

1 **PIWI silencing mechanism involving the retrotransposon *nimbus* orchestrates resistance to**
2 **infection with *Schistosoma mansoni* in the snail vector, *Biomphalaria glabrata***

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25 **ABSTRACT**

26 **Background**

27 Schistosomiasis remains widespread in many regions despite efforts at its elimination. By
28 examining changes in the transcriptome at the host-pathogen interface in the snail *Biomphalaria*
29 *glabrata* and the blood fluke *Schistosoma mansoni*, we previously demonstrated that an early
30 stress response in juvenile snails, manifested by induction of heat shock protein 70 (Hsp 70) and
31 Hsp 90 and of the reverse transcriptase (RT) domain of the *B. glabrata* non-LTR-
32 retrotransposon, *nimbus*, were critical for *B. glabrata* susceptibility to *S. mansoni*. Subsequently,
33 juvenile *B. glabrata* BS90 snails, resistant to *S. mansoni* at 25°C become susceptible by the F2
34 generation when maintained at 32°C, indicating an epigenetic response.

35 **Methodology/Principal Findings**

36 To better understand this plasticity in susceptibility of the BS90 snail, mRNA sequences were
37 examined from *S. mansoni* exposed juvenile BS90 snails cultured either at 25°C (permissive
38 temperature) or 32°C (non-permissive). Comparative analysis of transcriptomes from snails
39 cultured at the non-permissive and permissive temperatures revealed that whereas stress related
40 transcripts dominated the transcriptome of susceptible BS90 juvenile snails at 32°C, transcripts
41 encoding proteins with a role in epigenetics, such as PIWI (*BgPiwi*), chromobox protein
42 homolog 1 (*BgCBx1*), histone acetyl transferase histone deacetylase (HDAC) and
43 metallotransferase (MT) were highly expressed in those cultured at 25°C. To further determine a
44 role for *BgPiwi* in *B. glabrata* susceptibility to *S. mansoni*, siRNA corresponding to the *BgPiwi*
45 encoding transcript was utilized to suppress expression of *BgPiwi*, rendering the resistant BS90
46 juvenile snail susceptible to infection at 25°C. Given transposon silencing activity of PIWI as a

47 facet of its role as guardian of the integrity of the genome, we examined the expression of the
48 *nimbus* RT encoding transcript at 120 min after infection of resistant BS90 *piwi*-siRNA treated
49 snails. We observed that *nimbus* RT was upregulated, indicating that modulation of the
50 transcription of the *nimbus* RT was associated with susceptibility to *S. mansoni* in *BgPiwi*-
51 siRNA treated BS90 snails. Furthermore, treatment of susceptible snails with the RT inhibitor
52 lamivudine, before exposure to *S. mansoni*, blocked *S. mansoni* infection concurrent with
53 downregulation of the *nimbus* RT transcript and upregulation of the *BgPiwi* encoding transcript
54 in the lamivudine-treated, schistosome-exposed susceptible snails.

55 **Conclusions and Significance**

56 These findings support a role for the interplay of *BgPiwi* and *nimbus* in the epigenetic
57 modulation of plasticity of resistance/susceptibility in the snail-schistosome relationship.

58

59 **KEYWORDS:** *Biomphalaria glabrata*, *Schistosoma mansoni*, Schistosomiasis,
60 resistance, susceptibility, plasticity, epigenetics, *Bg-piwi*, gene- silencing, *nimbus*,
61 reverse transcriptase, Lamivudine

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70 INTRODUCTION

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72 The freshwater snail, *Biomphalaria glabrata*, is an obligate intermediate host of the
73 trematode, *Schistosoma mansoni*, the causative agent of the neglected tropical disease
74 (NTD) schistosomiasis in neotropical regions. At least 600 million people, mainly in sub-
75 Saharan Africa, are at risk for schistosomiasis, a number that remains excessively high,
76 despite efforts to control transmission of the disease [1]. This disease causes widespread
77 chronic morbidity and male and female infertility. Specifically, infections caused by the
78 species, *Schistosoma haematobium* may result in bladder cancer and female genital
79 schistosomiasis. The latter exacerbates transmission of sexually transmitted diseases
80 including HIV (AIDS) [2].

81

82 The disease burden from schistosomiasis is probably underestimated, and it has been
83 suggested that the number of infected individuals exceeds 400 million [3]. On the other
84 hand, there are other estimations that claim 230 million people worldwide are infected
85 with *S. mansoni* [1]. These numbers are often underestimated due to the inability of
86 current diagnostic methods to detect light infections [4]. There are few defenses against
87 schistosomiasis, mainly because the residents of infected areas lack sufficient
88 infrastructure to properly combat this disease [3]. An integrated control approach,
89 implementing mass chemotherapy and molluscicides has made a difference in breaking
90 the complex life cycle of the parasite but without new effective drugs and vaccines to
91 prevent re-infection in treated areas, the cycle of repeated infection is the norm, thereby
92 making the long- term control of schistosomiasis elusive [5, 6]. Because of recent

93 projections made by the World Health Organization to eliminate schistosomiasis by 2025
94 and coupled with recent concerns of the spread of the disease into Mediterranean
95 countries of Europe, alternative approaches focusing mainly on blocking the development
96 of the parasite in the snail host are aggressively sought [7-9].

97
98 Molecular mechanism(s) involved in shaping the relationship between the parasite and its
99 obligate intermediate snail host, *Biomphalaria glabrata*, remains largely unknown. However,
100 information that will assist in clarifying some of the mechanisms of host/parasite interactions is
101 steadily being amassed. Reference genome sequences for all three organisms (human,
102 schistosome and snail) pertinent to transmission of schistosomiasis are now available [10-12].
103 Additionally, genes that underlie resistance and susceptibility phenotypes in *B. glabrata* to *S.*
104 *mansoni* infection are being identified [13-15], as are transcripts encoding larval (miracidia)
105 parasite proteins that are expressed at the snail/parasite interphase [16, 17]. Both snail and
106 parasite determinants are involved in a complex and dynamic innate defence system that either
107 rejects or sustains the successful development of the intra-molluscan stages of the parasite [13,
108 15].

109
110 Previously, we demonstrated that juvenile *B. glabrata*, that are either resistant or
111 susceptible to *S. mansoni*, display a differential stress response after early exposure to
112 wild type but not to irradiated *S. mansoni* miracidia. The stress response observed in the
113 susceptible juvenile snail was manifested by the early induction of transcripts encoding
114 heat shock proteins (Hsp) 70, Hsp90 and the reverse transcriptase (RT) domain of the *B.*
115 *glabrata* non-LTR-retrotransposon, *nimbus* [18]. Furthermore, the non-random

116 relocalization of the Hsp70 gene loci in interphase nuclei preceded transcription of the
117 corresponding Hsp70 transcript in the susceptible but not in the resistant snail, indicating
118 that in-coming schistosomes possess the ability to orchestrate in a rapid and systemic
119 fashion, the genome remodeling of juvenile susceptible snails soon after infection [19].
120 We also demonstrated that resistance in the juvenile BS90 snail stock was a temperature
121 dependent trait. Thus, when cultured at room temperature (25°C), juvenile BS90 snails
122 remained consistently resistant to infection. However, when cultured at 32°C for several
123 generations (F₁ to F₃), the progeny juvenile snails were phenotypically susceptible [20],
124 indicating a plastic epigenetic control over resistance.

125

126 Other studies that have used adult (>7 mm in diameter) snails instead of juveniles have
127 suggested that ability to alter the resistance of the BS90 to infection at elevated
128 temperature might be a strain-specific trait [21]. In recent studies, resistant BS90 snails
129 were found to be susceptible when exposed to *S. mansoni* as neonates [22]. In general,
130 adult snails are less susceptible to infection [23]. Furthermore, in some stocks of *B.*
131 *glabrata*, for example the 93375 strain, the juveniles are susceptible but become resistant
132 to the same strain of *S. mansoni* as young adults (at the onset of fecundity), and once egg
133 laying ceases and amoebocyte accumulations disappear in the pericardial wall, revert to
134 the susceptible phenotype [23, 24].

135

136 To further investigate the molecular basis of susceptibility plasticity, notably in the BS90 snail,
137 we have undertaken a comparative analysis of the transcriptomes of juvenile BS90 snails
138 cultured for several generations at either permissive (32°C) or non- permissive (25°C), aiming to

139 obtain leads for pathway(s) that lead either to resistance or susceptibility. This investigation and
140 the findings are detailed below.

141

142 **MATERIALS & METHODS**

143 **Snails**

144 The BS-90 snail is a wild-type pigmented snail that is resistant to *S. mansoni* (NMRI
145 strain) either as a juvenile or as an adult snail at 25°C [25]. The NMRI snail is an albino
146 susceptible snail that was derived from a cross between the wild type Puerto Rican snail
147 and a highly susceptible Brazilian albino snail [26, 27]. The BB02 snail is a susceptible
148 pigmented wild type snail from Brazil whose genomic DNA sequence was recently
149 reported [10]. The susceptible snails (NMRI and BBO2) are highly susceptible as
150 juveniles but the degree of susceptibility, especially in the NMRI stock, is variable as an
151 adult snail [28].

152

153 **Snail husbandry and *S. mansoni* infections**

154 BS90 stocks were cultured at 32°C as described [20]. Exposure of BS90 snails cultured at
155 32°C to miracidia were performed using juvenile progeny, F₁- F₂, <4 mm in diameter that
156 were bred at the elevated temperature. Briefly, BS90 snails were cultured either at 25°C
157 or 32°C in freshly made artificial pond water (www.afbr-bri.com) and fed *ad libitum*
158 with either romaine lettuce or snail gel food [29]. Juvenile snails or egg clutches were
159 transferred from 25°C to 32°C and maintained in groups of 3 or 4 in fresh water (100 ml)
160 in beakers maintained in a water bath at 32°C. The temperature inside the water bath was
161 monitored daily to maintain 32°C for the duration of the experiment. The snails were

162 cleaned weekly making sure that pre-warmed (32°C) water was used to clean the snails.
163 Detritus including dead snails and decayed lettuce leaves were removed daily. The egg
164 clutches from these snails (produced at 32°C) were collected and their progeny were
165 maintained at 32°C until they had grown to 3 to 4 mm in diameter (juvenile snails) before
166 exposure to miracidia at 25°C. The juvenile BS90 snails, 3 to 4 mm in diameter, were
167 maintained for two generations at either 32°C or 25°C and RNA prepared from 0 and
168 two-hour infected F₂ progeny as described [20]. Snails were exposed individually to the
169 10 to 12 miracidia in wells of a 12-well tissue culture plate (Greiner Bio-One, North
170 Carolina, USA) at room temperature. Miracidia were hatched from eggs recovered from
171 the livers of mice which had been infected with *S. mansoni* (NMRI strain) for seven
172 weeks [30].

173
174 Exposed snails (not used for RNA) were maintained at 25°C and examined for cercarial
175 shedding from four to 10 weeks later. Susceptible BBO2 and NMRI *B. glabrata* snails
176 utilized in this study were exposed as juveniles (described above) to freshly hatched
177 miracidia. To determine patency (cercarial shedding), individual snails were immersed in
178 one ml nuclease-free water in 12-well plates and directly exposed to a light source for 30
179 to 60 minutes at room temperature, after which snails were removed from the wells.
180 Cercariae released from individual snails were counted after adding a few drops of
181 Lugol's iodine solution to each well. After shedding, snails that were patent were
182 euthanized by immersion in 95% ethanol; non-shedding snails were incubated for up to
183 10 weeks at 25°C and checked weekly for patency.

184

185 **RNA sequencing, assembly, and annotation**

186 Total RNA was isolated by RNazol (Molecular Research Center, Inc. Cincinnati, OH)
187 from resistant (25°C) and susceptible (32°C) BS90 snails. BS90 snail transcriptome was
188 generated from polyA⁺ RNA isolated from pooled intact 2 hour exposed juvenile snails
189 maintained either at the non-permissive temperature (25°C), or at the permissive
190 temperature (32°C) on an Illumina HiSeq 100. Following RNAseq Illumina sequencing,
191 *de novo* assembly of the transcriptome was performed using Trinity. Functional
192 annotation of the contigs was performed with Trinotate that included Gene Ontology
193 assignments when possible, pFAM domain identification, transmembrane region
194 predictions (TmHMM) and signal peptide predictions (signalP). Differential expression
195 (DE) analyses were performed by using DESeq and identified differentially expressed
196 contigs between BS90 snails from the different categories. The analyses of these contigs
197 revealed the presence of known specific genes coding for several stress related and other
198 transcripts (Table 1). The complete transcriptome profiles of changes observed in
199 schistosome juvenile BS90 snails at either permissive (32°C) or non-permissive (25°C)
200 are provided as FASTQ files in the SRA database within NCBI with Bioproject ID
201 PRJNA687288.

202

203 **Two step qPCR**

204 Differential expression of the selected transcripts identified from the single pass RNAseq dataset
205 generated from resistant (25°C) and susceptible (32°C) juvenile BS90 snails were further
206 validated in other representative BS90 and susceptible snail stocks NMRI and BBO2 that were
207 either unexposed (0 hour) or exposed to *S. mansoni* for 30 min, 1, 2, 4 and 16 hours at 25°C.

208 cDNA was prepared from total RNA as described and contaminating genomic DNA removed by
209 treatment of the RNAs with DNase (RQI Promega WI) before performing the RT qPCR assays
210 (Ittiprasert, 2012 #7020). Quantitative real time PCR was performed with forward and reverse
211 gene specific primers corresponding to selected transcripts normalized against the expression of
212 the myoglobin gene as a reference, as described [31]. Oligonucleotide primers (forward and
213 reverse, Table 1) for transcripts identified by sequencing RNA of BS90 snails at either
214 permissive (32°C) or non-permissive temperatures (25°C) were designed from amino acid
215 sequences corresponding to the coding DNA sequence (CDS) of the following transcripts: piwi
216 like protein (*BgPiwi*), chromobox protein homolog 1 (*BgCBx1*), methyltransferase (*BgMT*),
217 Histone Acetyl Transferase (*BgHAT*) and histone deacetylase (*BgHDAC*). These CDS were
218 utilized to interrogate the reference *B. glabrata* genome sequences in GenBank by using the
219 Basic Local Alignment Search Tool, BLAST in NCBI. A standard BLASTp was performed to
220 further validate annotations of the selected transcripts followed by a SMART BLAST to explore
221 the phylogeny of the *B. glabrata* orthologs to other transcripts in the public domain. Amino acid
222 sequences of *B. glabrata* CDS showing significant (E value = $< 10^{-4}$ and with $> 25\%$ amino acid
223 sequence identity) homology to other transcripts were converted to the nucleotide sequences and
224 gene specific primers were designed using primer BLAST. Forward and reverse primers for
225 qPCR analysis were obtained from Eurofins Genomics (Louisville, KY) after the exclusion of
226 sequences for *S. mansoni* to avoid any possible amplification of parasite RNA during Real Time
227 PCR analysis. Two-step RT qPCR was utilized to quantitatively assess expression of the
228 selected transcripts using 500 ng of cDNA as template. SYBR Green PCR Master Mix kit
229 (Applied Bio systems, Thermo Fisher Scientific, Wolston Warrington, UK), with 15 μ M of
230 forward and reverse primers were used to evaluate the temporal expression of PIWI, Chromobox

231 protein homolog 1, HDAC, HAT and methyl transferase (MT). Each sample was run in triplicate
232 and reactions normalized against the constitutively expressed myoglobin reference gene in a
233 7300-thermal cycler (Applied Biosystems). Relative quantitative expression of the genes of
234 interest between resistant and susceptible snails was evaluated by the $\Delta\Delta C_t$ method. The
235 resulting fold change in expression of the genes of interest normalized against the signal for
236 myoglobin were calculated by using the formula,
237
$$\text{Fold difference} = 2^{-\Delta\Delta C_t} = 2^{-[(C_{t_{\text{gene, test}}} - C_{t_{\text{myoglobin, test}}}) - (C_{t_{\text{gene, control}}} - C_{t_{\text{myoglobin, control}}})]}$$
 [32]. Differences
238 were assessed using Student's *t* test, Welch's *t* test and 2-way analysis of variance (ANOVA)
239 wherever relevant by comparing the differential expression (delta-Ct value) of the transcripts
240 among treatment and control groups. A *p*-value of <0.05 was considered to be statistically
241 significant, with level of significance denoted as follows, ****, $p \leq 0.0001$, ***, $p \leq 0.001$, **, p
242 ≤ 0.01 , *, $p \leq 0.05$, and ns, $p > 0.05$

243

244 ***BgPIWI* transcript silencing by dsRNA and siRNA**

245 To investigate the functional role of *Bgpiwi* expression in *B. glabrata* susceptibility to
246 schistosome infection, the transcription of *Bgpiwi* was silenced by soaking juvenile BS90 snails
247 in either dsRNA-, or siRNA- PEI complexes [33]. Double-stranded (ds)RNA corresponding to
248 *Bgpiwi* was synthesized by using an *in vitro* transcription kit with a purified *Bgpiwi* PCR product
249 containing T7 sequences (sense and antisense) as template according to the manufacturer's
250 instructions (MEGAscriptT7, ThermoFisher Scientific Inc.) [34]. Off-target silencing of the
251 transcript encoding *Bgpiwi* in the resistant BS90 snail was evaluated by soaking snails in parallel
252 in universal mock siRNA- PEI complexes as control (MISSION siRNA Universal negative
253 control#1, Sigma Aldrich, St. Louis, MO). Knock-down of the *Bgpiwi* transcript in the resistant

254 BS90 snail (cultured at 25°C) was done as follows: juvenile snails were placed in 1.0 ml
255 nuclease-free dH₂O containing either 300 ng dsRNA: 1.0 µg PEI nanoparticle complexes or 775
256 ng siRNA: 1.0 µg PEI nanoparticle complexes. The complexes were prepared as follows: in a 1.5
257 ml capacity microcentrifuge tube, 1 µg of PEI, branched with average molecular weight 25000,
258 (Sigma Aldrich) in 500 µl nuclease-free H₂O was added slowly, drop-wise, to two different
259 siRNAs (Sigma Millipore) (start on target sequence CDS position 2393bp- sense:
260 GAACCAUUGUGGAUCAAAU/anti-sense: AUUUGAUCCACAAUGGUUC; (start on target
261 sequence at CDS position 2403bp sense: GGAUCAAAUAAUUACGAA/anti-sense:
262 UUUCGUAUUAAUUUGAUCC) diluted in 500 µl before mixing vigorously for 10 seconds at
263 room temperature. Both duplex siRNAs corresponding to *BgPiwi* transcript were utilized
264 simultaneously in a single tube. Samples of siRNA/PEI complexes (Total of 1.0 ml in
265 microcentrifuge tubes) were incubated at room temperature for 20 minutes before placing
266 individual juvenile snails in the mixtures. Holes were punched in lids of the closed
267 microcentrifuge tubes containing snails in siRNA/PEI complexes before incubating overnight at
268 room temperature. Control tubes, incubated in parallel, contained the following samples, a)
269 *Bgpiwi* siRNAs without PEI, b) PEI only without *Bgpiwi* siRNAs and c) Mission Universal
270 mock siRNA/PEI complexes (Sigma Millipore). RNA was isolated as described above from
271 washed transfected snails before utilizing for qPCR as described above. For each assay,
272 quantitative expressions of *Bgpiwi* and *nimbusRT* transcripts (normalized against myoglobin
273 expression) were evaluated with and without *S. mansoni* infection by qPCR using forward and
274 reverse primers corresponding to either transcript (Table 1). Transfected snails (with and
275 without infection) that were not investigated by RNA-based assays were maintained in at 25°C
276 as above and monitored for cercarial shedding at 4, 6, or 10 weeks later. The silencing of *Bgpiwi*

277 with dsRNA/PEI was evaluated in three, and with siRNA/PEI, in four biological replicates,
278 respectively.

279

280 **Treatment of susceptible BBO2 snails with reverse transcriptase inhibitor,**

281 **lamivudine**

282 Given that the central role of PIWI involves silencing of endogenous mobile elements,
283 such as nimbus, a non-LTR retrotransposon in the genome of *B. glabrata* [10, 35], we
284 examined the modulation of expression of the transcript encoding the RT domain of
285 *nimbus* in the following categories of susceptible BBO2 snails - a) normal snails, b)
286 snails treated overnight at room temperature with lamivudine at 100 ng/ml (Sigma
287 Aldrich, St. Louis, MO) and c) BS90 resistant snails treated with siRNA corresponding to
288 *Bgpiwi*/PEI complexes, as described above. Snails in these categories (a to c) were either
289 unexposed (0) or exposed (individually) to 10 miracidia for 2 hours at 25°C. Before
290 exposure, individual snails incubated in either lamivudine or *Bgpiwi* siRNA/PEI
291 complexes were washed twice with water before transfer to 2.0 ml water in 6-well tissue
292 culture plates to which freshly hatched miracidia (isolated from 7 weeks infected mouse
293 liver homogenate) was added and maintained for 120 min at room temperature. Exposed
294 and unexposed snails from either lamivudine-treated susceptible BBO2 or BS90
295 *siRNABgPiwi*/PEI- treated snails were either frozen immediately at -80°C in RNazol
296 until required for RNA isolation or, if not used for RNA preparation immediately,
297 transferred into 500 ml beakers containing aerated tap water and maintained as described
298 above at room temperature and evaluated for cercarial shedding at week 4, 6 or 10 post-
299 exposure. For comparison, susceptible snails were also either pre-treated as described

300 above, or after two weeks post-exposure, with another RT inhibitor BPPA (Santa Cruz
301 Biotechnology Inc., CA) that specifically inhibits the catalytic RT domain of human
302 telomerase (hTERT).

303

304 **Examining genome organization, relocation of the *piwi* locus, in susceptible and**
305 **resistant snails following exposure to *S. mansoni* miracidia**

306 Fluorescence *in situ* hybridisation (FISH) was performed using a probe derived from *B.*
307 *glabrata* bacterial artificial chromosome (BAC) libraries for the *piwi* locus. The DNA
308 probe was labelled by nick translation (BioNick Invitrogen, UK) as described [36, 37]
309 and incubating for 45-50 mins. The probe was precipitated with 1 µg of labelled BAC
310 DNA[36], 80 µg of *B. glabrata* genomic DNA and 9 µg of herring sperm DNA. These
311 components were dissolved in 48 µL of hybridisation mix at room temperature overnight,
312 this amount can be used for up to four slides. Preparation and fixation of samples
313 followed the protocol described previously [19]. Snail shells were crushed using a
314 microscope slide and the ovotestes excised using needle-nose forceps. Each ovotestis
315 was placed in a microcentrifuge tube containing 0.05 M KCl, macerated using a tissue
316 grinder (Axygen, UK) and incubated in solution for 30 min at room temperature.

317 Samples were then centrifuged at 200g for 5 min and supernatant discarded.

318 Methanol:acetic acid [3:1, v/v] was presented dropwise, with agitation, to the samples.

319 Once 0.5 mL of fixative was added, the samples were incubated at room temperature for
320 10 min before centrifuging again and discarding the supernatant, this fixation step was
321 repeated twice with the final fix volume being 100 µL. Slides were also prepared by
322 misting the slide with water vapour and then dropping 20 µL of a sample from a height

323 onto the slide and allowing the slide to dry on a slide drier. The slides were aged by
324 placing into a 70°C oven for 60 min then were taken through a dehydration series of 70%,
325 90% and 100% ethanol, spending 5 min in each solution. Slides were dried and warmed
326 up to 37°C on a slide dryer alongside 22x22 coverslips in preparation for probe addition.
327 Probe denaturation was performed at 75°C for 5 min and then allowed to reanneal for 20
328 min at 37°C before use. Hybridisation of samples and probe was performed using the
329 Top Brite automatic slide hybridiser (Resnova, Italy). Eleven µL of probe was presented
330 to a coverslip and the slide with the sample brought to the coverslip and the coverslip
331 sealed to the slide using rubber cement (Weldtite). The Top Brite was set for 37°C for 2
332 min up to 75°C for 2 min and then lowered to 37°C for 30 min, once the slides had
333 returned to 37°C they were transferred to a humidified chamber at 37°C for 72 hours.
334 Post hybridisation, the rubber cement was removed and coverslips allowed to detach in
335 the first wash. The washes were performed at 42°C in 2x SSC three times for 5 min each.
336 A blocking solution, made of 4% BSA (Sigma Aldrich, UK) in 2x SSC, was prepared.
337 After slides were removed from the third wash the excess solution was drained and 100
338 µL of blocking solution was added and the slides covered with parafilm. Slides were
339 maintained in a humidified chamber at room temperature for 30 min. Streptavidin-Cy3
340 was diluted 1:200 in 1% BSA in 2x SSC. After the blocking solution was removed, 100
341 µL of streptavidin-Cy3 solution was placed on each slide, covered in parafilm and
342 incubated in a humidified chamber at 37°C for 30 min. After the streptavidin-Cy3
343 incubation the slides were washed sequentially in 2x SSC for 5 min, 1x PBS + 0.1%
344 Tween 20 (Sigma Aldrich) for 1 min, and 1x PBS for 1 min. Lastly, the slides were

345 rinsed in sterile water before counterstaining with 4',6-diamidino-2-phenylindole (DAPI)
346 in mountant (H1200, Vectorshield).

347

348 **Image Analysis**

349 Images of nuclei were captured either with an Olympus BX41 fluorescence microscope
350 with a greyscale digital camera (Digital Scientific, UK) and the Smart Capture 3 software
351 (Digital Scientific, UK) or a Leica DM4000 using a Leica DFC365 FC camera and the
352 Leica Application Suite (LAS) imaging software. At least 50 nuclei were imaged for
353 each condition and processed via erosion script analysis [38] [39] to assess gene loci
354 positioning by using greyscale images and measuring the intensity of DAPI and
355 fluorescence in situ hybridization (FISH) signal, using the DAPI to outline the nuclei to
356 create five shells of equal area so that the intensity of the DAPI and FISH signal can be
357 measured and the FISH signal normalised for position by dividing by the DAPI signal,
358 averaged for the 50 images. Unpaired, equal variance, Student's *t* tests were performed
359 to ascertain significant differences in gene loci positioning within the different shells.

360

361 **RESULTS**

362

363 **Transcripts encoding PIWI (*BgPiwi*), HDAC (*BgHDAC*), chromobox protein**
364 **homolog 1 (*BgCBx1*), histone acetyl transferase (*BgHAT*) and metallotransferase**
365 **(*BgMT*) are differentially regulated in resistant BS90 and susceptible BBO2 snails**

366 In order to confirm and validate results of differential expression (DE) obtained from the De-
367 novo single-pass sequencing

368 of RNA isolated from *S. mansoni* exposed juvenile BS90 F₂ snails, which were cultured
369 either at non-permissive (25°C), or permissive (32°C), temperatures. We investigated the
370 expression of selected transcripts that have a role in epigenetics by two-step qRT-PCR
371 performed with RNA isolated from several different individual juvenile *B. glabrata* snails
372 that were either resistant (BS90 cultured at 25°C) or susceptible (BBO2 and BS90
373 cultured at 32°C) to *S. mansoni* infection. The investigation of temporal (0, 30 min, 1, 2,
374 4, and 16 hours) expression of *BgPiwi* in either juvenile resistant BS90 (25°C) or juvenile
375 susceptible BBO2 following exposure to *S. mansoni* by qPCR showed that the transcript
376 encoding the *B. glabrata* Piwi-like protein (Isoform 1 Accession number XP_013081375)
377 was upregulated between 30 min and 2 hour (2- to 7-fold) post-exposure in the resistant
378 BS90 but not in the susceptible BBO2 snail (Fig.1A). Figure 1B shows the temporal
379 expression of the transcript encoding *BgHDAC* (Accession Xp_0130754221.1). Results
380 likewise showed upregulation of this transcript (1.8-fold) in the juvenile resistant BS90
381 but not in the juvenile susceptible (BBO2) snails 2 hours after *S. mansoni* exposure.
382 Similarly, as shown in Figures 1C to 1E, results demonstrated that transcripts encoding,
383 the chromobox protein homolog 1 (Fig. 1C, *BgCBx1*), histone acetyl transferase (Fig. 1D,
384 *BgHAT*) and metallotransferase (Fig. 1E, *BgMT*) were also upregulated in BS90 resistant
385 but not the susceptible BBO2 snails, following *S. mansoni* infection. Inductions of up to
386 13- and 10-fold for transcripts encoding the chromobox protein homolog 1 and MT,
387 respectively (between 2 and 4 hours post exposure) were observed in exposed juvenile
388 resistant BS90 but not their exposed juvenile BBO2 susceptible counterparts.
389

390 **siRNA corresponding to *BgPiwi* knock down expression of the *piwi* encoding**
391 **transcript rendering resistant BS90 snails susceptible**

392 Since the findings revealed that the transcript encoding *BgPiwi* was upregulated in
393 juvenile resistant BS90 snails after *S. mansoni* infection, we proceeded to examine the
394 modulation of this (*BgPiwi*) transcript in the resistant (25°C) and the susceptible juvenile
395 (32°C) BS90 snails, with and without *S. mansoni* infection. The findings presented in
396 Figure 2A demonstrate that unlike resistant BS90 snails, cultured at 25°C, where
397 expression of the *BgPiwi* encoding was upregulated following (2 hour) *S. mansoni*
398 infection; in susceptible juvenile BS90 snails, cultured at 32°C, the *piwi* transcript was
399 downregulated, similarly to *S. mansoni* infection of the susceptible BBO2 snail (Fig. 1A).

400 Based on these data, and given the known role of PIWI in silencing endogenous
401 retrotransposable elements and previous results that revealed expression (upregulation) of
402 the RT domain of *nimbus* occurring in juvenile susceptible but not resistant snails in
403 response to *S. mansoni*, the functional role of PIWI in the epigenetics of *B. glabrata/S.*
404 *mansoni* susceptibility was investigated by silencing the expression of the transcript
405 encoding *BgPiwi* by using corresponding siRNAs. As shown in Figure 2B, investigation
406 of the expression of the transcript encoding *BgPiwi* in siRNA/PEI transfected snails with
407 (2 hours) and without (0 hours) *S. mansoni* exposure showed the knock-down of the
408 *BgPiwi* encoding transcript in snails transfected with siRNAs corresponding to *BgPiwi*,
409 but not to the universal mock siRNA. Use of *Bgpiwi* dsRNA/PEI complexes instead of
410 siRNA/PEI complexes to transfect BS90 snails, similarly, produced the knock-down of
411 the *piwi* encoding transcript as observed with using siRNAs. To determine the biological
412 effect of silencing *BgPiwi* in relation to *S. mansoni* infection in *BgPiwi* siRNA/PEI

413 transfected BS90 snails, schistosome exposed transfected non-transfected normal snails
414 and Universal mock siRNA/PEI transfected snails were left at room temperature and
415 evaluated at 4- and 6-weeks post-exposure. As shown in Figures 2C and 2D, BS90,
416 normally resistant snails, transfected with *BgPiwi* siRNA/PEI, shed cercariae at 4- and 6-
417 weeks post-exposure to *S. mansoni*.

418

419 **Knock-down of piwi encoding transcript with *siBgpiwi*/PEI complexes concurrently**
420 **upregulates the *nimbus* RT in transfected BS90 snails**

421 Because PIWI suppresses the expression of retrotransposable elements, such as the *B.*
422 *glabrata* non-LTR retrotransposable element *nimbus*, the transcription of the RT domain
423 of this element was investigated in either unexposed control (0) or *S. mansoni* (2 hour)
424 exposed BS90 snails that were transfected with either *Bgpiwi* siRNA or mock universal
425 siRNA (Fig. 3A). The same cDNA templates utilized in the qPCR assays shown in Figure
426 2B were utilized in the analysis of the expression of *nimbus* RT (Fig. 3A). Using gene
427 specific primers corresponding to the RT domain of *nimbus* results showed that in normal
428 BS90 snails that were unexposed (0) to *S. mansoni*, that similar to resistant BS90 that
429 were transfected with mock siRNA (UnisiRNA/PEI) expression of *nimbus* RT remained
430 low upon infection of the normal resistant BS90 snails as demonstrated previously [18].
431 However, in BS90 snails transfected with *Bgpiwi* siRNA before exposure, the *nimbus* RT
432 encoding transcript was upregulated when *Bgpiwi* transcript was silenced.

433

434 **Lamivudine RT inhibitor treatment of susceptible BBO2 snails differentially**
435 **regulates transcription of *nimbus* RT and *Bgpiwi***

436 To further examine the interplay between the expression of *Bgpiwi* and *nimbus* RT
437 encoding transcripts in relation to *S. mansoni* infection of *B. glabrata*. The susceptible
438 juvenile BBO2 snail was treated with the RT inhibitor drug, lamivudine, as described in
439 materials and methods prior to exposure to *S. mansoni*. As shown in Figure 4, panels A
440 and B, qPCR analysis of the same cDNA template prepared from BBO2 susceptible
441 snails that were either treated or untreated with different concentrations (100 ng/ml and
442 200 ng/ml) of lamivudine before exposure (for 2 hours) or not exposed (0 hour) to *S.*
443 *mansoni* were performed by utilizing primers corresponding to the *Bgpiwi* (4A) or
444 *nimbus* RT (4B) encoding transcripts. Figure 4A shows the expression of *Bgpiwi*
445 remained relatively unchanged with drug treatment while that of *nimbus* RT (Fig. 4B)
446 was downregulated with Lamivudine. Both concentrations of lamivudine, either 100 or
447 200 ng/ml, used to treat BBO2 snails prior to exposure knocked-down the expression of
448 the *nimbus* RT encoding transcript in lamivudine-treated snails.

449

450 **Lamivudine blocks schistosome infection of BBO2 snails**

451 Because the silencing of the *Bgpiwi* encoding transcript rendered the resistant BS90 snail
452 susceptible, concurrent with the down- and up-regulation of the *Bgpiwi* encoding
453 transcript and *nimbus* RT encoding transcripts, respectively, and since follow-up qPCR
454 analysis using the same cDNA templates showed that *nimbus* RT was knocked down by
455 lamivudine treatment of the susceptible BBO2 snail, the effect of this drug on the ability
456 of the susceptible snail to sustain a viable infection was examined (Figure 5). As shown
457 in Figure 5A, where juvenile BBO2 snails were either treated with 100 ng/ml of
458 lamivudine either before or after two weeks exposure to *S. mansoni* miracidia, snails

459 failed to shed cercariae when treated with lamivudine prior to exposure (Fig. 5A).
460 However, snails treated with BPPA (anthraquinone diacetate), another RT inhibitor that
461 specifically blocks the reverse transcriptase activity of the human homolog of telomerase
462 (hTERT) in *B. glabrata* shed cercariae when treated before exposure but not when treated
463 at 14 days post parasite exposure (Fig. 5B) in contrast to the outcome when snails were
464 treated with lamivudine before *S. mansoni* infection. Treatment of the snails with
465 lamivudine at day 14 post-exposure, however, showed some snails shed cercariae when
466 the drug was utilized later after infection.

467

468 **Relocalization of the *piwi* gene locus occurs in resistant *B. glabrata* snails upon** 469 **infection**

470 We had demonstrated that co-culture of snail Bge cells with live parasite [34, 36], and
471 infection of whole snails with live parasite, both lead to non-random gene loci relocation
472 within the interphase nuclei of the host snail cells, correlated with gene up-regulation [13,
473 19, 40], with notable differences in gene movement between susceptible and resistant
474 snails. We have been able to demonstrate again that gene loci change their non-random
475 nuclear location with changes in gene expression. A FISH probe containing the
476 sequences for *B. glabrata piwi* was employed to delineate the nuclear position of the *piwi*
477 gene loci in three snail strains, BS90 (resistant) and the two susceptible strains, BB02 and
478 NMRI (Figure 6). The nuclear positioning of the gene loci were analyzed using the
479 erosion analysis script for gene and chromosome positioning, we have used previously
480 for different species[41-43] and can be assigned to a peripheral (Figure 6A), intermediate
481 (Figure 6B) or internal (Figure 6C) location in cell nuclei. Notably, the *piwi* gene signal

482 is in different nuclear compartments for the resistant and susceptible snails. Indeed, in
483 BS90 (Figure 6D), the gene loci are found towards the nuclear interior and upon infection
484 there is a relocation towards the nuclear periphery at 30 minutes after infection (Figure
485 6D), which coincides with the increase in *piwi* transcripts (Figure 1A). By two hours, the
486 gene loci are relocating back to the nuclear interior (Figure 6D).

487
488 The two susceptible snail strains of BB02 (Figure 6E) and NMRI (Figure 6F) display a
489 similar gene loci location for *piwi*, an intermediate location, different from the resistant
490 BS90 strain (Figure 7D). Furthermore, the *piwi* gene locus does not change location until
491 4 hours post-infection, where in both snail strains, the gene locus is in the nuclear
492 interior, a location has been correlated with down-regulation of expression in BS90, and
493 in BB02 (Figure 1A). Together, these findings further support the notion that the parasite
494 is able to influence genome behavior within its host for its own advantage in an
495 epigenetic mechanism, through functional spatial positioning.

496

497 **DISCUSSION**

498 Significant progress has been made in recent years towards elucidating the molecular
499 basis of *S. mansoni* resistance/susceptibility in the intermediate snail vector *B. glabrata*.
500 From these studies, it has become clear that the snail and schistosome relationship is
501 complex and highly variable [13, 20, 44]. This study was undertaken to determine the
502 role of epigenetics in shaping the relationship between the snail and the schistosome. The
503 genetics of this interaction is well known and several molecular determinants that
504 underlie the innate defense anti-schistosome response in *B. glabrata* have been identified

505 as have sequences that are linked in the snail genome to resistance [14, 21, 45].

506 Epigenetics describes the inheritance of a reversible phenotype that is not influenced by

507 any change in the sequence of DNA. Transgenerational epigenetic inheritance induced by

508 environmental changes have recently reported the role of PIWI and small piRNA in

509 stress-induced genome modifications (see [46]). The impact of viral infection on the

510 siRNA and Argonaute /PIWI pathway has mainly been reported in insects[47, 48] .

511 However, little is known regarding genes and proteins involved in this insect Ago-

512 2/RNAi antiviral/stress defense response [49].

513

514 Variation in *B. glabrata* susceptibility to *S. mansoni* was investigated by examining the

515 regulation of key transcripts that play a role in epigenetics. Our approach was to use

516 representative juvenile snail stocks that are either resistant (BS90) or susceptible (BBO2)

517 to the NMRI strain of *S. mansoni*, to examine the temporal regulation of transcripts

518 encoding Piwi (*BgPiwi*), chromobox protein homolog 1 (*BgCBx1*), histone acetyl

519 transferase (HAT) histone deacetylase (HDAC) and metallotransferase (MT) were

520 examined in these snail stocks within 30 min to 16hr post- exposure to *S. mansoni*. The

521 differential expression of these transcripts was confirmed by comparing RNA-seq

522 datasets that were generated from non-permissive juvenile BS90 snails, cultured at room

523 temperature (25°C), and their permissive counterparts cultured for two generations at

524 32°C. The snail infections were carried out exclusively with juvenile snails. Thus, to

525 further validate the differential expression of these transcripts, resistant juvenile BS90

526 and susceptible juvenile BBO2 snails were exposed to *S. mansoni* before using real-time

527 qPCR to confirm their modulation pre- and post-parasite exposure. Transcripts encoding

528 PIWI (*BgPiwi*), chromobox protein homolog 1 (*BgCBx1*), histone acetyl transferase
529 (HAT) histone deacetylase (HDAC) and metallotransferase (MT) were upregulated (1.8
530 to 10-fold) in the resistant (BS90) snail as compared to their downregulation in the
531 susceptible juvenile snail (BBO2). Upregulation of the majority of these transcripts
532 occurred within the first 30 min of exposure to *S. mansoni*, peaking at 120 min before
533 subsiding. In earlier reports, we showed that the regulation of the RT domain of the *B.*
534 *glabrata* endogenous non-LTR-retrotransposable element, *nimbus*, was linked to the
535 early differential stress response observed between juvenile resistant and susceptible
536 snails [18]. Accordingly, induction of RT occurred concurrently with the upregulation of
537 the transcript encoding Hsp70 in the susceptible but not the resistant snail to *S. mansoni*.
538 Exposure of *B. glabrata* to irradiated attenuated miracidia, however, failed to induce
539 these stress-related transcripts in early-infected juvenile susceptible snails [25]. Given
540 those findings and those presented here showing that upregulation of the *BgPiwi*
541 encoding transcript, occurs in resistant BS90 snails residing at room temperature (where
542 they are resistant) but not in their susceptible counterparts residing at 32°C, we examined
543 the expression of the *Bgpiwi* transcript more closely in relation to the early expression of
544 *nimbus* in susceptible and resistant snails. To reiterate, changes in transcription regulation
545 were evident within the 30 minutes of infection. In this regard, these novel findings differ
546 from those described by others where molecular interactions between the snail and
547 schistosomes were performed much later after miracidia penetration at which time the
548 responses we now report might have waned.
549

550 The existence of several piRNA sequences in *B. glabrata* has been described but their
551 role in a piwi-piRNA mediated anti-parasite defense mechanism in the snail remains
552 elusive [50]. However, to determine whether a piwi gene-silencing mechanism that
553 involves *nimbus* RT plays a role in blocking transmission of schistosomes in *B. glabrata*,
554 we utilized a previously developed PEI-mediated soaking method to deliver two different
555 *BgPiwi* corresponding duplex siRNAs, simultaneously, into the resistant BS90 snail,
556 thereby knocking-down the expression of the PIWI encoding transcript. The RNAi
557 suppression of PIWI transcription, rendered these *Bgpiwi* siRNA/PEI transfected resistant
558 BS90 snails susceptible and thus able to shed cercariae. While the transcript encoding
559 *Bgpiwi* was reduced in siRNA/PEI transfected snails, in contrast, the expression of
560 *nimbus* RT-encoding transcript was upregulated, indicating that a gene silencing
561 mechanism mediated by the interplay of piwi and modulation of the transcription of
562 *nimbus* RT plays a major role in *B. glabrata* susceptibility to *S. mansoni*. To provide
563 further support for these findings, the susceptible BBO2 snail was treated with
564 lamivudine, a known RT inhibitor. Lamivudine is a RT nucleoside analog inhibitor that is
565 used to treat hepatitis B and HIV/ AIDS [51]. Treatment of either susceptible BBO2 or
566 NMRI snails prior to schistosome exposure, consistently, after several biological
567 replicates blocked *S. mansoni* infection in the snail. By contrast, BPPA, another RT
568 inhibitor that specifically targets the RT activity of telomerase, did not block infection in
569 *B. glabrata* by *S. mansoni* when snails were treated before parasite exposure. However,
570 in snails treated at 2 weeks after exposure, BPPA prevented infection unlike what was
571 observed with this treatment regimen with lamivudine. These findings indicated that the
572 mechanism of action of this *nimbus*/PIWI interplay occurs very early in the *S. mansoni*

573 and *B. glabrata* interaction. In ongoing studies, we have shown that an hTERT homolog
574 is absent in the *S. mansoni* genome with the snail ortholog showing significant identity at
575 the amino acid level to the human enzyme.

576

577 Work is currently underway to determine if using the same siRNA mediated gene
578 silencing strategy as described above will reveal the significance of the other transcripts
579 identified in this study and their functional role in epigenetics of the *S. mansoni/B.*
580 *glabrata* relationship. Previously, we showed that hypomethylation of the stress Hsp70
581 protein locus precedes the early upregulation of the Hsp70 encoding transcript in *S.*
582 *mansoni* exposed susceptible (NMRI) but not resistant (BS90) juvenile snails [52]. The
583 findings here that the transcript encoding MT was upregulated in juvenile BS90 resistant
584 but not susceptible BBO2 upon early parasite infection snails supports this earlier result
585 [52]. Ideal follow up experiments to further verify the involvement of all the transcripts
586 identified in this study in epigenetics of snail plasticity to *S. mansoni* susceptibility will
587 be to edit their CDS by a permanent gene mutation such as by CRISPR/Cas9 to knockout
588 their function. These approaches can likely contribute to deciphering epigenetic processes
589 that underlie the susceptibility of the snail to the parasite. Toward this objective,
590 molecular toolkits for gene editing in *B. glabrata* are being developed. CRISPR gene
591 editing has been used to edit the allograft inflammatory factor gene in the *Bge* embryonic
592 cell line from *B. glabrata* [53] and is finding utility in editing the schistosome genome
593 [54] [55]. Confirming earlier findings, we show that in intact juvenile resistant and
594 susceptible snails, within a short period post-exposure to *S. mansoni*, the non-random
595 movement of the *piwi* locus within interphase nuclei in relation to its active transcription

596 depending on the susceptibility phenotype of the snail)[19]. We have shown for the
597 schistosome mediated relocation of gene loci that movement in interphase nuclei (from
598 peripheral to interior location) occurs early after infection of susceptible, not resistant
599 snails, and precedes transcription of the gene loci in question. A soluble factor(s) within
600 excretory secretory products (ESPs) from the wild type miracidium that mediates the
601 systemic reorganization of the host genome is yet to be uncovered. Aside from
602 schistosomes, viruses are the only other pathogens that have been shown to also mediate
603 non-random gene relocation. However, schistosomes are the first metazoan parasites that
604 have been shown to possess the ability to manipulate the genome of the host in this
605 profound spatio-epigenetic fashion.

606

607 To conclude, although a molecular basis exists for *B. glabrata* susceptibility to *S.*
608 *mansoni*, it remains far from clear what precise pathways/mechanisms are responsible for
609 parasite survival or rejection in the early infected snail. This is the first study to show that
610 epigenetics, involving the interplay of PIWI and the endogenous non LTR-
611 retrotransposable *nimbus*, plays a role in the plasticity of snail susceptibility to *S.*
612 *mansoni*.

613

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622

623 **Figure Legends**

624 **Figure 1A**

625 qPCR analysis of RNA from resistant BS-90 (blue histogram) or susceptible BBO2 (gray
626 histograms) juvenile snails unexposed (0) or exposed for increasing intervals (30 seconds to 16
627 hours) to *S. mansoni* miracidia. Histograms show expression of the *BgPiwi* encoding transcript in
628 snails at each time point from five biological replicates. Note the increase in fold change in the
629 resistant BS-90 compared to the susceptible BBO2 snails after parasite infection. Significant
630 expression normalized against expression of the myoglobin encoding transcript was measured by
631 2-way ANOVA and is indicated by number of asterixis on each histogram where ****, indicates
632 the most significant value $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, ns $p > 0.05$.

633

634 **Figure 1B**

635 qPCR analysis of RNA from either resistant BS-90 (blue histogram) or susceptible BBO2 (gray
636 histograms) juvenile snails unexposed (0) or exposed for increasing intervals (30 seconds to 16
637 hours) to *S. mansoni* miracidia. Histograms show expression of the *BgHDAC* encoding transcript
638 in snails at each time point from five biological replicates. Note the increase in fold change in the
639 resistant BS-90 compared to the susceptible BBO2 snails after parasite infection. Significant
640 expression normalized against expression of the myoglobin encoding transcript was measured by

641 2-way ANOVA and is indicated by number of asterixis on each histogram where ****, indicates
642 the most significant value $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, ns $p > 0.05$.

643

644 **Figure 1C**

645 qPCR analysis of RNA from either resistant BS-90 (blue histogram) or susceptible BBO2 (gray
646 histograms) juvenile snails unexposed (0) or exposed for increasing intervals (30 seconds to 16
647 hours) *S. mansoni* miracidia. Histograms show expression of the *BgCBx* encoding transcript in
648 snails at each time point from five biological replicates. Note the increase in fold change in the
649 resistant BS-90 compared to the susceptible BBO2 snails after parasite infection. Significant
650 expression normalized against expression of the myoglobin encoding transcript was measured by
651 2-way ANOVA and is indicated by number of asterixis on each histogram where ****, indicates
652 the most significant value $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, ns $p > 0.05$.

653

654 **Figure 1D**

655 qPCR analysis of RNA from either resistant BS-90 (blue histogram) or susceptible BBO2 (gray
656 histograms) juvenile snails unexposed (0) or exposed for increasing intervals (30 seconds to 16
657 hours) to *S. mansoni* miracidia. Histograms show expression of the *BgHAT* encoding transcript
658 in snails at each time point from five biological replicates. Note the increase in fold change in the
659 resistant BS-90 compared to the susceptible BBO2 snails after parasite infection. Significant
660 expression normalized against expression of the myoglobin encoding transcript was measured by
661 2-way ANOVA and is indicated by number of asterixis on each histogram where ****, indicates
662 the most significant value $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, ns $p > 0.05$.

663

664 **Figure 1E**

665 qPCR analysis of RNA from either resistant BS-90 (blue histogram) or susceptible BBO2 (gray
666 histograms) juvenile snails unexposed (0) or exposed for increasing intervals (30 seconds to 16
667 hours) to *S. mansoni* miracidia. Histograms show expression of the *BgMT* encoding transcript in
668 snails at each time point from five biological replicates. Note the increase in fold change in the
669 resistant BS-90 compared to the susceptible BBO2 snails after parasite infection. Significant
670 expression normalized against expression of the myoglobin encoding transcript was measured by
671 2-way ANOVA and is indicated by number of asterixis on each histogram where ****, indicates
672 the most significant value $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, ns $p > 0.05$.

673

674 **Figure 2A**

675 qPCR analysis of RNA from either non permissive (25°C) resistant BS-90 (blue histogram) or
676 permissive (32°C) susceptible BS-90 (gray histograms) juvenile snails unexposed (normal) or
677 exposed for 2hr to *S. mansoni* miracidia. Histograms show expression of the *BgPiwi* encoding
678 transcript in these snails residing at different temperatures. Note the significant induction (8-fold
679 change) in 25°C non-permissive BS-90 snails compared the down regulation of the transcript in
680 permissive BS-90 snails residing at 32°C after parasite infection. Fold change was determined as
681 described in materials and methods. Significant expression normalized against expression of the
682 myoglobin encoding transcript was measured by 2-way ANOVA and is indicated by number of
683 asterixis on each histogram where ****, indicates the most significant value $p \leq 0.0001$, *** $p \leq$
684 0.001 , ** $p \leq 0.01$, * $p \leq 0.05$, ns $p > 0.05$.

685

686 **Figure 2B**

687 qPCR analysis of RNA from resistant BS-90 juvenile snails unexposed (gray) or exposed (blue)
688 for 2hr to *S. mansoni* miracidia. Histograms show expression of the *BgPiwi* encoding transcript
689 in normal BS-90 snails (control) or those transfected with *BgPiwi* siRNA. Note induction of the
690 *BgPiwi* encoding transcript occurs in *S. mansoni* exposed control BS-90 snails and the knock
691 down of the transcript in BS-90 (exposed and unexposed) snails transfected with *BgPiwi* siRNA.
692 In BS-90 snails transfected with mock UNIsiRNA, note the upregulation of the *BgPiwi* encoding
693 transcript in exposed snails similar to induction observed in control exposed snails. Fold change
694 was determined as described in materials and methods. Significant expression normalized against
695 expression of the myoglobin encoding transcript was measured by 2-way ANOVA and is
696 indicated by number of asterixis on each histogram where *****, indicates the most significant
697 value $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, ns $p > 0.05$.

698

699 **Figure 2C**

700 To determine the biological effect of silencing *BgPiwi* in relation to *S. mansoni* infection
701 in *BgPiwi* siRNA/PEI transfected BS-90 snails, schistosome exposed *BgPiwi* siRNA
702 transfected and non-transfected (not shown) snails were left at room temperature and
703 evaluated at 4- and 6- weeks post- exposure. Note that the BS-90 snail transfected with
704 *BgPiwi* siRNA shed cercariae at 4- and 6- weeks post- exposure to *S. mansoni*.

705

706 **Figure 3**

707 qPCR analysis of RNA from resistant BS-90 juvenile snails unexposed (gray) or exposed (blue)
708 for 2hr to *S. mansoni* miracidia. Histograms show expression of the *nimbusRT* encoding
709 transcript in normal BS-90 snails (control) or those transfected with *BgPiwi* siRNA. Note the

710 down regulation of the *nimbusRT* encoding transcript in *S. mansoni* exposed control BS-90 snails
711 and the upregulation of *nimbusRT* transcript in BS-90 (exposed and unexposed) snails
712 transfected with *BgPiwi* siRNA where transcript encoding *BgPiwi* has been knocked-down
713 (shown in Fig.2B). In BS-90 snails transfected with mock UNIsiRNA, note the down regulation
714 of the *nimbusRT* encoding transcript in exposed snails similar to that observed in control exposed
715 snails. Fold change was determined as described in materials and methods. Significant
716 expression normalized against expression of the myoglobin encoding transcript was measured by
717 2-way ANOVA and is indicated by number of asterixis on each histogram where ****, indicates
718 the most significant value $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, ns $p > 0.05$.

719

720

721 **Figure 4A**

722 qPCR analysis of RNA from susceptible BBO2 juvenile snails unexposed (gray) or exposed
723 (blue) for 2hr to *S. mansoni* miracidia. Histograms show expression of the *BgPiwi* encoding
724 transcript in normal BBO2 snails (0) or those treated with RT inhibitor Lamivudine (100 ng/ml
725 or 200ng/ml). Note the down regulation of the *BgPiwi* encoding transcript in exposed control (0)
726 snails and lamivudine treated snails (exposed and unexposed) snails. Fold change was
727 determined as described in materials and methods. Significant expression normalized against
728 expression of the myoglobin encoding transcript was measured by 2-way ANOVA and is
729 indicated by number of asterixis on each histogram where ****, indicates the most significant
730 value $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, ns $p > 0.05$.

731

732 **Figure 4B**

733 qPCR analysis of RNA from susceptible BBO2 juvenile snails unexposed (gray) or exposed
734 (blue) for 2hr to *S. mansoni* miracidia. Histograms show expression of the *nimbusRT* encoding
735 transcript in normal BBO2 snails (0) or those treated with RT inhibitor Lamivudine (100 ng/ml
736 or 200ng/ml). Note the upregulation of the *nimbusRT* encoding transcript in exposed control (0)
737 snails and down regulation of this transcript in Lamivudine treated snails (exposed and
738 unexposed) snails. Fold change was determined as described in materials and methods.
739 Significant expression normalized against expression of the myoglobin encoding transcript was
740 measured by 2-way ANOVA and is indicated by number of asterixis on each histogram where
741 ****, indicates the most significant value $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, ns p
742 > 0.05 .

743

744 **Figure 5A**

745 To determine the effect of lamivudine in relation to *S. mansoni* infection of susceptible
746 BBO2 snails, snails were treated before exposure with 100 ng/ml of the RT inhibitor,
747 maintained at room temperature, and evaluated for up to 6 weeks post-exposure. Note
748 that the BBO2 snails treated before infection with lamivudine failed to shed cercariae at 6
749 weeks post-exposure to *S. mansoni* unlike in untreated (control) snails. Also note that
750 BBO2 snails treated with the hTERT RT inhibitor BBPA before exposure, unlike
751 Lamivudine shed cercariae at 6 weeks post-exposure.

752 **Figure 5B**

753 The effect of treating BBO2 susceptible snails with 100 ng/ml of lamivudine at 14 days
754 post -exposure to *S. mansoni* was compared to the effect of treating susceptible BBO2
755 snails before exposure with 100 ng/ml of BPPA as described in materials and methods

756 and left at room temperature and evaluated for up to 6 weeks post- exposure. Note that
757 the BBO2 snails treated before *S. mansoni* exposure with 100 ng BPPA failed to shed
758 cercariae at 6- weeks post- exposure unlike in untreated (control) snails. Also note that
759 BBO2 snails treated with lamivudine at 14 days after infection shed cercariae at 6 weeks
760 post-exposure.

761

762 **Figure 6**

763 Nuclei (blue) were isolated from snail strains BS90, BB02 and NIMR ovo-testis and
764 subjected to 2D-fluorescence in situ hybridisation (FISH) with labelled probes for the
765 transposable element, *piwi* (green). Scale bar = 5 μ m. Using a bespoke nuclear
766 positioning script that creates five concentric shells of equal area, shells 1-5, with shell 1
767 being the nuclear periphery and shell 5 the nuclear centre, the percentage of fluorescent
768 green gene signal is measured in each shell for over 50 nuclei and divided by the
769 percentage of blue fluorescent signal for the DNA content (DAPI) in each shell. The data
770 are averaged and plotted as bar charts with standard error of the mean (SEM).

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787 **References**

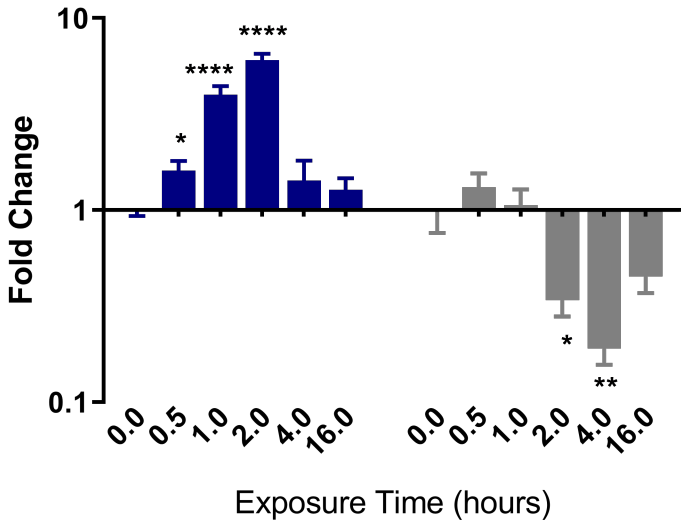
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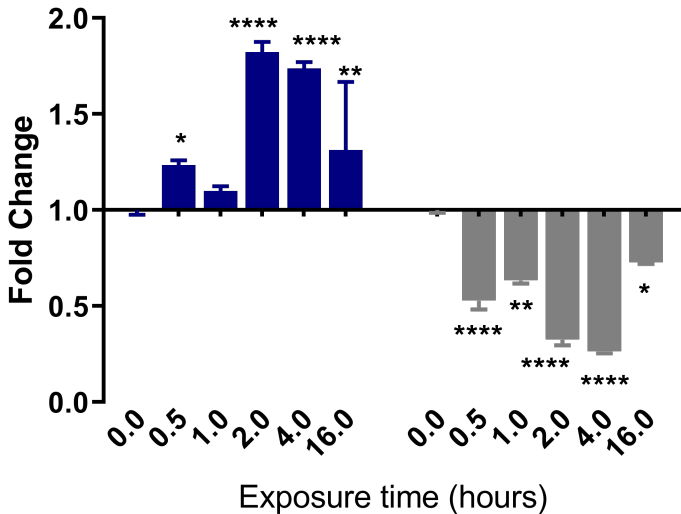
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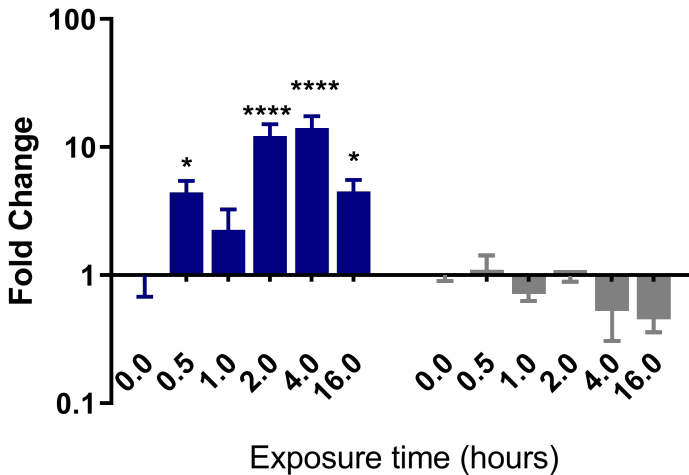
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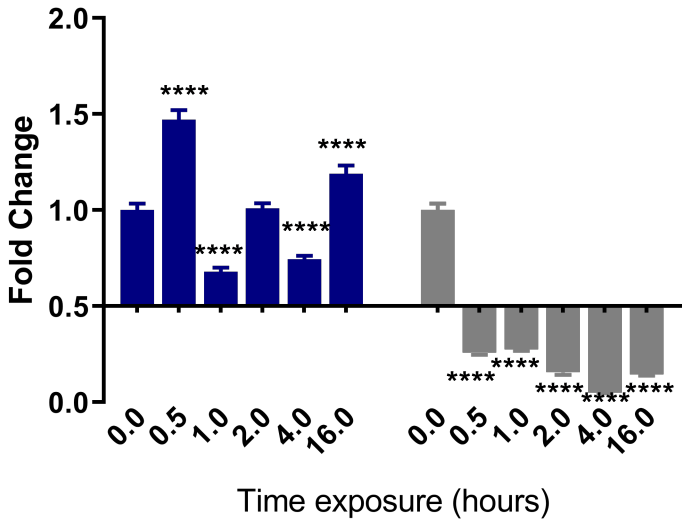
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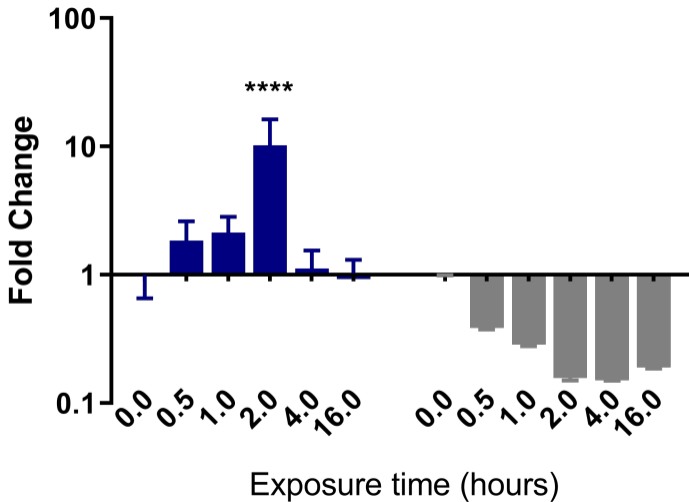
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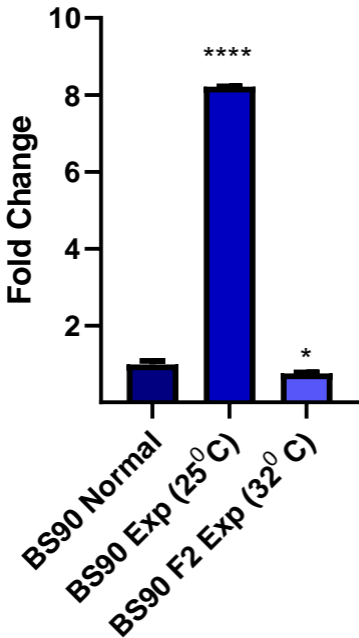


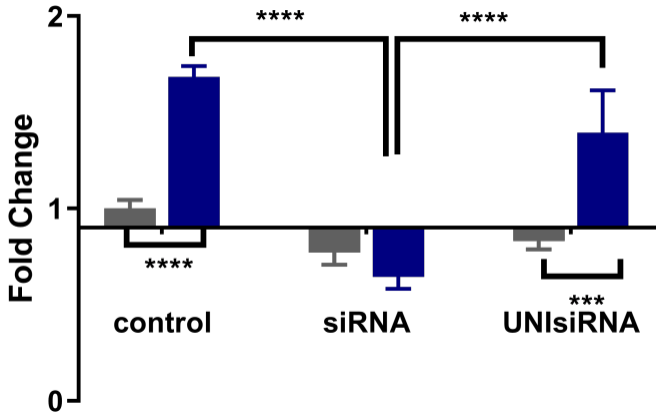






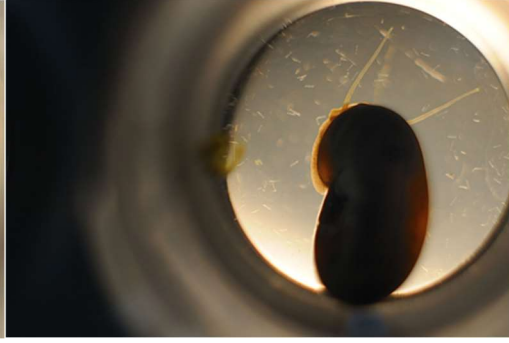




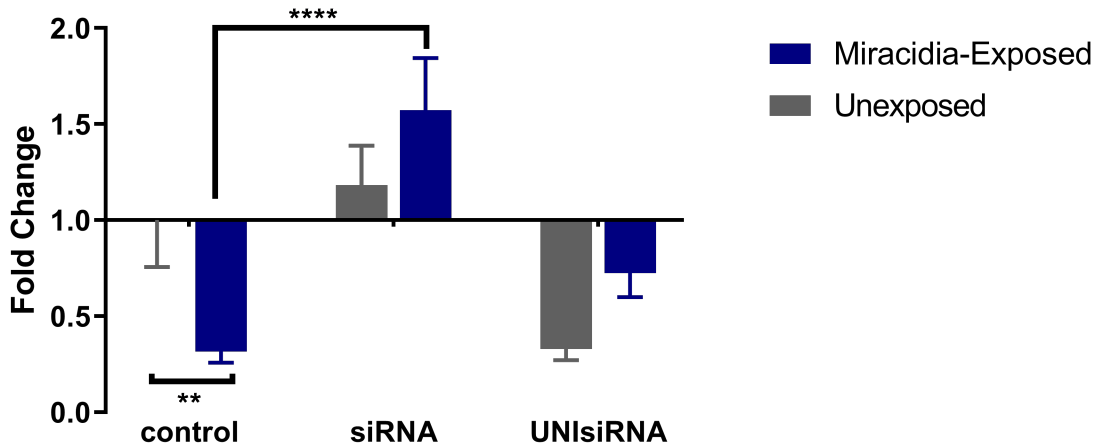


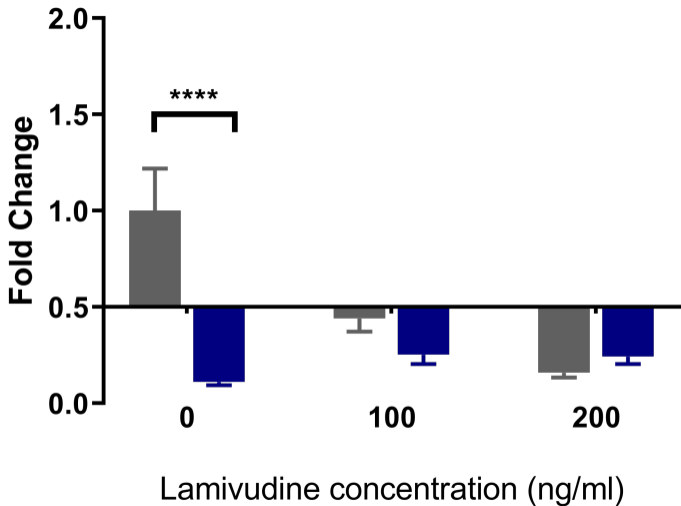


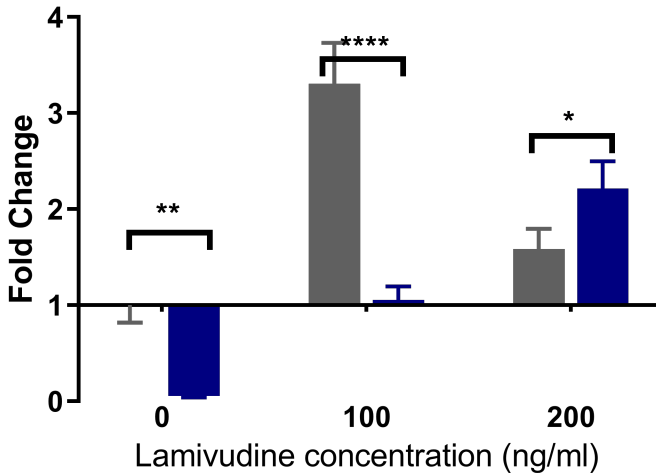
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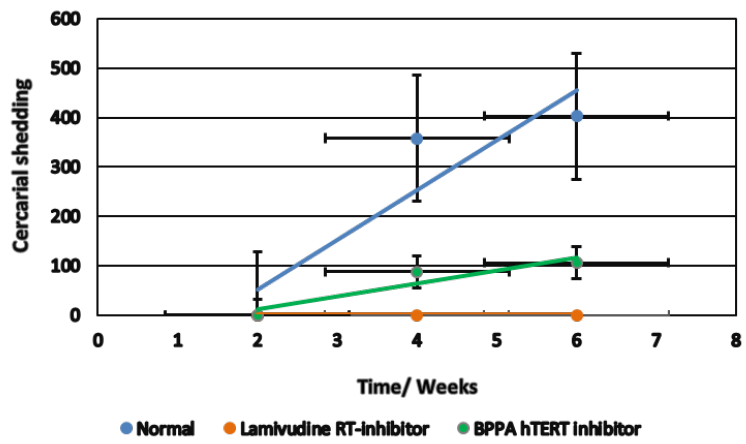
BS-90 PIWI/siRNA 6 weeks







5A



5B

