

1 **Recovery of primary sporocysts in vivo in the *Schistosoma mansoni* / *Biomphalaria***  
2 ***glabrata* model using a simple fixation method suitable for extraction of genomic DNA**  
3 **and RNA.**

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22

23 **Abstract**

24 Detailed studies of host/parasite interactions are currently limited because *in-situ* gene  
25 sequencing or monitoring of parasite gene expression is so far limited to genes presenting a  
26 high loci copy number in the Schistosome genome or a high level of expression. Indeed, how  
27 to investigate the host parasite molecular interplay when parasites are not directly accessible  
28 in vivo? Here we describe a method to circumvent this problem and to analyze DNA and  
29 RNA of *Schistosoma mansoni* during the interaction with its intermediate snail host  
30 *Biomphalaria glabrata*. We propose a technique for improved DNA and RNA extraction  
31 from the intra-molluscan stage of the parasite recovered after fixation of infected snails in  
32 RAILLET-HENRY solution. The extractions can be used for genetic analysis, transcription studies  
33 and microsatellite genotyping.

34  
35 **Keywords:** *Schistosoma mansoni*, *Biomphalaria glabrata*, intramolluscan stage, DNA/RNA  
36 extraction.

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## 39 1. Introduction

40 Schistosomiasis or bilharzia is a tropical parasitic disease affecting 200 million humans in  
41 74 countries, causing 200,000 deaths annually (WHO, 2002). It is the second most important  
42 tropical disease in terms of morbidity after malaria. Schistosomiasis is caused by flatworms of  
43 the genus *Schistosoma* (Platyhelminth, Digenea) (Chitsulo, et al., 2004, Gryseels, et al.,  
44 2006). The cycle of the parasite is complex. It requires humans as definitive hosts and  
45 freshwater snail species as intermediate hosts. Infection occurs in water by the free-living  
46 larval stages (cercaria for the definitive host and miracidia for the intermediate host).  
47 Significant attention has been paid to freshwater snails in the past because of both their  
48 medical and epidemiological importance as intermediate hosts for schistosome parasites.  
49 Moreover, the interaction between *Schistosoma mansoni* and the snail *Biomphalaria glabrata*  
50 provides a model of choice in evolutionary biology to investigate the host-parasite co-  
51 evolutionary dynamics but also invertebrate immune response (Baeza Garcia, et al., 2010,  
52 Lockyer, et al., 2008, Mone, et al., 2010, Roger, et al., 2008). While snail response to  
53 infection can be readily studied particularly at the molecular level (Adema, et al., 2010, Baeza  
54 Garcia, et al., 2010, Guillou, et al., 2007, Hanington, et al., 2010) it is not easy for the *S.*  
55 *mansoni* intramolluscan stage parasite that is not directly accessible. To avoid this problem, *in*  
56 *vitro* culture is often used in this model to obtain pure parasites and/or secretion products to  
57 identify the molecules involved in host-parasite interplay (Bender, et al., 2002, Coppin, et al.,  
58 2003, Guillou, et al., 2007, Roger, et al., 2008), to perform pharmacological investigations  
59 (Mattos, et al., 2006), or to study parasite development (Azzi, et al., 2009). However,  
60 miracidia to sporocyst *in vitro* transformation can be achieved only for a short period of time.  
61 For a longer period an artificial snail environment must be used involving sporocysts co-  
62 cultured with *Biomphalaria glabrata* embryonic cells (Bge cell line) (Coustau and Yoshino,  
63 2000, Taft, et al., 2009, Vermeire, et al., 2004). These approaches has been used with great

64 success in the past, however, the *in-vivo* response of the parasite to the authentic host snail  
65 environment was never investigated.

66 Here we describe a new efficient method for improved DNA and RNA extraction from the  
67 *S. mansoni* intra molluscan stage. We show that the method can be used for PCR  
68 amplification, analysis of gene expression and microsatellite genotyping approaches.

69

## 70 **2. Material and Methods**

### 71 *2.1. Biological material, Raillet-Henry fixation and parasite recovery*

72 *Schistosoma mansoni* (Guadeloupean strain) was maintained in its sympatric *Biomphalaria*  
73 *glabrata* strain and in hamsters (*Mesocricetus auratus*), as described previously (Théron, et  
74 al., 1997). Miracidia were hatched from eggs recovered from 60-day-infected hamster livers.  
75 The livers were homogenized and the eggs were filtered out, washed, and transferred to spring  
76 water. The miracidia were allowed to hatch under exposure to artificial light. For each  
77 experiment, snails (7-9 mm in diameter) were exposed individually to 20 miracidia. For  
78 primary sporocysts (SpI) detection and recovery, the snails were fixed 15 days post-exposure,  
79 according to a modified method, previously described (Mone, et al., 2010, Theron and  
80 Gerard, 1994). In brief, each infected snail was relaxed in pond water containing an excess of  
81 crystalline menthol for 6 h (menthol relaxation help in shell removing and snail anatomic  
82 observation), the snail body was recovered and fixed in modified Raillet-Henry's solution  
83 (0.6% NaCl; 2% acetic acid; 2% formalin). After 24 hours in fixative, a dissection of the  
84 head-foot zone was performed, and SpIs (Figure 1) were recovered, washed two times in  
85 Phosphate Buffer Saline (PBS) for 15 min on ice and kept at -80°C until use.

86 An alternative procedure can be used with a rapid fixation procedure when studying gene  
87 transcription. Infected snails were snap-frozen in liquid nitrogen and fixed directly with the

88 shell in modified Raillet-Henry's solution (0.6% NaCl; 2% acetic acid; 2% formalin). After  
89 24 hours in fixative, the shell is removed and SpI recovery was performed as above.

90

## 91 2.2. DNA and RNA extraction and reverse transcription

92 Genomic DNA (gDNA) was extracted from single Raillet-Henry fixed *S. mansoni* sporocysts  
93 according to the following protocol. Sixty  $\mu$ l of TE (Tris 10mM; EDTA 1mM; ph 8)  
94 containing 1.67mg/ml of Proteinase K (Merck) was added to the SpI. Samples were put 3  
95 hours at 55°C and vortexed each 15 min. Then samples were heated 10 min at 100°C for  
96 proteinase K inactivation. gDNA were kept at -20°C until use.

97 Total RNA was isolated from single or pool of 5 SpIs using the High Pure FFPE RNA  
98 microkit (Roche) according to manufacturer recommendations. Reverse transcription was  
99 performed using random hexamer primer and the RevertAid H minus First Strand cDNA  
100 Synthesis kit (Fermentas) following the manufacturer's protocol. cDNA were kept at -80°C  
101 until use. It as been largely documented that these new extraction methods have been now  
102 optimized for RNA extraction from formalin-fixed tissue to achieve high quality microarray  
103 and qRT-PCR downstream applications (April, et al., 2009, Ribeiro-Silva, et al., 2007,  
104 Serinsoz, et al., 2005).

105

## 106 2.3. PCR amplifications of gDNA and cDNA

107 gDNA PCR amplifications of fixed *S. mansoni* sporocyst were performed with the Advantage  
108 2 PCR Enzyme System (Clontech). To test PCR on gDNA as template, the SmPoMuc loci  
109 (Roger, et al., 2008, Roger, et al., 2008, Roger, et al., 2008) were amplified using specific  
110 primers designed to amplify specific fragment lengths (see Table 1 for primer sequences and  
111 PCR cycling conditions). PCR conditions were: 0.5 $\mu$ M primers; 1X final Advantage 2 PCR

112 buffer (2mM MgCl<sub>2</sub>); 0.4mM dNTPs; 1X final Advantage 2 polymerase and 5μl of gDNA in  
113 a total volume of 20μl of RNase, DNase free water.

114 cDNA PCR amplification of fixed *S. mansoni* sporocyst was performed with the GoTaq  
115 hotstart Enzyme (Promega). Five genes were tested (see Table 2 for gene name, primer  
116 sequences and PCR cycling conditions). PCR mix was: 0.4μM primers; 1.5mM MgCl<sub>2</sub>;  
117 0.2mM dNTPs, 1 unit GoTaq hot-start and 1μl of cDNA in a total volume of 20μl of RNase,  
118 DNase free water. All PCR products were separated by electrophoresis through 1% agarose  
119 gels, and visualized by staining with ethidium bromide.

120

#### 121 *2.4. PCR amplification of microsatellite loci*

122 Fifteen microsatellite markers, SmC1, SmDO11, SmDA28 (Curtis, et al., 2001), R95529,  
123 SmD57, SmD28, SmD25, SCMSMOXII, L46951 (Durand, et al., 2000), SmBR16, SmBR10,  
124 SmBR13 (Rodrigues, et al., 2007), SmS7-1 (Blair, et al., 2001), SmBR1, SmBR6 (Rodrigues,  
125 et al., 2002) were used in this study (Table 3). The relevant DNA fragments were amplified  
126 using PCR. Details on microsatellite sequences, primers and PCR conditions are available in  
127 Table 3. To maximise efficiency and minimize costs, these PCRs were performed in three  
128 multiplex reactions using the QIAGEN multiplex kit. The PCR amplifications of loci:  
129 R95529, SmC1, SmDO11, SmBR16 and SmD57 were grouped in the multiplex 1 (M1); loci:  
130 SmDA28, SmBR1, SmS7-1, SmD28, SCMSMOXII were grouped in the multiplex 2 (M2);  
131 and loci: SmD25, L46951, SmBR6, SmBR10 and SmBR13 were grouped in the multiplex 3  
132 (M3). These multiplex reactions were carried out according to the manufacturer's standard  
133 microsatellite amplification protocol in a final volume of 10μL and with 57°C as annealing  
134 temperature. PCR products were diluted in Sample Loading Solution (Beckman Coulter) with  
135 red-labelled size standard (CEQ™ DNA size standard kit, 400, Beckman Coulter) and  
136 electrophoresis was done on an automatic sequencer (CEQ™ 8000, Beckman Coulter).

137 Microsatellite sizes were determined using the fragment analyzer package of Beckman  
138 Coulter. To confirm that the fixation procedure did not modify the microsatellite length and  
139 thus did not affect negatively microsatellite analyses, we used a clonal strain of *S. mansoni*  
140 (Brazilian strain selected for locus homozygosity) to compare the microsatellite profile  
141 between fixed and non-fixed material. These samples were processed as described above.

142

### 143 *2.5. Ethical Statements*

144 Our laboratory has received the permit # A66040 for experiments on animals from both the  
145 French Ministry of Agriculture and Fishing and the French Ministry of National Education,  
146 Research and Technology. Housing, breeding and animal care of the hamster followed the  
147 ethical requirements of the French government. The experimenter possesses the official  
148 certificate for animal experimentation delivered by both ministries (Décret # 87-848 du 19  
149 octobre 1987).

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151

## 152 **3. Results**

### 153 *3.1. Sporocysts detection and dissection.*

154 After 24 hours in Raillet-Henry fixation, SpIs are readily observable as translucent white  
155 bodies within an opaque grey tissue background (Figure 1A). At higher magnifications we  
156 could see the spherical aspect of SpI (Figure 1B and C). Figure 1 shows a snail at 15 DPI.  
157 SpIs were at their optimal development (filled with secondary sporocysts (SpIIs)), the biggest  
158 reached 1mm in diameter and could be recovered by careful dissection of the snail head-foot  
159 zone under a binocular microscope. Dissecting SpIs before 6 DPI remains very difficult. It is  
160 important to recover the SpI without taking snail tissue in order to enrich the parasite signal  
161 for genomic DNA or transcript RNA extractions.

162

### 163 3.2. *S. mansoni* sporocysts fixation and genomic DNA amplification

164 We tested the possibility to amplify gDNA of single SpI after Raillet-Henry fixation. PCR  
165 products of up to 2 kb in length could be amplified from gDNA (columns 1, 2, 3; Figure 2).  
166 At 2.5 and 3 kb amplification did not work anymore or only occasionally for some individuals  
167 (see individual of column 3 at 2.5kb, Figure 2). The results were compared to a positive  
168 control (classical gDNA extraction) for which PCR amplifications worked for up to 3kb  
169 (column 4; Figure 2). To achieve this fragment length specific amplification, we design  
170 primers for SmPoMuc genes for *Schistosoma mansoni* polymorphic mucins. These mucins  
171 were specific of *S. mansoni* and no related genes were present in the snail host *B. glabrata*,  
172 thus no cross-amplifications did occur (data not showed).

173

### 174 3.3. *S. mansoni* sporocysts fixation and microsatellite amplification

175 Microsatellite PCR on DNA extracted from Raillet-Henry fixed SpIs shown good results  
176 (Figure 3). All microsatellite loci were amplified and fragment lengths were in agreement  
177 with the expected sizes (see Figure 3 and Table 3) based on previous studies done on DNA  
178 extracted from adults of the same parasite strain (Bech, et al., 2010, Durand, et al., 2000). In  
179 addition using a clonal strain of *S. mansoni* without microsatellite diversity we showed that  
180 microsatellite profiles were the same for fixed and non-fixed material (Table 4). The fixation  
181 procedure did not modify the microsatellite length and thus did not affect negatively  
182 microsatellite analyses.

183

### 184 3.4. *S. mansoni* sporocysts fixation and reverse-transcription PCR.

185 Five genes were selected to study the sensitivity of transcript amplification, (i) alpha tubulin  
186 is a highly expressed gene involved in microtubules (one of the main components of the

187 cytoskeleton) bio-synthesis, (ii) two genes involved in different metabolic pathways, the  
188 superoxide dismutase (SOD) that is part of the antioxidant defence against reactive oxygen  
189 species and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) involved mainly in  
190 glycolysis pathway ; (iii) calreticulin is a multifunctional calcium binding protein encoded by  
191 a single copy gene in *S. mansoni* genome (Berriman, et al., 2009). Finally (iv) the *S. mansoni*  
192 antigen 10-3, a developmentally regulated surface antigen known to be expressed specifically  
193 in cercaria, male and female worms, was tested. Transcript amplifications were tested for  
194 these five genes after menthol anaesthesia and Raillet-Henry fixation or after snail were snap-  
195 frozen in liquid nitrogen and Raillet-Henry fixation and the same results were obtained for  
196 both techniques. For the four first tested genes, PCR products could be obtained when 5 SpI  
197 were used for cDNA preparation (Column 1, Figure 4). cDNA extracted from a single fixed  
198 SpI gave only an amplification for alpha-tubulin (Column 2, Figure 4) probably due to the  
199 greater amount of transcript for this highly expressed gene. PCR amplifications were highly  
200 specific as no cross amplifications with the intermediate host *B. glabrata* cDNA could be  
201 observed (Column 4, Figure 4). All those four genes were known to be expressed in miracidia  
202 and sporocysts of *S. mansoni*. Antigen 10-3 known not to be expressed was also tested as an  
203 internal control and as expected gave no amplifications (Figure 4).

204

#### 205 **4. Discussion**

206 Identification of *S. mansoni* sporocyst intramolluscan stage using Raillet-Henry fixation is a  
207 classical technique used mainly to investigate prevalence and intensity or intramolluscan  
208 development of the larval stages in the *S. mansoni* / *B. glabrata* model (Sire, et al., 1998,  
209 Théron, et al., 1997, Theron, et al., 1998). However, recovery of these intramolluscan fixed  
210 parasite stages for extraction of DNA or RNA was never described. Indeed, study of genetic  
211 information in formalin-fixed tissues is often hampered by the impossibility to amplify the

212 desired DNA or RNA as a consequence of nucleic acid damage (Zimmermann, et al., 2008).  
213 It is thus important to dissect snails and to recover the parasites after a short period of fixation  
214 (less than 24 hours). Studying genomic or transcriptomic information of these fixed  
215 intramolluscan stages will be of interest in many fields of research including the molecular  
216 dialogue between parasite and the host's immune system, distribution of infrapopulation  
217 genotypes or gene expression during development and maturation from SpIs to cercariae. The  
218 present work describes an improved technique for DNA and RNA extraction from such  
219 samples and delivers proof for their use for the study of genomic DNA, transcript expression  
220 or microsatellite genotyping.

221 Raillet-Henry fixation permits to recover SpIs that are more than 6 days old. To isolate SpIs  
222 before 6 DPI was quite impossible due to the minute size of these parasites at these steps. The  
223 use of this technique to study post-miracidial stage or SpI within the first days of infection  
224 remains therefore difficult. This constitutes the main limitation of this approach. However  
225 recovery of SpIIs or cercariae is feasible (data not shown). Our extraction protocols are  
226 efficient on SpIs but also on all other intramolluscan parasite stages (data not shown).

227 However for gDNA a limited size of PCR amplification exists. Apparently, Raillet-Henry  
228 fixation results in gDNA breaks at around 2kb because amplification of fragment of 2.5 kb  
229 was difficult and of 3kb was impossible. Fragmentations in DNA extracted from formalin  
230 preserved samples has been described before and is based on nicks and double-strand breaks  
231 (Zimmermann, et al., 2008). Amplifications of microsatellite fragments of less than 500  
232 nucleotides worked very well using our protocols. There is no difference in microsatellite  
233 amplification between fixed and non-fixed material. Raillet-Henry fixation did not affect  
234 negatively the microsatellite analyses. The technique will allow for genotyping of single  
235 parasite intramolluscan stages and thus could permit reconstruction of the population structure  
236 of sporocyst infra-populations inside the snail. Finally, RNA extraction and RT-PCR showed

237 good results, cDNA transcript amplification from Raillet-Henry fixed material with or without  
238 nitrogen freezing works well. In our model this will facilitate the study of gene expression for  
239 all intra-molluscan parasite developmental stages at 6 DPI and older.

240 We hope that the technique developed herein paves the way to a better understanding of the  
241 host/parasite molecular dialogue by taking into account more easily an often neglected partner  
242 in this interaction, the parasite.

243

#### 244 **Acknowledgments**

245 We are grateful to Bernard Dejean and Anne Rognon for technical assistance. We thank  
246 Julien Portela for helping with picture taking. We gratefully acknowledge Dr. C. Grunau for  
247 correcting the English version of this paper and for his critical reading of the manuscript. The  
248 work received funding from the BiomGenIm (ANR-07-BLAN-0214-03) programs of the  
249 French National Agency for Research, CNRS, and UPVD. The funding agency had no role in  
250 the study design, data collection, data analysis, the decision to publish, or the manuscript  
251 preparation.

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- 254 1. Adema, C. M., Hanington, P. C., Lun, C. M., Rosenberg, G. H., Aragon, A. D., Stout,  
255 B. A., Lennard Richard, M. L., Gross, P. S., and Loker, E. S., 2010. Differential  
256 transcriptomic responses of *Biomphalaria glabrata* (Gastropoda, Mollusca) to bacteria  
257 and metazoan parasites, *Schistosoma mansoni* and *Echinostoma paraensei* (Digenea,  
258 Platyhelminthes). *Molecular immunology* 47, 849-860.
- 259 2. April, C., Klotzle, B., Royce, T., Wickham-Garcia, E., Boyaniwsky, T., Izzo, J., Cox,  
260 D., Jones, W., Rubio, R., Holton, K., Matulonis, U., Quackenbush, J., and Fan, J. B.,  
261 2009. Whole-genome gene expression profiling of formalin-fixed, paraffin-embedded  
262 tissue samples. *PloS one* 4, e8162.
- 263 3. Azzi, A., Cosseau, C., and Grunau, C., 2009. *Schistosoma mansoni*: developmental  
264 arrest of miracidia treated with histone deacetylase inhibitors. *Experimental*  
265 *parasitology* 121, 288-291.
- 266 4. Baeza Garcia, A., Pierce, R. J., Gourbal, B., Werkmeister, E., Colinet, D., Reichhart,  
267 J. M., Dissous, C., and Coustau, C., 2010. Involvement of the cytokine MIF in the  
268 snail host immune response to the parasite *Schistosoma mansoni*. *PLoS pathogens* 6.
- 269 5. Bech, N., Beltran, S., Portela, J., Rognon, A., Allienne, J. F., Boissier, J., and Theron,  
270 A., 2010. Follow-up of the genetic diversity and snail infectivity of a *Schistosoma*  
271 *mansoni* strain from field to laboratory. *Infection Genetic and Evolution* 10, 1039-  
272 1045.
- 273 6. Bender, R. C., Bixler, L. M., Lerner, J. P., and Bayne, C. J., 2002. *Schistosoma*  
274 *mansoni* sporocysts in culture: host plasma hemoglobin contributes to in vitro  
275 oxidative stress. *Journal of parasitology* 88, 14-18.
- 276 7. Berriman, M., Haas, B. J., LoVerde, P. T., Wilson, R. A., Dillon, G. P., Cerqueira, G.  
277 C., Mashiyama, S. T., Al-Lazikani, B., Andrade, L. F., Ashton, P. D., Aslett, M. A.,  
278 Bartholomeu, D. C., Blandin, G., Caffrey, C. R., Coghlan, A., Coulson, R., Day, T. A.,  
279 Delcher, A., DeMarco, R., Djikeng, A., Eyre, T., Gamble, J. A., Ghedin, E., Gu, Y.,  
280 Hertz-Fowler, C., Hirai, H., Hirai, Y., Houston, R., Ivens, A., Johnston, D. A.,  
281 Lacerda, D., Macedo, C. D., McVeigh, P., Ning, Z., Oliveira, G., Overington, J. P.,  
282 Parkhill, J., Pertea, M., Pierce, R. J., Protasio, A. V., Quail, M. A., Rajandream, M. A.,  
283 Rogers, J., Sajid, M., Salzberg, S. L., Stanke, M., Tivey, A. R., White, O., Williams,  
284 D. L., Wortman, J., Wu, W., Zamanian, M., Zerlotini, A., Fraser-Liggett, C. M.,  
285 Barrell, B. G., and El-Sayed, N. M., 2009. The genome of the blood fluke  
286 *Schistosoma mansoni*. *Nature* 460, 352-358.
- 287 8. Blair, L., Webster, J. P., and Barker, G. C., 2001. Isolation and characterization of  
288 polymorphic microsatellite markers in *Schistosoma mansoni* from Africa. *Molecular*  
289 *Ecology Notes* 1, 93-95.
- 290 9. Chitsulo, L., Loverde, P., and Engels, D., 2004. Schistosomiasis. *Nature reviews*  
291 *Microbiology* 2, 12-13.
- 292 10. Coppin, J. F., Lefebvre, C., Caby, S., Cocquerelle, C., Vicogne, J., Coustau, C., and  
293 Dissous, C., 2003. Gene expression changes in *Schistosoma mansoni* sporocysts  
294 induced by *Biomphalaria glabrata* embryonic cells. *Parasitology research* 89, 113-119.
- 295 11. Coustau, C., and Yoshino, T. P., 2000. Flukes without snails: advances in the in vitro  
296 cultivation of intramolluscan stages of trematodes. *Experimental parasitology* 94, 62-  
297 66.
- 298 12. Curtis, J., Sorensen, R. E., Page, L. K., and Minchella, D. J., 2001. Microsatellite loci  
299 in the human blood fluke *Schistosoma mansoni* and their utility for other schistosome  
300 species. *Molecular Ecology Notes* 1, 143-145.

- 301 13. Durand, P., Sire, C., and Theron, A., 2000. Isolation of microsatellite markers in the  
302 digenetic trematode *Schistosoma mansoni* from Guadeloupe island. *Molecular*  
303 *ecology* 9, 997-998.
- 304 14. Gryseels, B., Polman, K., Clerinx, J., and Kestens, L., 2006. Human schistosomiasis.  
305 *Lancet* 368, 1106-1118.
- 306 15. Guillou, F., Mitta, G., Galinier, R., and Coustau, C., 2007. Identification and  
307 expression of gene transcripts generated during an anti-parasitic response in  
308 *Biomphalaria glabrata*. *Developmental and comparative immunology* 31, 657-671.
- 309 16. Guillou, F., Roger, E., Mone, Y., Rognon, A., Grunau, C., Theron, A., Mitta, G.,  
310 Coustau, C., and Gourbal, B. E., 2007. Excretory-secretory proteome of larval  
311 *Schistosoma mansoni* and *Echinostoma caproni*, two parasites of *Biomphalaria*  
312 *glabrata*. *Molecular and biochemical parasitology* 155, 45-56.
- 313 17. Hanington, P. C., Lun, C. M., Adema, C. M., and Loker, E. S., 2010. Time series  
314 analysis of the transcriptional responses of *Biomphalaria glabrata* throughout the  
315 course of intramolluscan development of *Schistosoma mansoni* and *Echinostoma*  
316 *paraensei*. *International journal for parasitology* 40, 819-831.
- 317 18. Lockyer, A. E., Spinks, J., Kane, R. A., Hoffmann, K. F., Fitzpatrick, J. M., Rollinson,  
318 D., Noble, L. R., and Jones, C. S., 2008. *Biomphalaria glabrata* transcriptome: cDNA  
319 microarray profiling identifies resistant- and susceptible-specific gene expression in  
320 haemocytes from snail strains exposed to *Schistosoma mansoni*. *BMC genomics* 9,  
321 634.
- 322 19. Mattos, A. C., Kusel, J. R., Pimenta, P. F., and Coelho, P. M., 2006. Activity of  
323 praziquantel on in vitro transformed *Schistosoma mansoni* sporocysts. *Memorias do*  
324 *Instituto Oswaldo Cruz* 101 Suppl 1, 283-287.
- 325 20. Mone, Y., Gourbal, B., Duval, D., Du Pasquier, L., Kieffer-Jaquinod, S., and Mitta,  
326 G., 2010. A large repertoire of parasite epitopes matched by a large repertoire of host  
327 immune receptors in an invertebrate host/parasite model. *PLoS neglected tropical*  
328 *diseases* 4.
- 329 21. Mone, Y., Mitta, G., Duval, D., and Gourbal, B. E., 2010. Effect of amphotericin B on  
330 the infection success of *Schistosoma mansoni* in *Biomphalaria glabrata*. *Experimental*  
331 *parasitology* 125, 70-75.
- 332 22. Ribeiro-Silva, A., Zhang, H., and Jeffrey, S. S., 2007. RNA extraction from ten year  
333 old formalin-fixed paraffin-embedded breast cancer samples: a comparison of column  
334 purification and magnetic bead-based technologies. *BMC molecular biology* 8, 118.
- 335 23. Rodrigues, N. B., Loverde, P. T., Romanha, A. J., and Oliveira, G., 2002.  
336 Characterization of new *Schistosoma mansoni* microsatellite loci in sequences  
337 obtained from public DNA databases and microsatellite enriched genomic libraries.  
338 *Memorias do Instituto Oswaldo Cruz* 97 Suppl 1, 71-75.
- 339 24. Rodrigues, N. B., Silvia, M. R., Pucci, M. M., Minchella, D. J., Sorensen, R., Loverde,  
340 P. T., Romanha, A. J., and Oliveira, G., 2007. Microsatellite-enriched genomic  
341 libraries as a source of polymorphic loci for *Schistosoma mansoni*. *Molecular Ecology*  
342 *Notes* 7, 263-265.
- 343 25. Roger, E., Gourbal, B., Grunau, C., Pierce, R. J., Galinier, R., and Mitta, G., 2008.  
344 Expression analysis of highly polymorphic mucin proteins (Sm PoMuc) from the  
345 parasite *Schistosoma mansoni*. *Molecular and biochemical parasitology* 157, 217-227.
- 346 26. Roger, E., Grunau, C., Pierce, R. J., Hirai, H., Gourbal, B., Galinier, R., Emans, R.,  
347 Cesari, I. M., Cosseau, C., and Mitta, G., 2008. Controlled chaos of polymorphic  
348 mucins in a metazoan parasite (*Schistosoma mansoni*) interacting with its invertebrate  
349 host (*Biomphalaria glabrata*). *PLoS neglected tropical diseases* 2, e330.

- 350 27. Roger, E., Mitta, G., Mone, Y., Bouchut, A., Rognon, A., Grunau, C., Boissier, J.,  
351 Theron, A., and Gourbal, B. E., 2008. Molecular determinants of compatibility  
352 polymorphism in the *Biomphalaria glabrata*/*Schistosoma mansoni* model: new  
353 candidates identified by a global comparative proteomics approach. *Molecular and*  
354 *biochemical parasitology* 157, 205-216.
- 355 28. Roger, E., Mitta, G., Mone, Y., Bouchut, A., Rognon, A., Grunau, C., Boissier, J.,  
356 Theron, A., and Gourbal, B. E., 2008. Molecular determinants of compatibility  
357 polymorphism in the *Biomphalaria glabrata*/*Schistosoma mansoni* model: new  
358 candidates identified by a global comparative proteomics approach. *Molecular and*  
359 *biochemical parasitology* 157, 205-216.
- 360 29. Serinsoz, E., Bock, O., Kirsch, T., Haller, H., Lehmann, U., Kreipe, H., and Mengel,  
361 M., 2005. Compartment-specific quantitative gene expression analysis after laser  
362 microdissection from archival renal allograft biopsies. *Clinical nephrology* 63, 193-  
363 201.
- 364 30. Sire, C., Rognon, A., and Theron, A., 1998. Failure of *Schistosoma mansoni* to  
365 reinfect *Biomphalaria glabrata* snails: acquired humoral resistance or intra-specific  
366 larval antagonism? *Parasitology* 117 ( Pt 2), 117-122.
- 367 31. Taft, A. S., Vermeire, J. J., Bernier, J., Birkeland, S. R., Cipriano, M. J., Papa, A. R.,  
368 McArthur, A. G., and Yoshino, T. P., 2009. Transcriptome analysis of *Schistosoma*  
369 *mansoni* larval development using serial analysis of gene expression (SAGE).  
370 *Parasitology* 136, 469-485.
- 371 32. Theron, A., and Gerard, C., 1994. Development of accessory sexual organs in  
372 *Biomphalaria glabrata* as related to infection timing by *Schistosoma mansoni*:  
373 Consequences on the energy utilisation patterns by the parasite. *Journal of Molluscan*  
374 *Studies* 60, 23-31.
- 375 33. Théron, A., Pages, J. R., and Rognon, A., 1997. *Schistosoma mansoni*: distribution  
376 patterns of miracidia among *Biomphalaria glabrata* snail as related to host  
377 susceptibility and sporocyst regulatory processes. *Experimental parasitology* 85, 1-9.
- 378 34. Theron, A., Rognon, A., and Pages, J. R., 1998. Host choice by larval parasites: a  
379 study of *Biomphalaria glabrata* snails and *Schistosoma mansoni* miracidia related to  
380 host size. *Parasitology research* 84, 727-732.
- 381 35. Vermeire, J. J., Boyle, J. P., and Yoshino, T. P., 2004. Differential gene expression  
382 and the effects of *Biomphalaria glabrata* embryonic (Bge) cell factors during larval  
383 *Schistosoma mansoni* development. *Molecular and biochemical parasitology* 135,  
384 153-157.
- 385 36. WHO, 2002. TDR Strategic Direction for Research: Schistosomiasis. World Health  
386 Organization Information.
- 387 37. Zimmermann, J., Hajibabaei, M., Blackburn, D. C., Hanken, J., Cantin, E., Posfai, J.,  
388 and Evans, T. C., Jr., 2008. DNA damage in preserved specimens and tissue samples:  
389 a molecular assessment. *Frontiers in zoology* 5, 18.
- 390  
391  
392  
393  
394  
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396

**Table 1:** Primers used for genomic DNA PCR

<b>Fragment length</b>	<b>Forward primer name</b>	<b>Forward primer sequence</b>	<b>Reverse primer name</b>	<b>Reverse primer sequence</b>	<b>Cycling conditions</b>
1010 bp	Exon6F	TGAAGCTCAACTCAGTAAGCTGAAC	Exon5R	CTTGTATCGCCTTCGATTCCAATTC	Ta: 58°C - elong: 68°C, 3min 30 - 40 cycles
1422 bp	10483ex13.F2	ACGAGGATTAATGATTACAAATATGC	Exon11R	TAGATAATGTACTGCCCACTTTGTG	Ta: 58°C - elong: 68°C, 3min 30 - 40 cycles
1999 bp	InTron14/15.F	CACTTGTTTCATAAACACGTGTCTTC	Exon11R	TAGATAATGTACTGCCCACTTTGTG	Ta: 58°C - elong: 68°C, 3min 30 - 40 cycles
2517 bp	Exon11F	ATTTCTTCTAGAATGTCTGAG	InTron6/7.R	TAAAGGTGGAATATGCCAAACTCAC	Ta: 58°C - elong: 68°C, 3min 30 - 40 cycles
3052 bp	InT3/4-3.3.F	CTATGGACACTATGAACAATATTCG	InTron6/7.R	TAAAGGTGGAATATGCCAAACTCAC	Ta: 58°C - elong: 68°C, 3min 30 - 40 cycles

bp: base pairs

Ta: Primers annealing temperature in centigrade; elong: temperature in centigrade and duration of elongation in minutes.

**Table 2:** Primers used for cDNA PCR

Gene name	Accession No.	Forward primer	Reverse primer	Cycling conditions
Alpha tubulin	SCMSAT1A	AGCAGTTAAGCGTTGCAGAAATCA	TGACGAGGGTCACATTCACCAT	Ta: 53°C - elong: 72°C, 30sec - 40 cycles
Superoxide dismutase Cu/Zn (SOD)	XM_002580438	AGTGGACTCAAGGCTG	CCACGGCCTAAATCAT	Ta: 54°C - elong: 72°C, 30sec - 40 cycles
Glyceraldehydes-3-phosphate dehydrogenase (GAPDH)	XM_002576947	GCGAGGTTTCGACTGA	AACAACGAACATGGGTG	Ta: 55°C - elong: 72°C, 30sec - 40 cycles
Calreticulin	XM_002574439	ATACGCTCTGGGACAT	CCTTGCTTCTCGGCATTA	Ta: 54°C - elong: 72°C, 30sec - 40 cycles
<i>S.mansoni</i> antigen 10-3 (Ag10-3)	M22346.1	CACAAGGGTCTACTGCTAACGGA	CCTTTAACATGGAATTTATCAGTCTGG	Ta: 54°C - elong: 72°C, 30sec - 40 cycles

Ta: Primer annealing temperature in centigrade; elong: temperature in centigrade and duration of elongation in seconds.  
GenBank Accession numbers (No.) are indicated.

**Table 3:** Primers used for Microsatellite PCR

Multiplex	Microsatellite name	Accession No.	Range size (nt)	Repeat motif	Forward sequence	Reverse sequence	Cycling conditions
M1	R95529	R95529	228/275	(CAT)10	GTGATTGGGGTGATAAAG	CATGTTTCTTCAGTGTCC	Ta: 57°C - elong: 72°C, 1min - 35 cycles
	SmC1	AF325694	287-296	(AAT)6-16	TGACGAGGTTGACCATAATTCTAC	AACACAGATAAGAGCGTCATGG	Ta: 57°C - elong: 72°C, 1min - 35 cycles
	SmD57	AF202967	276-300	(TA)22(GA)9	TCCTTGATTCCACTGTTG	GCAGTAATCCGAAAGATTAG	Ta: 57°C - elong: 72°C, 1min - 35 cycles
	SmBR16	L04480	337-341	(TA)10	TGTGACTTTGATGCCACTGA	GGCCTGATACAATTCTCCGA	Ta: 57°C - elong: 72°C, 1min - 35 cycles
	SmDO11	AF325698	303-367	(GATA)20-37	TGTTTAAGTCGTCGGTGCTG	ACCCTGCCAGTTTAGCGTAG	Ta: 57°C - elong: 72°C, 1min - 35 cycles
M2	SmDA28	AF325695	91-115	(GATA)7-14	CATGATCTTAGCTCAGAGAGCC	AGCCAGTATAGCGTTGATCATC	Ta: 57°C - elong: 72°C, 1min - 35 cycles
	SmBR1	L81235	154	(AC)9	GAGTATACGGCTTCTTGGA	CGGAACGACAAGAAAATCAT	Ta: 57°C - elong: 72°C, 1min - 35 cycles
	SmS7-1	AF330105	184	(AC)17	TCCTCCTCTCTATTTTCTCTTTG	ATTACGATTGCACAGATACTTTTG	Ta: 57°C - elong: 72°C, 1min - 35 cycles
	SmD28	AF202966	240-244	(CAA)5	CATCACCATCAATCACTC	TATTCACAGTAGTAGGCG	Ta: 57°C - elong: 72°C, 1min - 35 cycles
	SCMSMOXII	M85305	283-295	(CAT)9CGT(CAT)6	TTCTACAATAATACCATCAAC	TTTTTTCTCACTCATATACAC	Ta: 57°C - elong: 72°C, 1min - 35 cycles
M3	SmBR10	DQ448293	109-133	(GATA)10	CATGATCTTAGCTCAGAGAGC	GTACATTTTATGTCAGTTAGCC	Ta: 57°C - elong: 72°C, 1min - 35 cycles
	L46951	L46951	168-174	(GAA)7	CAAACATATACATTGAATACAG	TGAATTGATGAATGATTGAAG	Ta: 57°C - elong: 72°C, 1min - 35 cycles
	SmBR13	DQ137790	205-225	(CTAT)16	GTCACAGATACCTGACGAGCTG	ACTCCCCAGCAATTGTGCC	Ta: 57°C - elong: 72°C, 1min - 35 cycles
	SmBR6	AF009659	272-278	(CTT)10	CTTAACAGACATACACGC	GAATACAGGCTATAATCTACA	Ta: 57°C - elong: 72°C, 1min - 35 cycles
	SmD25	AF202965	268-274	(CA)10	GATTCCAAGATTAATGCC	GCCATTAGATAATGTACGTG	Ta: 57°C - elong: 72°C, 1min - 35 cycles

Characteristics of *Schistosoma mansoni* strain Guadeloupe (GUA) microsatellite loci, including locus name, GenBank Accession number (No.), size of PCR products in nucleotides (nt), nature of repeated motifs, forward and reverse primer sequences, annealing temperature in centigrade (Ta); elong: temperature in centigrade and duration of elongation in minutes.

**Table 4:** Comparison of microsatellite sizes for fixed and non-fixed materials.

<b>Multiplex</b>	<b>Microsatellite name</b>	<b>Size (bp) for fixed material</b>	<b>Size (bp) for non-fixed material</b>
M1	R95529	275	275
	SmC1	290	290
	SmD57	296	296
	SmBR16	337	337
	SmDO11	332	332
M2	SmDA28	115	115
	SmBR1	154	154
	SmS7-1	184	184
	SmD28	240	240
	SCMSMOXII	295	295
M3	SmBR10	133	133
	L46951	168	168
	SmBR13	221	221
	SmBR6	272	272
	SmD25	292	292

Microsatellite locus sizes of *Schistosoma mansoni* Brazilian clonal strain.

## Legends to figures

**Figure 1.** Visualisation of implanted SpI in snail tissue at 15DPI after Raillet-Henry fixation. The SpI were readily observable as translucent white bodies within an opaque tissue background (A). Higher magnification showing the spherical aspect of SpI (B, C). *Biomphalaria glabrata* was exposed to 20 miracidia of *Schistosoma mansoni*.

**Figure 2.** PCR amplification of genomic DNA recovered from Raillet-Henry fixed sporocysts. Columns 1, 2 and 3 correspond to individual sporocysts fixed in Raillet-Henry. Column 4 is a PCR positive control using gDNA extracted from non fixed miracidia and column 5 correspond to the negative PCR control. Specific fragment lengths were amplified using specific primer couples designed on SmPoMuc gene (Roger, et al., 2008, Roger, et al., 2008, Roger, et al., 2008). See Table 1 for primer sequences and PCR conditions.

MW: molecular weight in kilo bases.

**Figure 3.** Multiplex microsatellite fragments profiles visualised after PCR amplifications and sequencing using the CEQ 8000 fragment analyzer package (Beckman Coulter). For each multiplex (M1, M2, M3) the name and size position of microsatellites were indicated (see Table 3 for primer sequences and PCR conditions). For SCMSMOXII, as the dye signal is faint compared to the others, we showed a higher magnification of this microsatellite in the upper right corner of the M2 picture.

nt: nucleotide; RFU: relative fluorescent units.

**Figure 4.** PCR amplification of cDNA transcripts recovered from Raillet-Henry fixed sporocysts. Column 1 corresponds to the pool of cDNA originating from 5 sporocysts fixed in

Raillet-Henry. Column 2 corresponds to the cDNA of 1 sporocyst fixed in Raillet-Henry. Column 3 corresponds to a positive control using cDNA originating from non fixed miracidia. Column 4 corresponds to *Biomphalaria glabrata* cDNA. Column 5 corresponds to retro-transcription negative control. Column 6 corresponds to PCR negative control. See Table 2 for primer sequences and PCR conditions.

MW: molecular weight in base pairs.







