg/ml). For each group, 20 snails (5-7 mm diameter) were exposed individually to 10

88 miracidia. Following exposure snails were kept in water at a constant temperature of 26°C and fed  
89 on lettuce *ad libitum*.

90 The infected or non-infected status of the exposed snails was detected by the observation of the  
91 mother sporocysts (Sp1) in the head-foot region. The snails were fixed 15 days post-exposure as  
92 described (Theron, et al., 2008). Briefly, the snails were relaxed in water containing an excess of  
93 crystalline menthol for 6 hours. The snail shell was removed and the body was fixed in modified  
94 Raillet-Henry's solution (930 ml distilled water, 6g sodium chloride, 50ml formol 40%, 20 ml  
95 acetic acid). The presence of Sp1 in each snail was determined by dissection of the head-foot zone  
96 and visual inspection. The Sp1 were readily observable as translucent white bodies within an  
97 opaque yellow tissue background.

98

#### 99 *Effect of antimicrobial substances on S. mansoni miracidia swimming behaviour*

100 Four groups of miracidia were formed and incubated during 3 hours in Volvic water containing  
101 different antimicrobial substances AM 1X, PnG (100 units/ml), StrS (0.1 mg/ml), and AmB (0.25  
102 µg/ml). An additional control experiment was done with untreated miracidia. The miracidia were  
103 filmed, their swimming behavior (sinuosity, rotation) was observed and their linear swimming  
104 speed was determined using the video analysis software Kinovea (<http://www.kinovea.org>).

105

#### 106 *Effect of antimicrobial substances on S. mansoni miracidia in vitro transformation to sporocysts* 107 *and sporocyst viability*

108 Miracidia were submitted to *in vitro* transformation to obtain primary sporocysts (Sp1).  
109 Miracidia were cultured for 24 h in sterile Chernin's balanced salt solution (CBSS) (Chernin, 1963),  
110 containing StrS (0.1 mg/ml) and PnG (100 units/ml) mixture (used as control), AM 1X, or AmB  
111 (0.25 µg/ml). The experiments were conducted two times in triplicate on 24 well plates with 20  
112 sporocysts per well.

113 The percentage of transformed miracidia was determined directly by the observation of remaining  
114 miracidia not transformed after 24 hours of culture. The viability of the Sp1 was assessed by two  
115 different methods: (i) Sp1 were observed with a light microscope and considered alive when their  
116 motility and/or the beating of the flame cells' flagella was observed; (ii) the Sp1 were exposed to  
117 trypan blue and observed using a light microscope. Sp1 were considered dead when intra-sporocyst  
118 trypan blue accumulation could be observed.

119

#### 120 *Effect of AmB on B. glabrata hemocyte Reactive Oxygen Species (ROS) production*

121 *Biomphalaria glabrata* hemocytes were recovered after hemolymph puncture as previously  
122 described (Bouchut, et al., 2006). Briefly, for each testing condition 500  $\mu$ l of hemolymph were  
123 recovered and centrifuged (600g, 10 min) to pellet the hemocytes. Hemocytes were washed three  
124 times in PBS (phosphate buffered saline) to remove hemoglobin. Hemocytes were then incubated in  
125 500  $\mu$ l of PBS (control), or PBS containing (i) 1 $\mu$ M Phorbol 12-Myristate 13-Acetate (PMA), (ii)  
126 0.25 $\mu$ M AmB, (iii) 1 $\mu$ M PMA + 0.25 $\mu$ M AmB. After 4 hours 500  $\mu$ l of Nitroblue tetrazolium  
127 (NBT) was added to each sample and incubated at 20°C for 60 min. The four samples were then  
128 centrifuged (600g, 10 min) and the hemocyte pellets were resuspended in 70% MeOH. Samples  
129 were centrifuged (600g, 10 min), the supernatant was discarded, 200  $\mu$ l of dimethylsulfoxide  
130 (DMSO)/2 M KOH solution was added and samples were mixed vigorously. Absorbance of  
131 supernatants was read in a spectrophotometer at 620 nm vs. a DMSO/KOH blank. For each  
132 condition, values were expressed as OD for  $5 \times 10^4$  hemocytes. The experiments were conducted two  
133 times in triplicate for each sample. The ROS-mediated (Reactive Oxygen Species) reduction of  
134 NBT results in the precipitation of insoluble blue formazan which can be quantified  
135 spectrophotometrically in hemocytes or in hemocytes after treatment with the antimycotic AmB and  
136 the respiratory burst stimulator PMA. This protocol was adapted from a published method  
137 (Anderson and Brubacher, 1995).

138

139 *Effect of AmB on S. mansoni sporocysts resistance to oxidative stress*

140 Miracidia were cultured 24 h in sterile Chernin's balanced salt solution (CBSS), containing  
141 StrS (0.1 mg/ml) and PnG (100 units/ml) mixture (used as control), or AmB (0.25 µg/ml). After 24  
142 hours of culture, increasing amounts of H<sub>2</sub>O<sub>2</sub> were added to the culture medium of primary  
143 sporocysts (Sp1). Five concentrations of H<sub>2</sub>O<sub>2</sub> were tested: 0, 200, 400, 800 and 1600 µM. After 4  
144 hours, the viability of Sp1 was assessed as described above

145 The experiments were conducted two times in triplicate on 24 well plates with 20 sporocysts per  
146 well.

147

148 *Effect of AmB on S. mansoni sporocysts total antioxidant capacity*

149 Quantitative measurement of the cumulative antioxidant capacity of sporocysts was determined  
150 following exposition to different AmB concentrations. Total antioxidant activity was measured  
151 using the Antioxidant Assay Kit (Sigma). For each test, 2000 sporocysts were *in-vitro* transformed  
152 and cultured in sterile Chernin's balanced salt solution (CBSS), containing 0; 0.25; 0.5; or 1.25  
153 µg/ml of AmB. After 24h fully transformed sporocysts were recovered by gentle centrifugation  
154 (800g, 5 minutes, 4°C), sonicated (3 pulses of 20 seconds), centrifuged to pellet cell debris (12000g,  
155 15 minutes, 4°C) and antioxidant capacity of supernatant was determined following manufacturer's  
156 recommendations. Protein amount in the supernatant was determined using Bradford protein  
157 quantification assay and used as a correcting factor. The experiment was done six times for each  
158 AmB concentration tested.

159

160 *Statistical analysis*

161 Results of miracidia infection, miracidia transformation and sporocyst viability were analyzed  
162 using a two-tailed Fisher's Exact test. Results of miracidia linear swimming speed and hemocyte  
163 ROS production were analyzed using the Kruskal-Wallis test. Results of AmB effects on sporocysts



164 resistance to oxidative stress and antioxidant capacity were analyzed using the Student T test.  
165 Levels of confidence lower than 0.05 were considered to be statistically significant.

166

## 167 RESULTS

### 168 *Effect of antimicrobial substances on S. mansoni miracidia infectivity*

169 We assayed the effect of miracidia treatment with antimicrobial substances on infectivity (  
170 Fig. 1). Miracidia treated with 1X AM and AmB display significant prevalence decrease ( $P < 0.006$   
171 and  $P < 0.005$  respectively) compared to the control. We observed a reduction of more than 50% of  
172 *S. mansoni* miracidia infectivity after those two treatments. Prevalence for the control was 65% and  
173 only 32.5% for 1X AM and 25% for AmB (Fig. 1). There is no significant difference between AM  
174 1X and AmB treatments, and neither StrS nor PnG had effects on miracidia infectivity compared to  
175 the control (Fig. 1). These results permit us to conclude that AmB is reducing *S. mansoni* miracidia  
176 infectivity and that no synergistic or antagonistic effects could be observed with the other  
177 antimicrobial substances tested here

### 178 *Effect of antimicrobial substances on S. mansoni miracidia swimming behavior*

179 Swimming behavior of miracidia was investigated after treatment with antimicrobial  
180 substances. Linear swimming speed (Fig. 2A), sinuosity and miracidia swimming rotation (data not  
181 show) did not differ from that of the control. The linear swimming speed was of 1.35, 1.18, 1.17,  
182 1.21, and 1.16 mm/s for the control, 1X AM, AmB, PnG, and StrS respectively. No significant  
183 differences were observed between those values (Kruskal-Wallis ;  $P = 0.63$ ). Thus, none of the  
184 molecules tested have an effect on swimming behavior of miracidia

185

### 186 *Effect of antimicrobial substances on S. mansoni miracidia in vitro transformation to sporocysts* 187 *and sporocysts viability*

188 In this experiment the use of antimicrobial molecules in the culture medium was necessary  
189 to avoid microbial contamination. Thus, a PnG/StrS mixture was used as control. Miracidia

190 transformation and sporocyst viability was compared between this control and 1X AM or AmB (see  
191 Fig. 2B). Miracidia transformation did not differ from that of the control. The percentage of  
192 transformed miracidia to sporocysts was 97, 96 and 98% for the control, 1X AM, and AmB,  
193 respectively. The sporocysts viability after 24 h in culture was very good with 93.2, 93.8, and  
194 91.9% of live sporocysts for the control, 1X AM, and AmB (Fig. 2B.1). Miracidia and sporocysts  
195 did not show any tegumental lysis or shape modifications when comparing the control and AmB  
196 (Fig. 2B.2). Motility, beating of the flame cells' flagella or trypan blue efflux were the same for all  
197 samples. To conclude, none of the molecules tested seem to have an effect on miracidia  
198 transformation and sporocyst viability.

199

#### 200 *Effect of AmB on B. glabrata hemocyte Reactive Oxygen Species (ROS) production*

201 Here we tried to quantify the ROS production of *B. glabrata* hemocytes after treatment with  
202 AmB in the presence or absence of PMA, a respiratory burst stimulator. The ROS produced by the  
203 hemocytes reduce NBT and the optical density (OD) of reduced NBT was quantified. Intracellular  
204 ROS production by AmB-treated and PMA-stimulated cells did not differ from that of  
205 untreated/unstimulated control cells (Fig. 2C). The OD of reduced NBT for  $5 \times 10^4$  hemocytes was  
206 0.084, 0.083, 0.088 and 0.095 for the control, PMA, AmB and AmB/PMA respectively (Fig. 2C).  
207 No significant differences were observed between those values (Kruskal-Wallis ;  $P=0.84$ ). It seems  
208 that AmB did not affect *B. glabrata* hemocyte ROS production, with or without burst stimulation.

209

#### 210 *Effect of AmB on S. mansoni sporocysts resistance to oxidative stress*

211 Resistance of Sp1 to oxidative stress was investigated after treatment with AmB. Here we  
212 used exposure to  $H_2O_2$  for 4 hours as oxidative stress. Different concentrations of  $H_2O_2$  were used.  
213 No significant differences between AmB-treated and untreated sporocysts (control) were observed  
214 for 0, 200, 400 and 800  $\mu M$  of  $H_2O_2$  (Fig. 3). However, for 1600  $\mu M$  a significant difference was  
215 noted (Student T-test;  $P=0.048$ ) (Fig. 3). Viability values were 95.5 and 99.3% for AmB-treated and

216 untreated, respectively. This suggests that Sp1 exposed to AmB would be less able to respond to  
217 oxidative stress.

218

#### 219 *Effect of AmB on S. mansoni sporocysts total antioxidant capacity*

220 Total antioxidant capacity of sporocysts was determined after exposition to increase concentrations  
221 of AmB (Fig. 4). Sporocysts antioxidant activity decreases when AmB concentrations increase.  
222 Antioxidant concentration was 214.02 $\mu$ M when no AmB was present in the culture medium and  
223 significantly decreased to 105.08 $\mu$ M when 1.25 $\mu$ g/ml of AmB was added to the culture medium  
224 (Student T-test; P=0.00107) (Fig. 4). To conclude it seems that AmB affect directly the total  
225 antioxidant capacity of sporocysts.

226

## 227 DISCUSSION

228 AmB is known to be an antifungal agent that binds to sterols (cholesterol and ergosterol) in  
229 the fungal cell wall, forming transmembrane channels resulting in osmotic lysis and death of the  
230 organism (HsuChen and Feingold, 1973, HsuChen and Feingold, 1973, Reuter, et al., 2003). On the  
231 other hand, several studies suggest that AmB has an effect on prokaryotes and eukaryotes  
232 (protozoan and metazoan) (Cruz, et al., 1980, Lachaud, et al., 2009, Olds, et al., 1981, Reuter, et al.,  
233 2003, Thomas, et al., 1973). The protective effect against those pathogens was linked mainly to  
234 AmB immunomodulatory effects and induction of macrophage activation (Beccari, et al., 1991,  
235 Kumar and Chakrabarti, 2000, Little, et al., 1978, Olds, et al., 1981, Wilson, et al., 1991, Wolf and  
236 Massof, 1990). However on helminths AmB was shown to act by two different ways. Alveolar  
237 echinococcosis caused by the parasitic cestode *Echinococcus multilocularis* was significantly  
238 reduced by AmB treatment (Reuter, et al., 2003). AmB seems to act directly on the parasite cell  
239 membrane. This destructive effect suppressed parasite growth and development (Reuter, et al.,  
240 2003). With the helminth *S. mansoni*, AmB treatment increases resistance in mice to a challenge  
241 with *S. mansoni* cercariae (Olds, et al., 1981). AmB treatment was effective in protecting mice by

242 killing schistosomula, the migrating larval stage of the parasite. This study demonstrated activation  
243 of murine macrophages by AmB, which contributes to enhanced mice resistance to infection (Olds,  
244 et al., 1981).

245 In our study we investigated the effect of AmB on the *S. mansoni* larval stage interacting  
246 with the invertebrate host *B. glabrata*. To our knowledge, we demonstrate for the first time a  
247 reduction of *S. mansoni* miracidia infectivity after AmB treatment (Fig. 1). We observed a reduction  
248 of Sp1 prevalence of more than 50% after treatment of miracidia with AmB (Fig. 1).

249 Based on previous work conducted on the AmB effect on helminthes, we tried to determine the  
250 mechanisms by which AmB reduces *S. mansoni* miracidia infectivity. A direct deleterious effect on  
251 miracidia was considered. We investigated the effect of AmB on parasite and host behavior and  
252 physiological traits but no significant differences were noted (Fig.2).

253 Thus AmB would not be able to impair (i) the capacity of *S. mansoni* miracidia to find and  
254 penetrate into snail host in aquatic environment, (ii) the capacity of *S. mansoni* miracidia to  
255 transform into sporocysts, (iii) the sporocysts growth, development and viability. The present  
256 results showed that AmB did not have any direct deleterious effect on miracidia and sporocysts.

257 To go further we looked at the immunomodulatory effect of AmB. In vertebrates AmB was known  
258 to activate macrophage oxidative burst by the production of reactive oxygen metabolites (Wilson, et  
259 al., 1991, Wolf and Massof, 1990). In invertebrate host of *S. mansoni*, *Biomphalaria glabrata*, the  
260 internal defense system is mediated by either humoral and/or cell components (Ataev and Coustau,  
261 1999, Bayne, et al., 2001). The most important effectors of immunity in molluscs are the circulating  
262 hemocytes. These phagocytic cells are able to distinguish foreign, non-self material, and  
263 encapsulate, kill and eliminate invaders such as parasite sporocysts (de Jong-Brink, et al., 2001,  
264 Hahn, et al., 2001). Several studies have indicated that reactive oxygen species (ROS), like  
265 hydrogen peroxide, produced by hemocytes play a crucial role in the killing of the parasite (Adema,  
266 et al., 1994, Bayne, et al., 2001, Dikkeboom, et al., 1988, Hahn, et al., 2001). In the present study  
267 we investigated the *in vitro* effect of AmB on *B. glabrata* hemocyte ROS production. ROS were

268 quantified by NBT reduction after hemocytes treatment with AmB alone or in combination with the  
269 respiratory burst stimulator PMA. Intracellular ROS production by AmB treated and PMA  
270 stimulated hemocytes did not differ from that of untreated/unstimulated control cells (Fig. 2C). It  
271 seems that AmB did not affect *B. glabrata* immune effectors. Unlike vertebrates it seems that AmB  
272 did not induce immunomodulatory effect in the invertebrate's phagocytic cells and did not activate  
273 hemocyte oxidative burst.

274 Decreased prevalence of *S. mansoni* sporocysts after AmB treatment observed in the present paper  
275 would thus neither be related to a direct deleterious effect on the parasite's membrane nor to  
276 activation of invertebrate host immunity. However, AmB may induce oxidative damage in  
277 conjunction with other oxidizing agents (Brajtburg, et al., 1985). Thus we investigated the  
278 resistance of Sp1 to H<sub>2</sub>O<sub>2</sub> oxidative stress after treatment with AmB. A significant difference was  
279 noted between AmB-treated and untreated sporocysts (Fig. 3). This suggests that Sp1 exposed to  
280 AmB are less able to respond to oxidative stress. To go further and clarify the influence of AmB on  
281 parasite antioxidant activity, we measure the total antioxidant capacity of the sporocysts after  
282 treatment with AmB. The results show that AmB treatment causes a decrease of the sporocyst  
283 antioxidant capacity in a dose-dependant manner. Living organisms have a large number of  
284 antioxidants, including macro and micro molecules, and enzymes that play a central role in  
285 preventing oxidative stress. AmB affects the global antioxidant capacity of the parasite and thus  
286 directly reduces its ability to respond to oxidative stress.

287 It is known that miracidia and sporocysts use antioxidant molecules and ROS scavengers to protect  
288 themselves against hemocyte-mediated cytotoxicity (Bernal, et al., 2006, Connors, et al., 1991,  
289 Guillou, et al., 2007, Knudsen, et al., 2005, Perez-Sanchez, et al., 2006, Roger, et al., 2008). If  
290 parasite antioxidant molecules were used to overcome AmB oxidant effects, those molecules would  
291 not be available and effective against ROS snail host activities. Thus sporocysts would not be able  
292 to protect themselves from a second oxidative stress that occurred when sporocysts are confronted  
293 by snail hemocytes ROS products (Bayne, 2009, Bayne, et al., 2001, Bender, et al., 2005).

294

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299

300

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542

543 **Legends to figures:**

544 **Figure 1:**

545 Prevalence and abundance of *S. mansoni* sporocysts in *B. glabrata* exposed to miracidia  
546 treated with different antimicrobial substances. The snails were individually exposed to 10  
547 miracidia treated either by 1X AM, 100U/ml PnG, 10mg/ml StrS, or 0.25µg/ml AmB. The control  
548 was untreated miracidia. The asterisks indicate treatment for which the decrease of prevalence is  
549 significant ( $p < 0.05$ ).

550

551 **Figure 2**

552 Influence of the antimicrobial substances on *S. mansoni* and *B. glabrata* behavioral and  
553 physiological traits. (A) Linear swimming speed of miracidia exposed to 1X AM, 100U/ml PnG,  
554 10mg/ml StrS, or 0.25µg/ml AmB. (B.1) Viability % of sporocysts after 24 hours in CBSS medium  
555 containing a mixture of 100U/ml PnG and 10mg/ml StrS used as control or 1X AM or 0.25µg/ml  
556 AmB. (B.2) *S. mansoni* miracidia and cultivated sporocysts in presence or absence of 0.25µg/ml  
557 AmB. Miracidia and sporocysts were exposed to AmB for 3 hours and 24 hours respectively. The  
558 detached ciliated plates in the sporocysts culture were indicated with black arrows. Scale bars are 35  
559 µm. (C) AmB (0.25µg/ml) effect on ROS production by *B. glabrata* hemocytes stimulated or not by  
560 PMA. The ROS production was assessed by the reduction of nitroblue tetrazolium measured  
561 spectrophotometrically at 620 nm.

562

563 **Figure 3**

564 Sporocyst viability 4 hours after exposure to different H<sub>2</sub>O<sub>2</sub> concentrations. The sporocysts  
565 were treated with AmB (0.25µg/ml) for 24 hours in CBSS medium or untreated. The asterisk  
566 indicate significant difference ( $p < 0.05$ ) of viability between AmB treated and untreated sporocysts.

567

568 **Figure 4:**

569 Total antioxidant capacity of sporocysts exposed to different concentrations of AmB. The  
570 sporocysts were treated with AmB (0.25; 0.5; 1.25 $\mu$ g/ml) for 24 hours in CBSS medium or  
571 untreated. The asterisk indicate significant difference ( $p < 0.05$ ) of antioxidant concentration between  
572 AmB treated and untreated sporocysts.

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Figure 1

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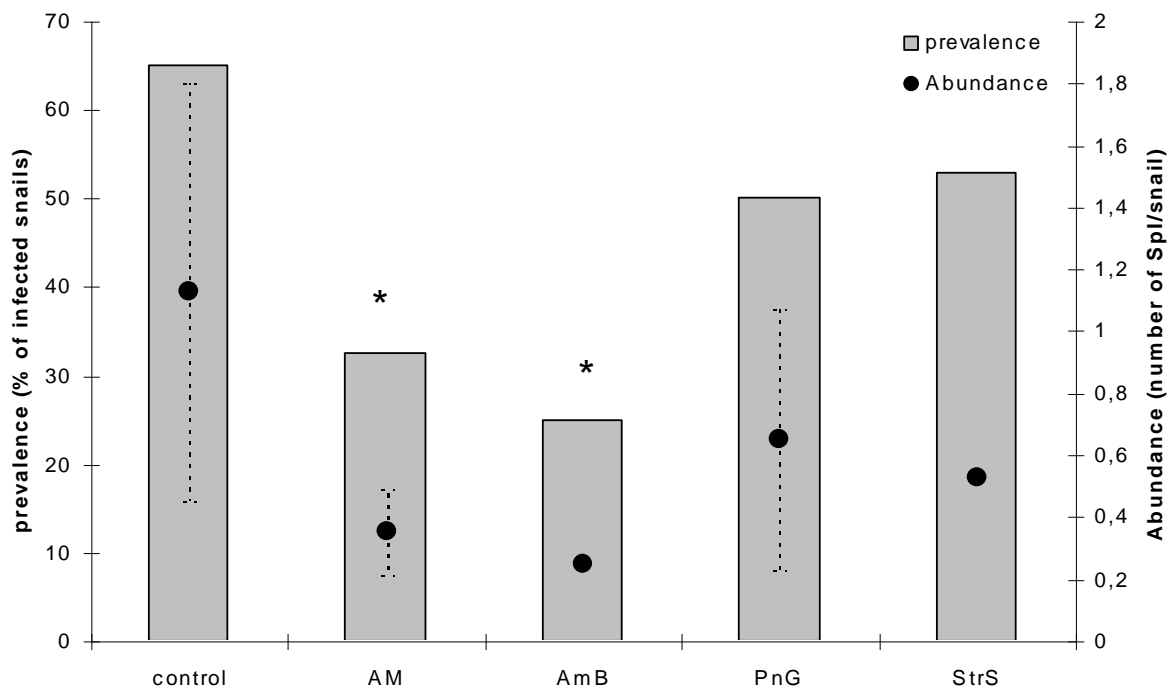
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594 **Figure 2**

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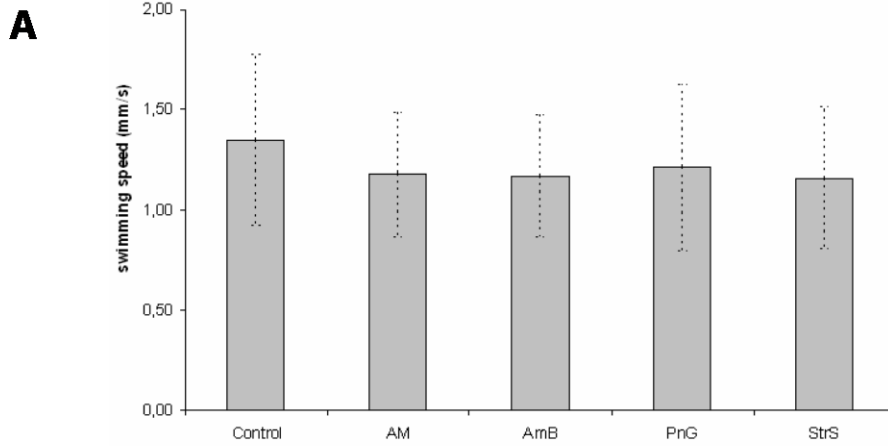
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**B.1**

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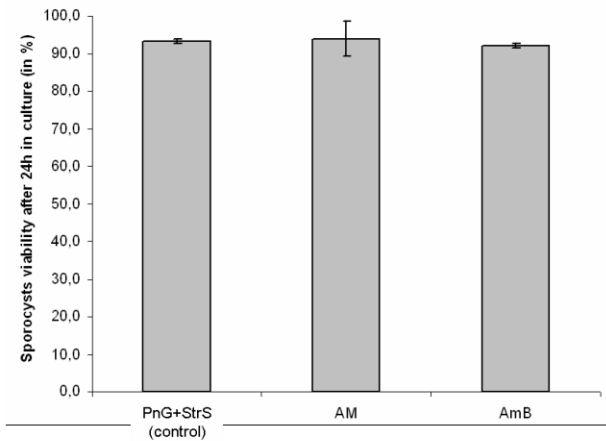
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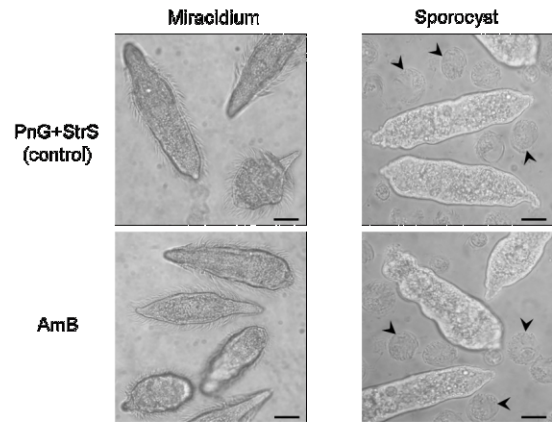
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**B.2**



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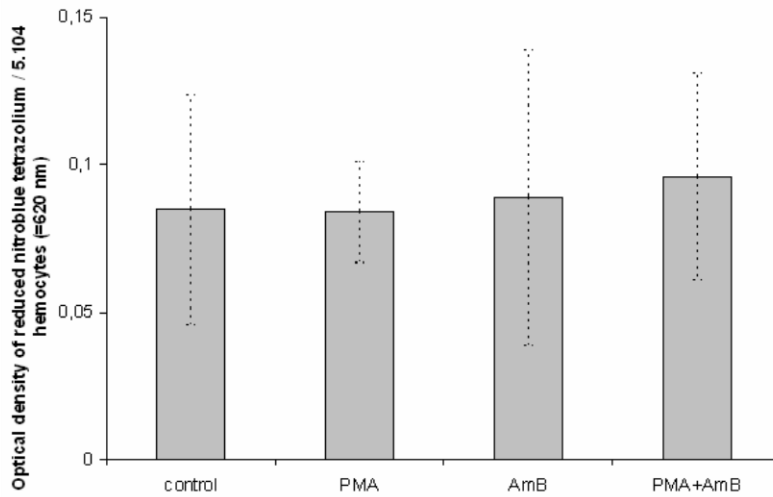
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**C**



619 Figure 3

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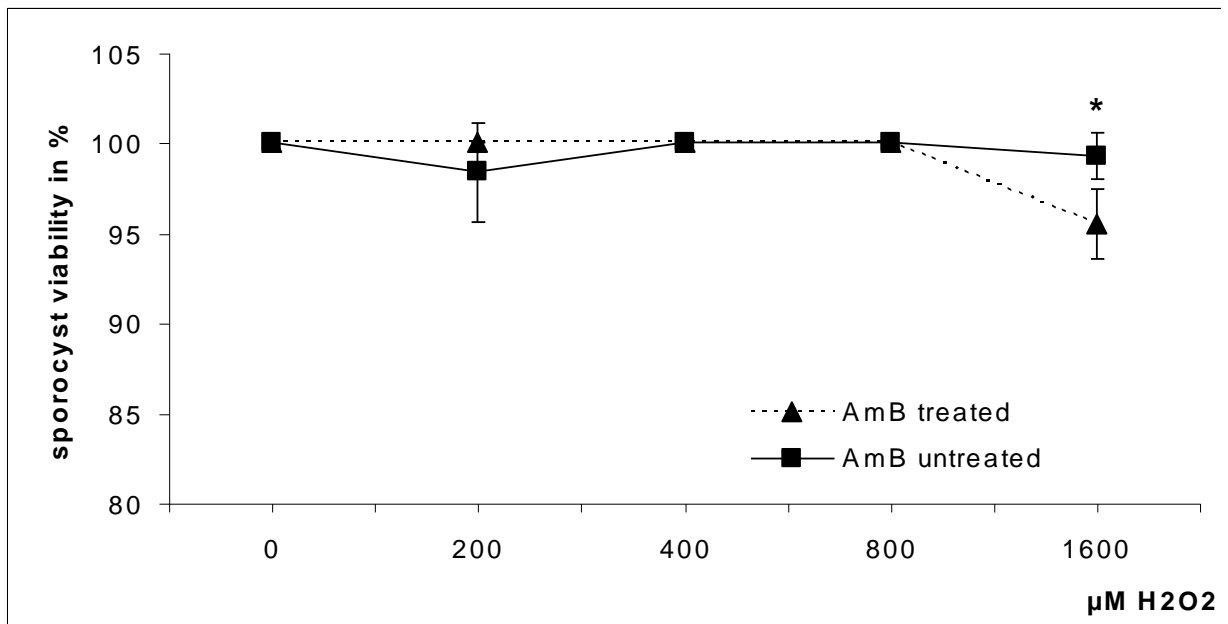
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Figure 4

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