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ABSTRACT

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

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

INTRODUCTION

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

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

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

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

MATERIALS AND METHODS

Biological material

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

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

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

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

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

Effect of antimicrobial substances on S. mansoni miracidia swimming behaviour

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

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

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

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

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

Biomphalaria glabrata hemocytes were recovered after hemolymph puncture as previously described (Bouchut, et al., 2006). Briefly, for each testing condition 500 µl of hemolymph were recovered and centrifuged (600g, 10 min) to pellet the hemocytes. Hemocytes were washed three times in PBS (phosphate buffered saline) to remove hemoglobin. Hemocytes were then incubated in 500 µl of PBS (control), or PBS containing (i) 1µM Phorbol 12-Myristate 13-Acetate (PMA), (ii) 0.25µM AmB, (iii) 1µM PMA + 0.25µM AmB. After 4 hours 500 µl of Nitroblue tetrazolium (NBT) was added to each sample and incubated at 20°C for 60 min. The four samples were then centrifuged (600g, 10 min) and the hemocyte pellets were resuspended in 70% MeOH. Samples were centrifuged (600g, 10 min), the supernatant was discarded, 200 µl of dimethylsulfoxide (DMSO)/2 M KOH solution was added and samples were mixed vigorously. Absorbance of supernatants was read in a spectrophotometer at 620 nm vs. a DMSO/KOH blank. For each condition, values were expressed as OD for $5x10^4$ hemocytes. The experiments were conducted two times in triplicate for each sample. The ROS-mediated (Reactive Oxygen Species) reduction of NBT results in the precipitation of insoluble blue formazan which can be quantified spectrophotometrically in hemocytes or in hemocytes after treatment with the antimycotic AmB and the respiratory burst stimulator PMA. This protocol was adapted from a published method (Anderson and Brubacher, 1995).

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

Miracidia were cultured 24 h in sterile Chernin's balanced salt solution (CBSS), containing StrS (0.1 mg/ml) and PnG (100 units/ml) mixture (used as control), or AmB (0.25 µg/ml). After 24 hours of culture, increasing amounts of H_2O_2 were added to the culture medium of primary sporocysts (Sp1). Five concentrations of H_2O_2 were tested: 0, 200, 400, 800 and 1600 µM. After 4 hours, the viability of Sp1 was assessed as described above

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

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

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

Statistical analysis

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

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

RESULTS

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

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

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

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

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

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

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

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

Here we tried to quantify the ROS production of *B. glabrata* hemocytes after treatment with AmB in the presence or absence of PMA, a respiratory burst stimulator. The ROS produced by the hemocytes reduce NBT and the optical density (OD) of reduced NBT was quantified. Intracellular ROS production by AmB-treated and PMA-stimulated cells did not differ from that of untreated/unstimulated control cells (Fig. 2C). The OD of reduced NBT for $5x10^4$ hemocytes was 0.084, 0.083, 0.088 and 0.095 for the control, PMA, AmB and AmB/PMA respectively (Fig. 2C). No significant differences were observed between those values (Kruskal-Wallis ; P=0.84). It seems that AmB did not affect *B. glabrata* hemocyte ROS production, with or without burst stimulation.

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

Resistance of Sp1 to oxidative stress was investigated after treatment with AmB. Here we used exposure to H_2O_2 for 4 hours as oxidative stress. Different concentrations of H_2O_2 were used. No significant differences between AmB-treated and untreated sporocysts (control) were observed for 0, 200, 400 and 800 µM of H_2O_2 (Fig. 3). However, for 1600 µM a significant difference was 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 214.02µM when no AmB was present in the culture medium and
223 significantly decreased to 105.08µM when 1.25µg/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

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

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

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

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

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

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

Decreased prevalence of *S. mansoni* sporocysts after AmB treatment observed in the present paper would thus neither be related to a direct deleterious effect on the parasite's membrane nor to activation of invertebrate host immunity. However, AmB may induce oxidative damage in conjunction with other oxidizing agents (Brajtburg, et al., 1985). Thus we investigated the resistance of Sp1 to H_2O_2 oxidative stress after treatment with AmB. A significant difference was noted between AmB-treated and untreated sporocysts (Fig. 3). This suggests that Sp1 exposed to AmB are less able to respond to oxidative stress. To go further and clarify the influence of AmB on parasite antioxidant activity, we measure the total antioxidant capacity of the sporocysts after treatment with AmB. The results show that AmB treatment causes a decrease of the sporocyst antioxidant capacity in a dose-dependant manner. Living organisms have a large number of antioxidants, including macro and micro molecules, and enzymes that play a central role in preventing oxidative stress. AmB affects the global antioxidant capacity of the parasite and thus directly reduces its ability to respond to oxidative stress.

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

ACKNOWLEDGMENTS

This work was supported by the CNRS. We thank Bernard Dejean, and Anne Rognon, for technical assistance. We are grateful to Dr. Michael Freitag and Dr. Christoph Grunau for their critical reading of the manuscript.

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Legends to figures:

Figure 1:

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

Figure 2

Influence of the antimicrobial substances on *S. mansoni* and *B. glabrata* behavioral and physiological traits. (A) Linear swimming speed of miracidia exposed to 1X AM, 100U/ml PnG, 10mg/ml StrS, or 0.25µg/ml AmB. (B.1) Viability % of sporocysts after 24 hours in CBSS medium containing a mixture of 100U/ml PnG and 10mg/ml StrS used as control or1X AM or 0.25µg/ml AmB. (B.2) *S. mansoni* miracidia and cultivated sporocysts in presence or absence of 0.25µg/ml AmB. Miracidia and sporocysts were exposed to AmB for 3 hours and 24 hours respectively. The detached ciliated plates in the sporocysts culture were indicated with black arrows. Scale bars are 35 µm. (C) AmB (0.25µg/ml) effect on ROS production by *B. glabrata* hemocytes stimulated or not by PMA. The ROS production was assessed by the reduction of nitroblue tetrazolium measured spectrophotometrically at 620 nm.

Figure 3

Sporocyst viability 4 hours after exposure to different H_2O_2 concentrations. The sporocysts were treated with AmB (0.25µg/ml) for 24 hours in CBSS medium or untreated. The asterisk indicate significant difference ($p<0.05$) of viability between AmB treated and untreated sporocysts.

Figure 4:

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

Figure 1

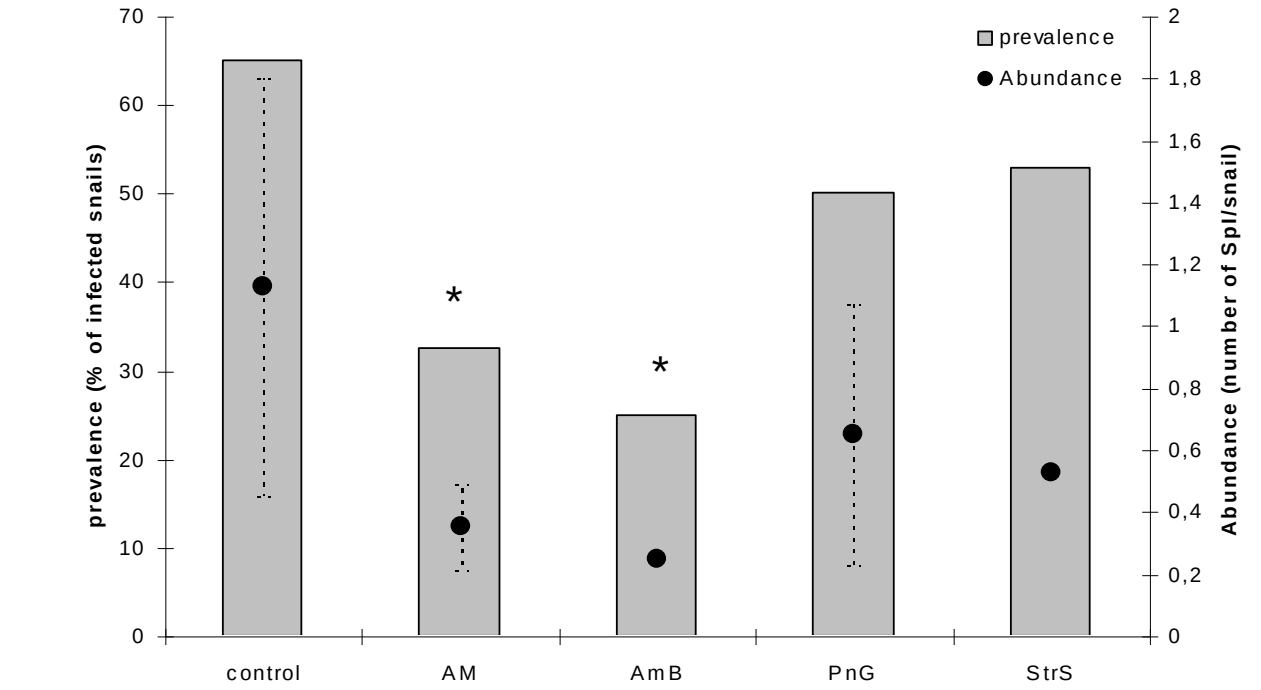

prevalence
Abundance
prevalence (% of infected snails)
Abundance (number of Spl/snail)
70
60
50
40
30
20
10
0
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
*
control
AM
AmB
PnG
StrS

Figure 2

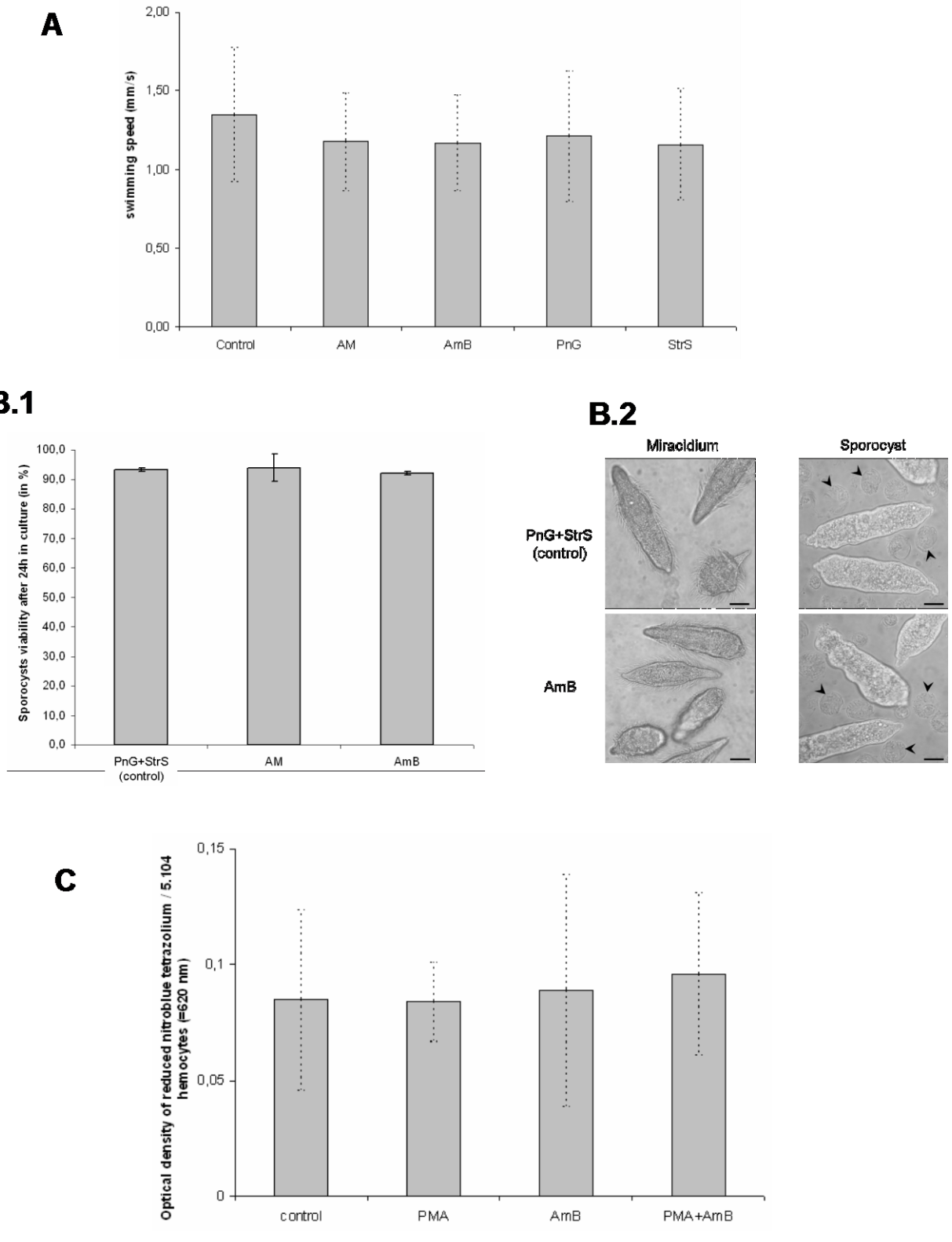
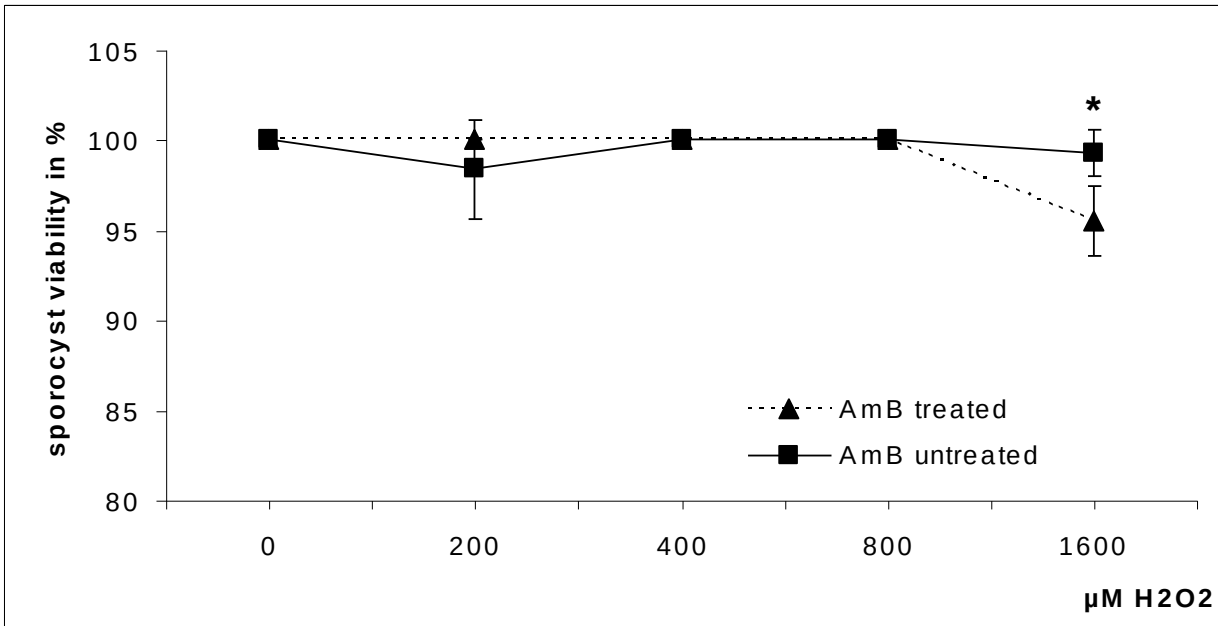

A
swimming speed (mm/s)
2,00
1,50
1,00
0,50
0,00
Control
AM
AmB
PnG
StrS
B.1
Sporocysts viability after 24h in culture (in %)
100,0
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
PnG+StrS (control)
AM
AmB
B.2
Miracidium
Sporocyst
PnG+StrS (control)
AmB
C
Optical density of reduced nitroblue tetrazolium / 5.10⁴ hemocytes (=620 nm)
0,15
0,1
0,05
0
control
PMA
AmB
PMA+AmB

Figure 3


105
100
95
90
85
80
sporocyst viability in %
*
AmB treated
AmB untreated
0
200
400
800
1600
µM H2O2

642 Figure 4

643

644