

An example of molecular co-evolution: Reactive oxygen species (ROS) and ROS scavenger levels in Schistosoma mansoni/Biomphalaria glabrata interactions.

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1	An example of molecular co-evolution: reactive oxygen species (ROS) and ROS scavenger
2	levels in Schistosoma mansoni/Biomphalaria glabrata interactions

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23 Abstract

The co-evolution between hosts and parasites involves huge reciprocal selective pressures 24 25 on both protagonists. However, relatively few reports have evaluated the impact of these reciprocal 26 pressures on the molecular determinants at the core of the relevant interaction, such as the factors 27 influencing parasitic virulence and host resistance. Here, we address this question in a host-parasite 28 model that allows co-evolution to be monitored in the field: the interaction between the mollusk, 29 Biomphalaria glabrata, and its trematode parasite, Schistosoma mansoni. Reactive oxygen species 30 (ROS) produced by the hemocytes of B. glabrata are known to play a crucial role in killing S. 31 mansoni. Therefore, the parasite must defend itself against oxidative damage caused by ROS using 32 ROS scavengers in order to survive. In this context, ROS and ROS scavengers are involved in a coevolutionary arms race, and their respective production levels by sympatric host and parasite could 33 34 be expected to be closely related. Here, we test this hypothesis by comparing host oxidant and 35 parasite antioxidant capabilities between two S. mansoni/B. glabrata populations that have coevolved independently. As expected, our findings show a clear link between the oxidant and 36 antioxidant levels, presumably resulting from sympatric co-evolution. We believe this work 37 provides the first supporting evidence of the Red Oueen Hypothesis of reciprocal evolution for 38 39 functional traits at the field-level in a model involving a host and a eukaryotic parasite.

40

41 *Keywords*: Host-parasite co-evolution, *Schistosoma mansoni*, *Biomphalaria glabrata*, Reactive
42 oxygen species (ROS), ROS scavengers

43

44 **1. Introduction**

Understanding the co-evolution of host-parasite interactions represents a challenge in 45 evolutionary biology. Parasites cause substantial deleterious effects on their hosts, and therefore 46 47 represent a major driving force in their evolution (Howard, 1991). Similarly, the host immune defenses represent the major selective pressure driving the evolution of parasites. For parasites to 48 49 survive and develop in the host they must adapt to the host-defense system or they will die. This 50 parallel co-evolution of host-parasite interactions can be viewed as an arms race in which both the 51 host and the parasite develop mechanisms to circumvent the weapons developed by their opponent. 52 In this context of reciprocal co-evolution, illustrated by Van Valen (1974), under the Red Queen 53 Hypothesis it is assumed that the parasitic genes responsible for infectivity will evolve alongside the host defense genes, resulting in adaptation of the interactions between local host and parasite 54 populations (Dybdahl and Storfer, 2003). To date, however, only a few studies have sought to 55 verify this prediction and convincing experiments have only been reported for models involving 56 viruses, bacteria and unicellular eukaryotes (Lohse et al., 2006; Forde et al., 2008). 57

Demonstrating co-evolution in an animal host-parasite system is not straightforward and 58 most prior discussions of such processes have been indirect, as in studies describing local 59 adaptation when compatibility is higher between sympatric host-parasite combinations than 60 between allopatric combinations (Gasnier et al., 2000; Gagneux et al., 2006; Munoz-Antoli et al.), 61 62 or studies that have focused on only one trait of the interaction, such as host resistance (Green et al., 2000) or parasite infectivity (Little et al., 2006). Moreover it is important to take into account that 63 64 non-co-evolutionary mechanisms could also explain correlations between the traits of interacting species and that the absence of correlated traits is not evidence for an absence of co-evolution 65 (Nuismer et al., 2007, 2010; Yoder and Nuismer, 2010). However we assume that the direct 66 67 examination of reciprocal selection in both the host and the parasite could provide supporting 68 evidence of co-evolution.

69 Two relatively recent studies investigated this reciprocal response more thoroughly. In the first, reciprocal changes in resistance and infectivity were identified for co-evolving Potamopyrgus 70 71 snail hosts and their trematode parasites; however, while these changes were identified using 72 prevalence phenotypes, they were not supported by the studied functional markers (Koskella and 73 Lively, 2007). The second report provided experimental support for the reciprocity of adaptation 74 costs, rapid genetic changes and increased genetic diversity during the co-evolution of a 75 multicellular host, the nematode Caenorhabditis elegans, and its pathogenic bacteria, the Gram-76 positive bacterium, Bacillus thuringiensis (Schulte et al., 2010). In both papers, co-evolution was 77 studied using laboratory strains selected by experimental evolutionary approaches, and only the 78 second paper focused on molecular changes induced by the co-evolutionary process. Both papers 79 were based on an "over time" approach in which the evolution of phenotypes was monitored over 80 the course of experimental laboratory generations. Other empirical studies have been based on a 81 "point time" approach in which the pattern of co-variations between host-parasite populations or 82 strains that co-evolved independently were assessed at a single experimental time point (Forde et 83 al., 2004; Morgan et al., 2005).

84 Here, we investigated the reciprocal evolution of molecular mechanisms directly at the core of the host-parasite interaction in a natural system of co-evolution, by comparing host and parasite 85 86 populations that have co-evolved independently. As a model, we used the interaction between the 87 trematode, *Schistosoma mansoni* (responsible for human intestinal schistosomiasis), and its mollusk 88 intermediate host, Biomphalaria glabrata. This interaction is a model of choice for the study of 89 potential co-evolutionary dynamics (Webster and Davies, 2001; Webster et al., 2004; Beltran and 90 Boissier, 2008; Beltran et al., 2008; Bouchut et al., 2008; Roger et al., 2008a; Roger et al., 2008b; 91 Roger et al., 2008c; Steinauer, 2009).

During its intramolluskal stage, the parasite must cope with the snail's immune system. One of the main immune effectors in mollusks are the reactive oxygen species (ROS) produced by hemocytes (the circulating immune cells of snails) (Hahn et al., 2000; de Jong-Brink et al., 2001;

95 Hahn et al., 2001b; Mourao et al., 2009b). Previous studies conducted by Hahn and co-workers demonstrated that hydrogen peroxide (H₂O₂) plays a crucial role in the killing of S. mansoni 96 97 sporocysts (Hahn et al., 2001a, b). Furthermore, hemocytes from S. mansoni-resistant snails were 98 shown to generate significantly more H_2O_2 than susceptible snails, perhaps due at least in part to the 99 former having constitutively elevated levels of the mRNA encoding the copper/zinc superoxide dismutase (Cu-ZN SOD) (Goodall et al., 2004; Bender et al., 2005; Bender et al., 2007). To resist 100 101 ROS-mediated attacks, the S. mansoni larvae produce ROS-detoxifying enzymes (Vermeire et al., 102 2006; Guillou et al., 2007; Vermeire and Yoshino, 2007; Roger et al., 2008c; Wu et al., 2009), 103 several of which appear to be secreted by sporocysts (Guillou et al., 2007; Wu et al., 2009). 104 Supporting this, a recent report showed that antioxidant enzymes produced by S. mansoni 105 sporocysts are directly involved in protecting the pathogen against immune cell-mediated oxidative 106 stress (Mourao et al., 2009b).

In this context, the snail-produced ROS and the parasite-produced ROS scavengers are involved in a co-evolutionary arms race, and we can hypothesize that their production levels will be closely related. Here, we tested this hypothesis by comparing host oxidant and parasite antioxidant abilities for two *S. mansoni/B. glabrata* strains that have evolved independently, originated from different geographic endemic zones and which are found to display significant differences in compatibility.

113

114 **2. Materials and methods**

115 2.1 Ethics statement

Our laboratory has received the permit # A66040 for experiments on animals from both the French Ministry of agriculture and Fishing and the French Ministry of National Education, Research and Technology. Housing, breeding and animal care of the mice followed the ethical requirements of French government. The experimenter possesses the official certificate for animal experimentation delivered by both ministries (Décret # 87–848 du 19 octobre 1987; number of the authorization
007083).

122

123 2.2. Biological materials

Two strains of *S. mansoni* were used in this study: a Brazilian strain (*Sm*BRE) and a Guadeloupean strain (*Sm*GH2). Each strain was maintained: (i) in their sympatric strain of *B. glabrata* (*Bg*BRE and *Bg*GUA, respectively); and (ii) in hamsters (*Mesocricetus auratus*), as described previously (Théron et al., 1997).

Miracidia from *Sm*BRE and *Sm*GH2 were hatched from eggs axenically recovered from 60day-infected hamster livers, according to the previously described procedure (Roger et al., 2008c). Briefly, livers were collected and kept overnight at 4°C in sterile saline solution (NaCl 150 mM) containing an antibiotic/antimycotic mixture (penicillin 100 units/ml, streptomycin 0.1 mg/ml, amphotericin B 0.25 μ g/ml; Sigma). The livers were then homogenized and the eggs were filtered out, washed, and transferred to spring water. The miracidia were allowed to hatch out under illumination.

135

136 2.3. Schistosome-snail compatibility: snail exposure, infection rates and intensities

The compatibilities of the tested snail-schistosome combinations were evaluated by monitoring the infection rates (% of snails infected) and the intensity of infection (number of mother sporocysts (SpI) developed) among snails individually challenged with different numbers of miracidia. As the miracidial dose increased, a larger fraction of the phenotypic diversity in the parasitic isolate was sampled; thus, dose-response curves are much more informative than singledose challenges when examining the dynamics of compatibility between two host-parasite combinations (Théron et al., 2008).

144 For each experiment, snails (7-9 mm in diameter) were exposed individually to a fixed 145 number of miracidia in approximately 10 ml of water for 8 h. Following exposure to miracidia, the snails were replaced in their original containers until their infection status (presence of SpI) was assessed. For the detection of SpI, the snails were fixed 15 days post-exposure, following previously described methods (Gerard et al., 1995; Moné et al., 2010b). In brief, each snail was relaxed in pond water containing an excess of crystalline menthol for 6 h, the body was removed and fixed in modified Raillet-Henry's solution, exhaustive dissection of the head-foot zone was performed, and the number of SpI present in each snail (readily observable as translucent white bodies within an opaque gray tissue background) was determined.

Dose-response curves were obtained by challenging individual snails (30-40 snails per treatment) with doses of 1, 10, 20, 30 and 50 miracidia. Compatibility was measured for the two sympatric combinations (*Sm*BRE versus BgBRE and *Sm*GH2 versus BgGUA) and the two allopatric combinations (*Sm*BRE versus BgGUA and *Sm*GH2 versus BgBRE).

157

158 2.4. Cytotoxicity of H₂O₂ on S. mansoni sporocysts

H₂O₂ cytotoxicity was measured using the Roche Cytotoxicity Detection Kit (Roche 159 160 Diagnostics, Mannheim, Germany), which is based on the measurement of lactate dehydrogenase 161 (LDH) activity released from dead and lysed cells into the supernatant. Four hundred miracidia each of SmBRE and SmGH2 were submitted to in vitro transformation to obtain primary sporocysts 162 (Sp1). Briefly, the miracidia were cultured for 24 h in sterile Chernin's balanced salt solution 163 (CBSS) (Chernin, 1963), containing an antibiotic/antimycotic mixture (penicillin 100 units/ml, 164 streptomycin 0.1 mg/ml, amphotericin B 0.25 µg/ml; Sigma). The sporocysts were then exposed to 165 166 four different concentrations of H₂O₂ (0, 75, 150 and 200 µM) for 2 h and cytotoxicity was examined according to the manufacturer's instructions. As a positive control, we measured LDH 167 release from Sp1 that had been lysed with the provided lysis solution (high control, HC); this was 168 169 taken as 100% LDH release. To correct for the background, we measured LDH levels in Sp1-free 170 H₂O₂-treated culture medium (substance control, SC). All measured values were assayed in

171 triplicate. The percentage of specific H₂O₂-induced LDH release was determined as: % cvtotoxicity

172 = $[(experimental result - SC)/(HC - SC)] \times 100.$

173

174 2.5. Effect of H_2O_2 on S. mansoni sporocyst mortality

Two independent experiments were conducted in triplicate on 24-well plates containing 20 Sp1 (representing *Sm*BRE or *Sm*GH2) per well. The Sp1 were in-vitro transformed as described above (see Materials and methods section 2.2), and exposed to 0, 200, 400, 800 or 1,600 μ M of H₂O₂ (Hydrogen peroxide 35%, FLUKA, Germany) for 4 h. Mortality was assessed under a light microscope, with the Sp1 considered "dead" when we failed to observe motility and/or the beating of the flame-cell flagella.

181

182 2.6. The total antioxidant capacity of S. mansoni sporocysts

183 The cumulative (total) antioxidant capacity of the sporocysts was quantified for the two 184 parasite strains, SmBRE and SmGH2, using an Antioxidant Assay Kit (Sigma). For each test, 2,000 sporocysts were in-vitro transformed as described above. After 24 h, fully transformed sporocysts 185 were recovered by gentle centrifugation (800 g, 5 min, 4° C). The samples were then disrupted by 186 sonication (three pulses of 20 s each) and pelleted by centrifugation (12,000 g, 15 min, 4°C), and 187 188 the antioxidant capacity of each supernatant was determined following the manufacturer's 189 recommendations. The amount of protein in each supernatant was determined using a Bradford 190 protein assay kit and used as a correcting factor. The experiment was performed six times per strain.

191

192 2.7. Reverse Transcription-quantitative PCR (RT-qPCR)

RT-qPCR analyses were conducted to compare the expression of parasite antioxidant enzymes suspected to play key roles in the detoxification of host-induced oxidative stress. Realtime PCR analyses were performed using a LightCycler 2.0 system (Roche Applied Science) and a LightCycler Faststart DNA Master SYBR Green I kit (Roche Applied Science). Total RNA extractions from miracidia were performed using the Trizol Reagent (Life Technologies, USA) and

198 the manufacturer's protocol. Reverse transcription was performed according to previously described 199 procedures (Guillou et al., 2004). qPCR amplification was performed using 2.5 µl of cDNA in a 200 final volume of 10 µl containing 3 mM MgCl₂, 0.5 µM of each primer and 1 µl of master mix. The 201 primers were designed using either the LightCycler probe design software or the web-based 202 Primer3 plus interface (http://www.bioinformatics.nl/cgibin/ primer3plus/primer3plus.cgi) and are 203 given in Table 1. The following Light-Cycler run protocol was used: denaturation at 95°C for 10 204 min, followed by 40 cycles of amplification and quantification at 95°C for 10 s, 60°C for 5 s and 205 72°C for 16 s, a melting curve of 60-95°C with a heating rate of 0.1°C/s and continuous 206 fluorescence measurement, and then a cooling step to 40°C. For each reaction, the cycle threshold 207 (Ct) was determined using the "Fit Point Method" of the LightCycler Software, version 3.3. The 208 PCR reactions were performed in duplicate and the mean Ct value was calculated. For each sample, 209 the expression level of the target gene was normalized with regard to the expression of two 210 constitutively expressed genes (28S rRNA and α tubulin). The expression ratio (R) was calculated 211 according to the formula: $R = 2(\Delta Ct)$, where ΔCt represents Ct (target gene) – Ct (constitutively 212 expressed gene).

213

214 2.8. ROS detection in single cells

215 The cell-permeable fluorescent oxygen probe, 1-pyrenebutyric acid (PBA), can be used for 216 the measurement of free radicals in solution (Oter and Ribou, 2009) and in living cells (Ribou et al., 217 2004; Rharass et al., 2006), with the fluorescence intensity and lifetime of PBA decreasing 218 proportionately to the free-radical concentration. Measurement of the fluorescent lifetime offers 219 many advantages over intensity based measurements when working in vivo, not the least that the 220 measurements are independent of the absolute intensity of emitted light and the fluorophore 221 concentration, thereby avoiding artifacts arising from optical losses. Moreover, these probes do not 222 require a reaction with ROS, are usually stable and the fluorescent lifetime is not modified by probe 223 degradation or variations in its intracellular accumulation.

224

225 2.8.1. Staining and fixation

226 Hemolymph samples were recovered from *Bg*BRE and *Bg*GUA snails, and aliquots (150 µl) 227 were put in a Sykes-Moore chamber. After 4 h, the adhered hemocytes were rinsed with Hank's 228 buffered salt solution (HBSS) and stained for 20 min with PBA (Acros Organics, Belgium; 0.10 229 µM in 1% ethanol). The hemocytes were then rinsed three times and placed in HBSS for 230 measurements. For fixation experiments, hemocytes were treated as described above except that 231 after the final rinsing step, the cells were killed by incubation for 10 min in Baker solution (10% 232 paraformaldehyde in 1% aqueous calcium chloride). In the latter case, the experiments were 233 performed within 1 h after cell killing, in order to avoid probe reorganization (Ribou et al., 2004).

234

235 2.8.2. ROS quantification by fluorescent-lifetime measurement of single cells

236 The fluorescent decay of single living cells loaded with PBA was recorded using time-237 resolved microfluorimetry, as previously described (Ribou et al., 2003). Briefly, a laser (nitrogen 238 laser NL100; Stanford Research Systems, USA) delivered monochromatic 337-nm pulses, each 239 with a half-amplitude pulse-width of 3 ns, and an objective (40×; Unitron) was used to concentrate 240 the excitation beam on the microscopic sample. Emitted photons were collected and focused on a photomultiplier 1P28 (Hamamatsu Corporation, Japan). A diaphragm placed on the emission 241 242 pathway allowed the selection of signals from single cells, while a 404-nm bandpass filter (half bandwidth; 40 nm) also located along the emission pathway was used to select the pyrene emission. 243 244 Each signal was digitalized by a digital oscilloscope (TDS 3032C; Tektronix, USA). The 245 fluorescent decay of single PBA-loaded cells selected by the 404-nm filter could be resolved into 246 three exponential curves. The time constants (i.e. lifetimes) and amplitude values of each 247 exponential curve in the decay were obtained using the downhill simplex method (Nelder and 248 Mead, 1965). The first two decays corresponded to the intrinsic fluorescence of the cell attributed to 249 the reduced form of NAD(P)H. The third long-time constant (> 100 ns), which was characteristic of pyrene derivatives, was related to the ROS concentration through the Stern-Volmer equation (Stern and Volmer, 1919) that describes collisional fluorescent quenching of a probe (i.e. PBA) by a
quencher (i.e., free radicals). The method has been described in several papers (Ribou et al., 2003;
Ribou et al., 2004; Rharass et al., 2006). We calculated the variation of intracellular ROS
concentrations as follows:

$$[ROS] / [ROS]_{m} = [t_{m} (t_{0} - t)] / [t(t_{0} - t_{m})]$$
Equation 1

where t is the fluorescent lifetime measured for 108 single hemocytes originating from eight BgBRE snails and 111 cells from eight BgGUA snails; t_m is the mean of all lifetimes; and t₀ is the fluorescent lifetime in the absence of ROS (measured from dead hemocytes fixed with Baker solution). In this equation, [ROS]_m is the mean of the concentrations from all tested cells (219 cells). We assumed that fixation ended all cellular activity and ROS production. In the presented data, the mean ROS concentration has been assigned an arbitrary value of 1.

262

263 2.9. H_2O_2 production and release by B. glabrata hemocytes

264 The H_2O_2 production by hemocytes was measured using Amplex[®] Red (Invitrogen). Hemolymph was collected from the head-foot regions of BgBRE and BgGUA snails (7-11 mm in 265 diameter) as previously described (Bouchut et al., 2006), and the number of hemocytes per µl of 266 hemolymph was quantified using a cell counter (Z Series Coulter Counter; Beckman Coulter); 267 226.2 ± 50.6 cells /µl and 241.2 ± 102.1 cells /µl were obtained for BgBRE and BgGUA, 268 respectively. The Hemolymph of four snails was pooled and 45,000 hemocytes per well were 269 270 dispensed to a 96-well plate for each strain. The hemocytes were allowed to adhere and spread for 1 h at 26°C. The plate was then centrifuged (600g for 10 min), the plasma was removed, the adhered 271 272 hemocytes were washed three times with HBSS, and the wells were treated with Amplex® Red 273 reaction mixture (100 µL per well, prepared according to the manufacturer's instructions). Optical 274 density was measured with a microplate reader at 570 nm during the following 1 h (at 5, 10, 15, 20, 275 30, 40, 50, 60 min). The results are expressed as Amplex Red O.D. at 570 nm / 45,000 cells.

278 The plasma content of superoxide anion was monitored via the superoxide-mediated reduction of nitroblue tetrazolium (NBT), which results in the precipitation of an insoluble blue 279 280 formazan that can be quantified spectrophotometrically. Briefly, hemolymph was collected from 281 BgBRE and BgGUA snails as described above. Hemocytes were removed by centrifugation (1,500 282 g for 15 min), and then 50 µL of plasma from each snail was mixed with 50 µl of 0.1% NBT 283 (Sigma) dissolved in PBS; (Na₂HPO₄ 8.41 mM, NaH₂PO₄ 1.65 mM, NaCl 45.34 mM, pH 7.45). 284 Formazan blue formation was measured with a microplate reader at 620 nm over the course of 3 h 285 (at 5, 10, 15, 30, 60, 90, 180 min). NBT-free plasma was used as a control, and triplicate 286 experiments were conducted for 10 individuals per strain.

287

288 2.11. Statistical analyses

289 The normality of our experimental data was assessed using the Shapiro-Wilk normality test 290 (Shapiro and Wilk, 1965). Our data on the effect of H₂O₂ cytotoxicity on S. mansoni sporocysts 291 (LDH test), B. glabrata hemocyte H₂O₂ production, and superoxide anion plasma content were all found to be normally distributed (P > 0.05), and were subsequently analyzed using the student's t-292 293 test. Our data on the effect of H₂O₂ on S. mansoni sporocyst mortality and the total antioxidant 294 capacity of sporocysts were not normally distributed (P < 0.05), and were subsequently analyzed 295 using the Mann-Whitney test. The results of the ROS concentration assays in each mollusk strain 296 were analyzed using the Mann-Whitney test. The Kolmogorov-Smirnov two-samples test was 297 utilized to determine whether the ROS concentrations were similarly distributed in hemocytes from 298 *Bg*BRE and *Bg*GUA.

299

300 **3. Results**

301 3.1. Effect of H_2O_2 on S. mansoni sporocysts

302 Two different assays were conducted to test the effect of H_2O_2 on the two strains of S. 303 mansoni sporocysts (SmBRE and SmGH2) (Fig. 1). First, an LDH test was used to examine the 304 cytotoxicity of H₂O₂ on sporocysts of each strain. Our results revealed that the susceptibility to 305 H_2O_2 was significantly higher for SmGH2 than SmBRE (Fig. 1A). When exposed to 200 μ M H_2O_2 , SmGH2 sporocysts showed 25.8% cytotoxicity (i.e., 25.8% of the cells had lysed and released their 306 307 LDH content), whereas no changes were observed for SmBRE at the same H₂O₂ concentration (Fig. 308 1A). However, although cell lysis occurred in SmGH2, the sporocysts were still alive at this 309 concentration. To investigate possible between-strain differences in mortality, we next exposed 310 sporocytes to increasing concentrations of H₂O₂ and examined motility and the beating of the 311 flame-cell flagella, which were taken as distinguishing between living and dead larvae. No 312 difference between the two strains was observed until the concentration of H_2O_2 reached 1,600 μ M 313 (Fig. 1B). At this concentration, 31.2% and 2.6% of the SmGH2 and SmBRE sporocysts were dead, 314 respectively; this difference is statistically significant (student's *t*-test; P = 0.017). These results 315 suggest that SmGH2 sporocysts are more susceptible to H₂O₂ than SmBRE sporocysts.

316

317 3.2. The total antioxidant capacity of S. mansoni sporocysts

To investigate potential differences in the constitutive antioxidant abilities of sporocysts from *Sm*BRE and *Sm*GH2, we measured the cumulative antioxidant activities of these two strains (Fig. 2). Our results revealed that the antioxidant ability of *Sm*GH2 was significantly lower than that of *Sm*BRE (approximately 13% less; Mann-Whitney test, P = 0.0001).

322

323 3.3. Reverse Transcription-quantitative PCR of ROS-scavenger expression among S. mansoni
324 strains

RT-qPCR was used to compare the expression of parasite antioxidant enzymes suspected to play key roles in the detoxification of host-induced oxidative stress (Table 1) (Guillou et al., 2007). Notably, Cu-Zn SOD (Smp_176200.2) was found to be expressed at a significantly higher level in *Sm*BRE than in *Sm*GH2 (2.7-fold; *P* = 0.017) (Fig. 3). In contrast, no difference was observed in the
expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Smp_056970.1), GST
omega (Smp_152710.1), GST 28 kD (Smp_054160), GST 26 kD (Smp_163610), glyoxalase II
(Smp_091010) or thioredoxin peroxidase (TPX, Smp_158110) (data not shown).

332

333 3.4. Intracellular ROS measurements in single B. glabrata hemocytes

334 We monitored intracellular ROS levels in single hemocytes, using PBA. This method allows 335 global ROS to be measured without interference from the reactive hydroxyl radical or H₂O₂. PBA 336 fluorescent lifetimes were measured for 108 and 111 individual hemocytes originating from eight 337 BgBRE and eight BgGUA snails, respectively. BgBRE hemocytes produced significantly (11.2%) more ROS than BgGUA hemocytes (Mann-Whitney test; P = 0.009) (Fig. 4A). Fig. 4B shows the 338 339 distribution of hemocytes from both strains according to their ROS concentrations, which were 340 calculated from the ratio given in equation 1 (see Materials and methods section 2.8.2.). Although the cells from both BgBRE and BgGUA samples were distributed around the mean ROS 341 342 concentration, their distributions were significantly different (Kolmogorov-Smirnov test, P =343 0.012). Among the hemocytes producing more than 1.5-fold of the mean ROS concentration, 68.8% 344 were from BgBRE strain, while only 31.2% were from BgGUA. Conversely, among the hemocytes 345 that showed the lowest ROS concentrations (< 0.7-fold of the mean ROS concentration) 38.1% were from BgBRE and 62% were from BgGUA (Fig. 4B). By recording the fluorescent lifetimes of 346 single cells loaded with PBA, we also obtained the relative concentrations of free and bound 347 348 NAD(P)H (an indicator of metabolic change) in each cell. However there was no significant 349 difference in the quantity of bound and free NAD(P)H (mean ratio = 0.60 for both strains; data not shown), suggesting that the strains had similar levels of metabolism. Thus, the only molecular 350 351 difference observed between the two strains was the level of ROS production.

352

353 3.5. H_2O_2 production and release by B. glabrata hemocytes

We assessed H₂O₂ production and secretion by hemocytes of both strains using Amplex Red. Fig. 5 shows the cumulative amount of H₂O₂ constitutively released by hemocytes of each strain over 1 h. The maximum level of H₂O₂ production was reached at 20 min for *Bg*GUA and at 40 min for *Bg*BRE after addition of Amplex Red substrate. In total, *Bg*BRE hemocytes produced significantly more (1.44-fold; mean value) H₂O₂ than *Bg*GUA hemocytes (student's *t*-test; *P* < 0.05) (Fig. 5).

360

361 *3.6. Biomphalaria glabrata superoxide anion plasma content*

To our knowledge, all spectrophotometric methods currently available for the determination of H₂O₂ are based on the measurement of red or orange pigments, making these methods unsuitable for use on *B. glabrata* plasma samples, which are already tinted red by hemoglobin. Consequently, we used NBT to measure the amount of superoxide anion (O₂^{-;}; a precursor of H₂O₂) produced in both strains. As shown in Fig. 6, at 3 h after addition of NBT *Bg*BRE plasma contained significantly more (44% more) superoxide anion than *Bg*GUA plasma (student's *t*-test; *P* = 0.0007).

369 3.7. Compatibility of sympatric and allopatric S. mansoni/B. glabrata combinations

370 Sympatric pairings of S. mansoni and B. glabrata originating from Brazil and Guadeloupe 371 were previously shown to display different levels of compatibility that remained remarkably stable 372 across laboratory generations (Théron et al., 2008). We first used dose-response curves obtained by 373 challenging snails with increasing doses of miracidia to confirm that similar differences could be 374 observed between our strains. At doses of 10 or more miracidia/snail, SmBRE/BgBRE showed an infection rate of 100%, while SmGH2/BgGUA had an infection rate of approximately half that, at 375 376 around 55%. Interestingly, differences were also observed for the number of parasites (SpI) that 377 develop within the snails. The infection intensity rose gradually as the challenge doses increased for SmBRE/BgBRE, reaching 16.18 \pm 0.86 parasites/snail at the 50-miracidia dose. In contrast, the 378

infection intensity for SmGH2/BgGUA remained low regardless of the challenge dose, varying between 1.6 ± 0.20 and 3.2 ± 0.64 parasites/snail (Fig. 7).

When we tested the heterologous combinations, we found that the *Sm*BRE/*Bg*GUA pairing showed a substantial level of compatibility, with infection rates of 80-90% (not significantly different from the 100% achieved by the *Sm*BRE/*Bg*BRE pairing), but with lower parasite intensities $(9.8 \pm 0.89 \text{ for the 50-miracidia dose})$ compared with the sympatric combination (16.18 ± 0.86 parasites/snail at the 50-miracidia dose). In contrast, the *Sm*GH2/*Bg*BRE combination showed very little infectivity, with infection rates < 6% and ~1 parasite/snail regardless of the challenge dose (Fig. 7).

388

389 3.8. ROS, ROS scavengers and compatibility in sympatric and allopatric S. mansoni/B. glabrata
390 combinations

391 The above-described results indicated that levels of ROS and ROS scavengers were correlated in both sympatric combinations, with high-level ROS/ROS scavenger production in the 392 393 Brazilian combination, but lower-level ROS/ROS scavenger production in the Guadeloupean 394 combination. If high levels of S. mansoni ROS scavenger are correlated with better resistance of the 395 intramolluskan stage of the parasite (as we hypothesized), we would expect SmGH2 to have a 396 relatively low ability to infect the allopatric BgBRE snails, while SmBRE would have a high ability 397 to infect the allopatric BgGUA snails. This hypothesis was verified in our model, as shown in Fig. 398 8.

399

400 **4. Discussion**

401 Snail-schistosome compatibility and infection rates result from a complex interplay between 402 the host's defense mechanisms and the parasite's infectivity strategies. Due to selective pressures 403 exerted by the parasite on the host and vice versa, co-evolutionary dynamics may be observed 404 (Janzen, 1980; Howard, 1991). Between-population or between-strain differences in the outcomes 405 of such evolutionary processes may be expected due to differences in the epidemiological and 406 environmental conditions, and/or genetic architectures. Such differential selection patterns could 407 explain, at least in part, the geographic and/or strain-specific compatibility variations seen in snail-408 schistosome interactions (Théron et al., 2008). At present, however, there is relatively little 409 empirical evidence demonstrating reciprocal molecular adaptations in both host and parasite.

Here, we investigated the interaction between *S. mansoni* and the snail, *B. glabrata*, as this interaction is a popular model for the study of co-evolutionary dynamics (Beltran and Boissier, 2008; Beltran et al., 2008; Bouchut et al., 2008; Roger et al., 2008a; Roger et al., 2008b; Roger et al., 2008c; Steinauer, 2009). We confirmed that there are different levels of compatibility between two geographic strains of *S. mansoni* and their sympatric snail hosts, *B. glabrata* (Fig. 7) that both have co-evolved independently. We compared the host oxidant and parasite antioxidant abilities that appear to form the core of the attack/defense interactions of these two pairings.

417 ROS are the main effectors of the snail immune system; they are highly reactive and can trigger irreversible cell damage. Indeed, ROS produced by the hemocytes of *B. glabrata* are known 418 419 to play a crucial role in the killing of S. mansoni (Hahn et al., 2000; Hahn et al., 2001a, b; Bender et 420 al., 2005; Bayne, 2009). Conversely, S. mansoni possess antioxidant systems capable of 421 counteracting the ROS produced by their host's immune system. Schistosoma mansoni is exposed 422 to ROS in both their intermediate (snail) and definitive (human or mammalian) hosts, and produce 423 oxidative-stress scavengers in their excretory-secretory products (ESP) during all stages of their life 424 cycle (Mei and LoVerde, 1997; Curwen et al., 2004; Zelck and Von Janowsky, 2004; Knudsen et 425 al., 2005; van Balkom et al., 2005; Bernal et al., 2006; Dzik, 2006; Perez-Sanchez et al., 2006; Cass et al., 2007; Guillou et al., 2007; Mourao et al., 2009a; Wu et al., 2009). Therefore, the success or 426 failure of host invasion by S. mansoni depends at least in part on its ability to defend itself against 427 428 oxidative damage (Mourao et al., 2009a). In this system, therefore, ROS and ROS scavengers 429 should be involved in a co-evolutionary arms race, and we would expect their respective production 430 levels in sympatric host/parasite combinations to be closely related.

431 As previous studies have established that H_2O_2 is the main ROS involved in killing S. 432 mansoni sporocysts, probably due to its stability and capacity to cross cell membranes (Hahn et al., 433 2001b; Bienert et al., 2006), we studied the susceptibility of two strains of S. mansoni to H₂O₂. Our 434 results showed a clear intrinsic difference between parasites isolated from two different geographic regions: the intramolluskan stages of Guadeloupean S. mansoni (SmGH2) were more sensitive to 435 H₂O₂ than those of the Brazilian strain (SmBRE) (Fig. 1). Moreover, we observed a difference in 436 437 antioxidant potential between strains, with SmGH2 displaying a lower level of antioxidant activity 438 than SmBRE (Fig. 2). Thus, SmBRE has a more efficient antioxidant system, which would seem to 439 explain its higher level of resistance to H₂O₂-mediated oxidative damage.

440 In order to identify the molecular pathways involved in these differential antioxidant properties, we investigated the strain-specific transcription levels of genes encoding various 441 442 antioxidant enzymes, including GAPDH (Smp 056970.1), GST omega (Smp 152710.1), GST28 443 (Smp 054160), GST26 (Smp 163610), glyoxalase II (Smp 091010), thioredoxin peroxidase, and Cu-Zn SOD (Smp 176200.2) (Guillou et al., 2007; Vermeire and Yoshino, 2007; Roger et al., 444 2008c; Mourao et al., 2009a; Wu et al., 2009). Among these candidates, only the Cu-Zn SOD 445 mRNA displayed differential expression, with expression levels that were 2.7-fold higher in 446 447 SmBRE than in SmGH2 (Fig. 3). This finding is consistent with our protein-level results from a 448 previous proteomic study (Roger et al., 2008c), and these observations collectively suggest that Cu-449 Zn SOD plays a key role in the antioxidant strategy of S. mansoni. The involvement of Cu-Zn SOD 450 in ROS detoxification is a recurring and intriguing question, because it is capable of dismutating the 451 superoxide anion (O₂[•]) to produce H₂O₂ (Zelck and Von Janowsky, 2004; Guillou et al., 2007; 452 Mourao et al., 2009a). The hypothesis currently used to explain the role of S. mansoni Cu-Zn SOD 453 in ROS detoxification is based on a suspected peroxidative function (Yim et al., 1993; Yim et al., 454 1996; Kim and Kang, 1997; Bayne et al., 2001). In short, it has been proposed that S. mansoni Cu-Zn SOD could use its own dismutation product (H_2O_2) to produce hydroxyl radicals (HO^{\bullet}) that are 455 less toxic for sporocysts (Bayne et al., 2001). 456

457 In a co-evolutionary context, the between-strain differences in ROS susceptibility and antioxidant activity of these S. mansoni strains suggest that there could be comparable differences 458 459 in the ROS production capabilities of the host snail strains. To test this hypothesis, we investigated 460 ROS production by the two snail strains. First, we used a fluorescence-based method (Rharass et al., 2006) to investigate the hemocyte production of free-radicals such as nitric oxide and superoxide 461 462 anion. This approach revealed that BgBRE snails produced more free radicals than BgGUA snails 463 (Fig. 4A). Moreover, a distribution analysis of free-radical concentrations in single hemocytes 464 showed that the cells producing higher concentrations of ROS came from BgBRE individuals, while 465 those producing lower levels of free radicals were from *Bg*GUA snails (Fig. 4B).

466 However, although our results revealed that global ROS production differed between 467 BgBRE and BgGUA, oxidants can differ in their reactivity and efficient parasite killing requires 468 that the host produce the right oxidant (Bayne et al., 2001). As previous studies have demonstrated 469 the crucial role of hydrogen peroxide (H₂O₂) in the killing of S. mansoni sporocysts (Hahn et al., 2001b; Goodall et al., 2004; Bender et al., 2005; Bender et al., 2007), we investigated potential 470 471 differences in hemocyte H₂O₂ production between the snail strains. Our results showed that hemocytes from BgBRE constitutively produced more H_2O_2 than those from BgGUA (Fig. 5). We 472 473 then examined the H₂O₂ content of plasma from these snails. As technical restrictions make it 474 impossible to directly measure H_2O_2 in plasma, we measured the superoxide anion, which is a precursor of H₂O₂ (Selkirk et al., 1998). Our results confirmed that BgBRE plasma contained 475 significantly more superoxide anion than BgGUA plasma (Fig. 6). All of these data were obtained 476 477 from hemocytes harvested from uninfected snails and without cell stimulation. Notably, no difference in ROS production was observed when these hemocytes were stimulated by the addition 478 479 of phorbol 12-myristate 13-acetate (PMA) to culture medium (data not shown).

480 Taken together, our data show that: (i) the production of ROS in general and H_2O_2 (the main 481 ROS acting against *S. mansoni* sporocysts) in particular differ between the two snail strains; and (ii) 482 this H_2O_2 production seems to be correlated with the level of ROS scavengers produced by 483 sympatric parasites. BgBRE snails produce higher amounts of H_2O_2 and interact naturally with 484 *SmBRE*, which have better resistance against oxidative stress, while BgGUA snails produce less 485 H_2O_2 and are sympatric with *Sm*GH2, which is more susceptible to ROS. If our hypothesis is 486 accurate, therefore, we would expect our cross-infection experiments to reveal differences: (i) in the 487 infective potential of our two *S. mansoni* strains; and (ii) in the resistance potential of our two *B.* 488 *glabrata* strains.

489 Indeed, the results of the infection and cross-infection experiments showed significant 490 differences in the infection rates and intensities (Fig. 7). The factors and mechanisms underlying 491 these differences are not yet known, but may include historical epidemiological conditions, 492 differential selective pressures in the transmission areas, genotypic diversities in the host and 493 parasitic isolates, recognition mechanisms developed through the matching-phenotypes model, and 494 intraspecific competition among sporocysts (for details, see (Théron et al., 1997; Théron and 495 Coustau, 2005; Théron et al., 2008; Bech et al.). Notably, the host-parasite combination 496 characterized by the higher infection rates and parasite intensities (SmBRE/BgBRE) was also 497 characterized by a higher ROS-production capacity by the host and a higher ROS-scavenging 498 ability by the parasite. In contrast, the host-parasite combination with lower infection rates and 499 parasite intensities (SmGH2/BgGUA) showed lower ROS production by the host and lower ROS 500 scavenging by the parasite (Fig. 8). These observations argue for the presence of reciprocal 501 adaptation between the ROS and ROS scavenger traits. This was further supported by the results 502 from our allopatric cross-infections. The SmGH2 strain, which had co-evolved with its sympatric 503 snail (BgGUA) to produce lower levels of ROS, could not effectively infect high-ROS-producing BgBRE snails (Figs. 7 and 8). Conversely, the SmBRE strain, which had co-evolved with a host that 504 produced more ROS (BgBRE), could easily infect low-ROS-producing BgGUA snails (Figs. 7 and 505 506 8). Interestingly, however, the infection success of SmBRE was lower for the allopatric combination 507 than the sympatric pairing (Figs. 7 and 8), suggesting that the oxidative factors probably act in 508 combination with other factors to determine the outcome of the B. glabrata/S. mansoni interaction.

509 Within hosts, immune effectors exert the main selective pressure on parasites (Loker and 510 Adema, 1995; Damian, 1997). However, another factor that helps to define the interaction is the 511 efficiency of parasite recognition by snail immune receptors, and the ability of the parasite to escape 512 this recognition. We previously discovered a group of polymorphic antigens of S. mansoni (the S. 513 mansoni polymorphic mucins, SmPoMucs) (Roger et al., 2008a; Roger et al., 2008b; Roger et al., 514 2008c), and recently showed that these antigens are recognized by diversified *B. glabrata* immune 515 receptors (the fibrinogen-related proteins, FREPs) (Moné et al., 2010a). These reports on the 516 molecular interactions underlying snail-schistosome compatibility suggest that co-evolutionary 517 (reciprocal adaptation) processes probably occur through a combination of changes in general 518 resistance (ROS/ROS scavengers) and more specific interactions (FREPs/SmPoMucs). In non-519 specific resistance/infectivity interactions involving density-dependant forces (e.g., the number of 520 developing parasites within the host), co-evolution leads to global increases in the amount of 521 attack/defense products, such as the interplay of ROS and ROS scavengers described herein. In highly specific genotype-by-genotype interactions, such as recognition/evasion processes, however, 522 523 co-evolution leads to increases in the diversification and/or polymorphisms among specific molecules, as observed for FREPs and SmPoMucs. 524

525 Even if the success of infection is not exclusively based on the levels of ROS and ROS 526 scavengers, our model of dynamic co-evolution predicts that a change in parasite virulence or host 527 resistance would be associated with life history trade-offs (reallocation of resources), with increased 528 production of a molecule under co-evolutionary pressure yielding indirect negative consequences 529 for other functions (development, growth, fecundity, reproductive rate, etc.) (Green et al., 2000; 530 Lohse et al., 2006; Forde et al., 2008). Indeed, this kind of trade-off has been observed in our 531 model, as a previous study showed that cumulative cercarial production was two-fold higher for the 532 SmGH2/BgGUA combination than for the SmBRE/BgBRE (Théron et al., 1997). This could 533 indicate that *Sm*BRE has made a tradeoff by investing in the production of ROS scavengers at the 534 expense of producing cercariae.

In summary, host-parasite interactions are dynamic biological systems in which the host's defense mechanisms face the parasite's infectivity mechanisms, leading to a co-evolutionary arms race (Combes, 2000; Howard and Jack, 2007).

538 Developing correlation approaches to studying co-evolution have some limitations. Indeed 539 the correlations between traits of interacting species cannot always provide unequivocal evidence 540 for co-evolution. Reciprocity could also occur and an absence of correlated traits is not evidence for 541 an absence of co-evolution (Nuismer et al., 2007; Nuismer et al., 2010; Yoder and Nuismer, 2010). 542 Non-co-evolutionary mechanisms could explain correlations between the traits of interacting 543 species. For example, the correlation could result from a colonization process in which a parasite 544 species with new potential arrived in a new environment and is more well-adapted to the sympatric 545 interacting species. In other interaction models correlated traits could evolve if the abiotic or biotic 546 environments favour similar traits in both of the interacting species. For example, a biotic selection 547 that affects only one of the interacting species can itself cause trait matching. This can occur if interactions have potent fitness consequences for only one of the species or if the outcome of 548 interactions depends on the phenotype of only one of the species. These one-way interactions can 549 550 generate correlations that are indistinguishable from those that evolve due to co-evolutionary processes (Nuismer et al., 2007; Nuismer et al., 2010; Yoder and Nuismer, 2010). In our model of 551 552 interest B. glabrata could be infected by a lot of pathogens species (other than S. mansoni) that 553 represent a selective pressure that could enhance snail ROS production. In this context, schistosomes for which the specificity for the intermediate snail host is very high will still succeed 554 555 in infecting the snails, only if they are able to circumvent ROS by increasing their ROS scavenger production. 556

However, Nuismer et al. (2007, 2010) state that correlation could occur if interactions are mediated by a mechanism of phenotype matching such as what takes place for host-parasite interactions. This phenotype matching process was proposed for our *S. mansoni / B. glabrata* model of interest (Théron and Coustau, 2005).

561 Therefore whatever are the mechanisms involved in the apparition of trait correlation between two interacting species, our present results reveal the existence of phenotypic matching 562 563 between host and parasitic strains in terms of their attack (ROS production) and defense (ROS 564 scavenging) traits. To our knowledge, this work provides the first example of a clear link between 565 the level of oxidant and antioxidant molecules possibly resulting from sympatric co-evolution, and 566 provides supporting evidence for a field illustration of the Red Queen Hypothesis (Van Valen, 567 1974) and its predictions of a functional trait in a metazoan host/parasite model. Detailed 568 mechanistic studies will be conducted in multiple populations to fully confirm the link between 569 correlated traits and the Red Queen context.

570

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580 **References**

581 Bahia, D., Avelar, L., Mortara, R.A., Khayath, N., Yan, Y., Noel, C., Capron, M., Dissous, C.,

582 Pierce, R.J., Oliveira, G., 2006. SmPKC1, a new protein kinase C identified in the platyhelminth
583 parasite *Schistosoma mansoni*. Biochem. Biophys. Res. Commun. 345, 1138-1148.

584 Bayne, C.J., 2009. Successful parasitism of vector snail *Biomphalaria glabrata* by the human blood

fluke (trematode) *Schistosoma mansoni*: a 2009 assessment. Mol. Biochem. Parasitol. 165, 8-18.

- 586 Bayne, C.J., Hahn, U.K., Bender, R.C., 2001. Mechanisms of molluscan host resistance and of 587 parasite strategies for survival. Parasitology 123 Suppl, S159-167.
- 588 Bech, N., Beltran, S., Portela, J., Rognon, A., Allienne, J.F., Boissier, J., Theron, A., 2010. Follow-
- up of the genetic diversity and snail infectivity of a *Schistosoma mansoni* strain from field to
 laboratory. Infect. Genet. Evol. 10, 1039-1045.
- Beltran, S., Boissier, J., 2008. Schistosome monogamy: who, how, and why? Trends Parasitol. 24,
 386-391.
- Beltran, S., Cezilly, F., Boissier, J., 2008. Genetic dissimilarity between mates, but not male
 heterozygosity, influences divorce in schistosomes. PLoS ONE 3, e3328.
- 595 Bender, R.C., Broderick, E.J., Goodall, C.P., Bayne, C.J., 2005. Respiratory burst of Biomphalaria
- 596 glabrata hemocytes: Schistosoma mansoni-resistant snails produce more extracellular H2O2 than
- susceptible snails. J. Parasitol. 91, 275-279.
- 598 Bender, R.C., Goodall, C.P., Blouin, M.S., Bayne, C.J., 2007. Variation in expression of 599 *Biomphalaria glabrata* SOD1: a potential controlling factor in susceptibility/resistance to 600 Schistosoma mansoni. Dev. Comp. Immunol. 31, 874-878.
- 601 Bernal, D., Carpena, I., Espert, A.M., De la Rubia, J.E., Esteban, J.G., Toledo, R., Marcilla, A.,
- 602 2006. Identification of proteins in excretory/secretory extracts of Echinostoma friedi (Trematoda)
- from chronic and acute infections. Proteomics 6, 2835-2843.
- Bienert, G.P., Schjoerring, J.K., Jahn, T.P., 2006. Membrane transport of hydrogen peroxide.
 Biochim. Biophys. Acta 1758, 994-1003.
- Bouchut, A., Roger, E., Gourbal, B., Grunau, C., Coustau, C., Mitta, G., 2008. The compatibility
- polymorphism in invertebrate host/trematodes interactions: research of molecular determinants.Parasite 15, 304-309.
- 609 Bouchut, A., Sautiere, P.E., Coustau, C., Mitta, G., 2006. Compatibility in the Biomphalaria
- 610 glabrata/Echinostoma caproni model: Potential involvement of proteins from hemocytes revealed
- 611 by a proteomic approach. Acta Trop. 98, 234-246.

- 612 Cass, C.L., Johnson, J.R., Califf, L.L., Xu, T., Hernandez, H.J., Stadecker, M.J., Yates, J.R., 3rd,
- Williams, D.L., 2007. Proteomic analysis of *Schistosoma mansoni* egg secretions. Mol. Biochem.
 Parasitol. 155, 84-93.
- 615 Chernin, E., 1963. Observations on hearts explanted in vitro from the snail Australorbis glabratus.
- 616 The Journal of parasitology 49, 353-364.
- 617 Combes, C., 2000. Selective pressure in host-parasite systems. J. Soc. Biol. 194, 19-23.
- 618 Curwen, R.S., Ashton, P.D., Johnston, D.A., Wilson, R.A., 2004. The Schistosoma mansoni soluble
- 619 proteome: a comparison across four life-cycle stages. Mol. Biochem. Parasitol. 138, 57-66.
- 620 Damian, R.T., 1997. Parasite immune evasion and exploitation: reflections and projections.
- 621 Parasitology 115 Suppl, S169-175.
- de Jong-Brink, M., Bergamin-Sassen, M., Solis Soto, M., 2001. Multiple strategies of schistosomes
- to meet their requirements in the intermediate snail host. Parasitology 123 Suppl, S129-141.
- Dybdahl, M.F., Storfer, A., 2003. Parasite local adaptation: Red Queen versus Suicide King. Trends
 in ecology & evolution 18, 523-530.
- Dzik, J.M., 2006. Molecules released by helminth parasites involved in host colonization. Acta
 Biochim. Pol. 53, 33-64.
- _____
- Forde, S.E., Thompson, J.N., Bohannan, B.J., 2004. Adaptation varies through space and time in a
 coevolving host-parasitoid interaction. Nature 431, 841-844.
- Forde, S.E., Thompson, J.N., Holt, R.D., Bohannan, B.J., 2008. Coevolution drives temporal
 changes in fitness and diversity across environments in a bacteria-bacteriophage interaction.
 Evolution 62, 1830-1839.
- 633 Gagneux, S., DeRiemer, K., Van, T., Kato-Maeda, M., de Jong, B.C., Narayanan, S., Nicol, M.,
- 634 Niemann, S., Kremer, K., Gutierrez, M.C., Hilty, M., Hopewell, P.C., Small, P.M., 2006. Variable
- 635 host-pathogen compatibility in Mycobacterium tuberculosis. Proc. Natl. Acad. Sci. U. S. A. 103,
- 636 2869-2873.

- Gasnier, N., Rondelaud, D., Abrous, M., Carreras, F., Boulard, C., Diez-Banos, P., Cabaret, J.,
 2000. Allopatric combination of *Fasciola hepatica* and *Lymnaea truncatula* is more efficient than
 sympatric ones. Int. J. Parasitol. 30, 573-578.
- 640 Gerard, C., Balzan, C., Theron, A., 1995. Spatial distribution patterns of the sporocyst 641 infrapopulation of *Schistosoma mansoni* within its mollusc host (*Biomphalaria glabrata*): an 642 unusual phenotype of aggregation. J. Parasitol. 81, 310-312.
- Goodall, C.P., Bender, R.C., Broderick, E.J., Bayne, C.J., 2004. Constitutive differences in Cu/Zn
 superoxide dismutase mRNA levels and activity in hemocytes of *Biomphalaria glabrata* (Mollusca)
 that are either susceptible or resistant to *Schistosoma mansoni* (Trematoda). Mol. Biochem.
- 646 Parasitol. 137, 321-328.
- 647 Green, D.M., Kraaijeveld, A.R., Godfray, H.C., 2000. Evolutionary interactions between
 648 *Drosophila melanogaster* and its parasitoid *Asobara tabida*. Heredity 85 Pt 5, 450-458.
- Guillou, F., Mitta, G., Dissous, C., Pierce, R., Coustau, C., 2004. Use of individual polymorphism
 to validate potential functional markers: case of a candidate lectin (BgSel) differentially expressed
 in susceptible and resistant strains of *Biomphalaria glabrata*. Comp. Biochem. Physiol. B Biochem.
- 652 Mol. Biol. 138, 175-181.
- Guillou, F., Roger, E., Moné, Y., Rognon, A., Grunau, C., Theron, A., Mitta, G., Coustau, C.,
 Gourbal, B.E., 2007. Excretory-secretory proteome of larval *Schistosoma mansoni* and *Echinostoma caproni*, two parasites of *Biomphalaria glabrata*. Mol. Biochem. Parasitol. 155, 4556.
- Hahn, U.K., Bender, R.C., Bayne, C.J., 2000. Production of reactive oxygen species by hemocytes
- of *Biomphalaria glabrata* : carbohydrate-specific stimulation. Dev. Comp. Immunol. 24, 531-541.
- Hahn, U.K., Bender, R.C., Bayne, C.J., 2001a. Involvement of nitric oxide in killing of Schistosoma
- 660 mansoni sporocysts by hemocytes from resistant *Biomphalaria glabrata*. J. Parasitol. 87, 778-785.

- 661 Hahn, U.K., Bender, R.C., Bayne, C.J., 2001b. Killing of Schistosoma mansoni sporocysts by
- hemocytes from resistant *Biomphalaria glabrata*: role of reactive oxygen species. The Journal ofparasitology 87, 292-299.
- Howard, J.C., 1991. Immunology. Disease and evolution. Nature 352, 565-567.
- Howard, J.C., Jack, R.S., 2007. Evolution of immunity and pathogens. Eur. J. Immunol. 37, 1721-1723.
- Janzen, D.H., 1980. When is it coevolution ? Evolution 34, 611-612.
- Kim, S.M., Kang, J.H., 1997. Peroxidative activity of human Cu,Zn-superoxide dismutase. Mol.
 Cells 7, 120-124.
- 670 Knudsen, G.M., Medzihradszky, K.F., Lim, K.C., Hansell, E., McKerrow, J.H., 2005. Proteomic
- analysis of *Schistosoma mansoni* cercarial secretions. Mol. Cell. Proteomics 4, 1862-1875.
- Koskella, B., Lively, C.M., 2007. Advice of the rose: experimental coevolution of a trematode
 parasite and its snail host. Evolution 61, 152-159.
- 674 Little, T.J., Watt, K., Ebert, D., 2006. Parasite-host specificity: experimental studies on the basis of
- 675 parasite adaptation. Evolution 60, 31-38.
- 676 Lohse, K., Gutierrez, A., Kaltz, O., 2006. Experimental evolution of resistance in *Paramecium*677 *caudatum* against the bacterial parasite *Holospora undulata*. Evolution 60, 1177-1186.
- 678 Loker, E.S., Adema, C.M., 1995. Schistosomes, Echinostomes and Snails: Comparative
 679 Immunobiology. Parasitology today 11, 120-124.
- 680 Mei, H., LoVerde, P.T., 1997. *Schistosoma mansoni*: the developmental regulation and 681 immunolocalization of antioxidant enzymes. Exp. Parasitol. 86, 69-78.
- Moné, Y., Gourbal, B., Duval, D., Du Pasquier, L., Kieffer-Jaquinod, S., Mitta, G., 2010a. A Large
- 683 Repertoire of Parasite Epitopes Matched by a Large Repertoire of Host Immune Receptors in an
- 684 Invertebrate Host/Parasite Model. PLoS Negl. Trop. Dis. 4, e813.
- Moné, Y., Mitta, G., Duval, D., Gourbal, B.E., 2010b. Effect of amphotericin B on the infection
- 686 success of Schistosoma mansoni in Biomphalaria glabrata. Exp. Parasitol. 125, 70-75.

- Morgan, A.D., Gandon, S., Buckling, A., 2005. The effect of migration on local adaptation in a
 coevolving host-parasite system. Nature 437, 253-256.
- Mourao, M.d.M., Dinguirard, N., Franco, G.R., Yoshino, T.P., 2009a. Role of the Endogenous
 Antioxidant System in the Protection of *Schistosoma mansoni* Primary Sporocysts against
 Exogenous Oxidative Stress. PLoS Negl. Trop. Dis. 3, e550.
- 692 Mourao, M.M., Dinguirard, N., Franco, G.R., Yoshino, T.P., 2009b. Phenotypic screen of early-
- developing larvae of the blood fluke, *Schistosoma mansoni*, using RNA interference. PLoS Negl.
 Trop. Dis. 3, e502.
- Munoz-Antoli, C., Marin, A., Trelis, M., Toledo, R., Esteban, J.G., 2010. Sympatric and allopatric
- 696 experimental infections of the planorbid snail Gyraulus chinensis with miracidia of Euparyphium
- 697 *albuferensis* (Trematoda: Echinostomatidae). J. Helminthol., 1-5.
- Nelder, J.A., Mead, R., 1965. A Simplex Method for Function Minimization. Comput. J. 7, 308-313.
- Nuismer, S.L., Gomulkiewicz, R., Ridenhour, B.J., 2010. When is correlation coevolution? Am.
 Nat. 175, 525-537.
- Nuismer, S.L., Ridenhour, B.J., Oswald, B.P., 2007. Antagonistic coevolution mediated by
 phenotypic differences between quantitative traits. Evolution 61, 1823-1834.
- Oter, O., Ribou, A.C., 2009. Quenching of long lifetime emitting fluorophores with paramagnetic
 molecules. J Fluoresc 19, 389-397.
- 706 Perez-Sanchez, R., Ramajo-Hernandez, A., Ramajo-Martin, V., Oleaga, A., 2006. Proteomic
- analysis of the tegument and excretory-secretory products of adult *Schistosoma bovis* worms.
 Proteomics 6 Suppl 1, S226-236.
- 709 Rharass, T., Vigo, J., Salmon, J.M., Ribou, A.C., 2006. Variation of 1-pyrenebutyric acid
- 710 fluorescence lifetime in single living cells treated with molecules increasing or decreasing reactive
- 711 oxygen species levels. Anal. Biochem. 357, 1-8.

- Ribou, A.C., Vigo, J., Kohen, E., Salmon, J.M., 2003. Microfluorometric study of oxygen
 dependence of (1"-pyrene butyl)-2-rhodamine ester probe in mitochondria of living cells. J.
 Photochem. Photobiol. B 70, 107-115.
- Ribou, A.C., Vigo, J., Salmon, J.M., 2004. Lifetime of fluorescent pyrene butyric acid probe in
 single living cells for measurement of oxygen fluctuation. Photochem. Photobiol. 80, 274-280.
- 717 Roger, E., Gourbal, B., Grunau, C., Pierce, R.J., Galinier, R., Mitta, G., 2008a. Expression analysis
- of highly polymorphic mucin proteins (Sm PoMuc) from the parasite Schistosoma mansoni. Mol.
- 719 Biochem. Parasitol. 157, 217-227.
- 720 Roger, E., Grunau, C., Pierce, R.J., Hirai, H., Gourbal, B., Galinier, R., Emans, R., Cesari, I.M.,
- 721 Cosseau, C., Mitta, G., 2008b. Controlled chaos of polymorphic mucins in a metazoan parasite
- 722 (*Schistosoma mansoni*) interacting with its invertebrate host (*Biomphalaria glabrata*). PLoS Negl.
 723 Trop. Dis. 2, e330.
- Roger, E., Mitta, G., Mone, Y., Bouchut, A., Rognon, A., Grunau, C., Boissier, J., Theron, A.,
 Gourbal, B.E., 2008c. Molecular determinants of compatibility polymorphism in the *Biomphalaria glabrata/Schistosoma mansoni* model: New candidates identified by a global comparative
 proteomics approach. Molecular and Biochemical Parasitology 157, 205-216.
- Schulte, R.D., Makus, C., Hasert, B., Michiels, N.K., Schulenburg, H., 2010. Multiple reciprocal
 adaptations and rapid genetic change upon experimental coevolution of an animal host and its
 microbial parasite. Proceedings of the National Academy of Sciences 107, 7359-7364.
- Selkirk, M.E., Smith, V.P., Thomas, G.R., Gounaris, K., 1998. Resistance of filarial nematode
 parasites to oxidative stress. Int. J. Parasitol. 28, 1315-1332.
- Shapiro, S.S., Wilk, M.B., 1965. An analysis of variance test for normality (complete samples).
 Biometrika 52, 591-611.
- 735 Steinauer, M.L., 2009. The sex lives of parasites: investigating the mating system and mechanisms
- of sexual selection of the human pathogen *Schistosoma mansoni*. Int. J. Parasitol. 39, 1157-1163.

- 737 Stern, O., Volmer, M., 1919. The extinction period of fluorescence. Physikalische Zeitschrift 20,
 738 183-188.
- Théron, A., Coustau, C., 2005. Are *Biomphalaria* snails resistant to *Schistosoma mansoni*? J.
 Helminthol. 79, 187-191.
- Théron, A., Coustau, C., Rognon, A., Gourbiere, S., Blouin, M.S., 2008. Effects of laboratory
 culture on compatibility between snails and schistosomes. Parasitology 135, 1179-1188.
- 743 Théron, A., Pages, J.R., Rognon, A., 1997. Schistosoma mansoni: distribution patterns of miracidia
- among *Biomphalaria glabrata* snail as related to host susceptibility and sporocyst regulatory
 processes. Exp. Parasitol. 85, 1-9.
- van Balkom, B.W.M., van Gestel, R.A., Brouwers, J.F.H.M., Krijgsveld, J., Tielens, A.G.M., Heck,
- A.J.R., van Hellemond, J.J., 2005. Mass Spectrometric Analysis of the *Schistosoma mansoni*Tegumental Sub-proteome. J. Proteome Res. 4, 958-966.
- Van Valen, L., 1974. Molecular evolution as predicted by natural selection. Journal of Molecular
 Evolution 3, 89-101.
- 751 Vermeire, J.J., Taft, A.S., Hoffmann, K.F., Fitzpatrick, J.M., Yoshino, T.P., 2006. Schistosoma
- *mansoni*: DNA microarray gene expression profiling during the miracidium-to-mother sporocyst
 transformation. Mol. Biochem. Parasitol. 147, 39-47.
- Vermeire, J.J., Yoshino, T.P., 2007. Antioxidant gene expression and function in in vitrodeveloping *Schistosoma mansoni* mother sporocysts: possible role in self-protection. Parasitology
 134, 1369-1378.
- Webster, J.P., Davies, C.M., 2001. Coevolution and compatibility in the snail-schistosome system.
 Parasitology 123 Suppl, S41-56.
- 759 Webster, J.P., Gower, C.M., Blair, L., 2004. Do hosts and parasites coevolve? Empirical support
- 760 from the *Schistosoma system*. Am. Nat. 164 Suppl 5, S33-51.

- Wu, X.J., Sabat, G., Brown, J.F., Zhang, M., Taft, A., Peterson, N., Harms, A., Yoshino, T.P., 2009.
 Proteomic analysis of *Schistosoma mansoni* proteins released during in vitro miracidium-tosporocyst transformation. Mol. Biochem. Parasitol. 164, 32-44.
- Yim, M.B., Chock, P.B., Stadtman, E.R., 1993. Enzyme function of copper, zinc superoxide
 dismutase as a free radical generator. J. Biol. Chem. 268, 4099-4105.
- 766 Yim, M.B., Kang, J.H., Yim, H.S., Kwak, H.S., Chock, P.B., Stadtman, E.R., 1996. A gain-of-
- 767 function of an amyotrophic lateral sclerosis-associated Cu,Zn-superoxide dismutase mutant: An
- roc. Natl.
- 769 Acad. Sci. U. S. A. 93, 5709-5714.
- Yoder, J.B., Nuismer, S.L., 2010. When does coevolution promote diversification? Am. Nat. 176,
 802-817.
- Zelck, U.E., Von Janowsky, B., 2004. Antioxidant enzymes in intramolluscan *Schistosoma mansoni*and ROS-induced changes in expression. Parasitology 128, 493-501.
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- 776 **Figure legends**
- 777

Fig. 1. Effect of hydrogen peroxide (H₂O₂) on *Schistosoma mansoni* sporocysts. (A) Cytotoxicity among *S. mansoni* sporocysts 2 h after exposure to different H₂O₂ concentrations. The asterisk indicates a significant difference (P < 0.05) in the cytotoxic effect of H₂O₂ on *S. mansoni* Guadeloupean strain (*Sm*GH2) versus *S. mansoni* Brazilian strain (*Sm*BRE) sporocysts. (B) Percent sporocyst mortality after 4 h exposure to different H₂O₂ concentrations. The asterisk indicates a significant difference (P < 0.05) in the mortality rates of *Sm*GH2 versus *Sm*BRE sporocysts.

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Fig. 2. Constitutive total antioxidant capacities of *Schistosoma mansoni* Guadeloupean strain (*Sm*GH2) and *S. mansoni* Brazilian strain (*Sm*BRE) sporocysts. Values are expressed as μ M of antioxidant activity per 10 μ g of sporocyst proteins. The asterisk indicates a significant difference (*P* < 0.05).

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Fig. 3. Ratios of Zn-Cu superoxide dismutase (Zn-Cu SOD, Smp_176200.2) transcript levels in the two strains of *Schistosoma mansoni* miracidia (Guadeloupean strain, *Sm*GH2 and Brazilian strain, *Sm*BRE). Ratios were determined using real-time quantitative PCR and are expressed relative to the expression levels of 28s rRNA and α -tubulin. The histogram represents the average values of duplicates \pm S.D. The expression ratio was calculated according to the formula: R = 2(Δ Ct), where Δ Ct represents Ct (target gene) – Ct (constitutively expressed gene).

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802 Fig. 4. Intracellular ROS measurements in single Biomphalaria glabrata hemocytes (A) Global 803 reactive oxygen species (ROS) concentration in each snail strain. The histogram represents 804 the ROS concentrations in arbitrary units (-fold mean) for the Biomphalaria glabrata 805 Guadeloupean strain, BgGUA and Brazilian strain, BgBRE. The asterisk indicates a significant difference (P < 0.05) in ROS production by hemocytes of the two snail strains. 806 807 (B) ROS concentrations in hemocyte populations from BgGUA and BgBRE snails. The 808 histograms represent the fluorescent lifetimes of 1-pyrenebutyric acid (PBA)-loaded 809 hemocytes from eight each of BgGUA and BgBRE; 108 single hemocytes from eight 810 BgBRE and 111 hemocytes from eight individuals of BgGUA snails were assessed. The x811 axis represents the fluorescent lifetime in nanoseconds, while the *y* axis corresponds to the 812 number of cells.

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Fig. 5. Hydrogen peroxide (H₂O₂) production by *Biomphalaria glabrata* hemocytes. Cumulative production of H₂O₂ was measured using Amplex Red. The data are presented as the mean (\pm S.D.) of Amplex Red absorbance at 570 nm (A_{570nm}) per 45,000 cells over five replicates. The asterisk indicates a significant difference (P < 0.05) in H₂O₂ production from hemocytes of *B. glabrata* Guadeloupean strain, *Bg*GUA, versus Brazilian strain, *Bg*BRE.

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Fig. 6. Constitutive superoxide anion plasma content in *Biomphalaria glabrata* Guadeloupean strain, BgGUA, versus Brazilian strain, BgBRE. The superoxide anion plasma content was assessed by spectrophotometric measurement (620 nm) of nitroblue tetrazolium (NBT) reduction. At 3 h after initiation of the reaction, the BgBRE plasma contained significantly more superoxide anion than that from BgGUA (the asterisk indicates a significant difference; P < 0.05).

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- 827 Fig. 7. Infection intensities in sympatric allopatric rates and and Schistosoma 828 mansoni/Biomphalaria glabrata combinations. The percentage of snails infected and the intensity of infection: number of mother sporocysts (SpI) developed (n SpI) was measured 829 830 after individual snails were challenged with different miracidial doses (1, 10, 20, 30 or 50 831 miracidia (Mi)).
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- Fig. 8. Schematic representation of our reactive oxygen species (ROS)-based co-evolutionary
 hypothesis. The percentage of prevalence is indicated for each *Biomphalaria glabrata/Schistosoma mansoni* combination. The number of arrows represents the
 differential host oxidant (ROS) or parasite antioxidant (ROS scavenger) capabilities. *Biomphalaria glabrata* Brazilian strain, *Bg*BRE, and Guadeloupean strain, *Bg*GUA; *S. mansoni* Brazilian strain, *Sm*BRE, and Guadeloupean strain, *Sm*GH2.
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Table 1

Cana Nama and SchistoDR ID	Amplicon Longth	Smn scaffold	Forward primer $(5^{2} \text{ to } 3^{2})$
Gene Ivanic and Schistoph ID	Amplicon Length	Ship_scanou	Forward printer (5 to 5)
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Smp_056970	125	000155	TGGCCGTGGAGCGATGCAAA
Glutathione S-transferase omega Smp_152710	148	000154	ACAGCTCTAGTTGTCGACCAAAC
Glutathione S-transferase 28 kD (GST 28) Smp_054160	128	000143	CGGACGCGGACGTGCTGAAT
Glutathione-S-transferase 26 kD (GST 26) Smp_163610	104	000249	GCAAAGCTGGTGGTTTGGGGC
Glyoxalase II Smp_091010	124	000428	ATGGCCTTCATTGCTTTGGACAGA
Thioredoxin peroxidase (TPX) Smp_158110	101		CAAAGGCCTTGTACAACCAACTC
Superoxide dismutase (SOD) Smp_176200.2	100	000615	AGTGGACTCAAGGCTG

Primer sequences for *Reverse Transcription-quantitative PCR* in this study.

Gene names are given according to the SchistoDB accession numbers (<u>http://schistodb.net/schistodb20/</u>). Their respective scaffolds are included in the table. Smp_163610 primer sequences are given according to the mRNA sequence of the gene (XM_002582157.1) due to inconsistency in the genome assembly. α -tubulin and 28S primers sequences were previously published (Bahia et al., 2006; Roger et al., 2008a).







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