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## Epigenetic and phenotypic variability in populations of *Schistosoma mansoni* - a possible kick-off for adaptive host/parasite evolution

Celine Cosseau, E. Roger, A. Azzi, Anne Rognon, Christoph Grunau, Jérôme Boissier, S. Gourbière, G. Mitta

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1 **Epigenetic and phenotypic variability in populations of *Schistosoma mansoni* – a**  
2 **possible kick-off for adaptive host/parasite evolution**

3 C. Cosseau, A. Azzi (1), A. Rognon, J. Boissier, S. Gourbière, E. Roger (2), G. Mitta  
4 and C. Grunau (3)

5 Parasitologie Fonctionnelle et Evolutive  
6 UMR 5244 CNRS/Université de Perpignan/EPHE  
7 52, Avenue Paul Alduy  
8 66860 Perpignan  
9 France

10 Tel +33-4-68-66-21-80

11 Fax +33-4-68-66-22-81

12 1) both first authors contributed equally

13 2) Current address:

14 U 547 Inserm - Université de Lille 2

15 Institut Pasteur de Lille

16 1, rue du Professeur A. Calmette,

17 59019 - Lille,

18 France

19 3) corresponding author

20

21 **Abstract**

22 Epigenetics, the science of heritable but modifiable information, is now a well-  
23 accepted component of many research fields. Nevertheless, epigenetics has not yet  
24 found broad appreciation in one of the most exciting fields of biology: the  
25 comprehension of evolution. This is surprising, since the reason for the existence of  
26 this alternative information-transmitting system lies certainly in the evolutionary  
27 advantage it provides. Theoretical considerations support a model in which epigenetic  
28 mechanisms allow for increasing phenotypic variability and permit populations to  
29 explore the adaptive landscape without modifications of the genotype. The data  
30 presented here support the view that modulating the epigenotype of the human  
31 bloodfluke *Schistosoma mansoni* by treatment of larvae with histone deacetylase  
32 inhibitor leads indeed to an increase of phenotypic variability. It is therefore  
33 conceivable that environmentally induced changes in the epigenotype release new  
34 phenotypes on which selection can act and that this process is the first step in adaptive  
35 evolution.

36 **Introduction**

37 The genotype of an organism refers to its basic DNA sequence. The epigenotype  
38 refers to chemical modifications of that DNA and associated proteins, such as DNA-,  
39 or histone methylation that affect gene expression. The epigenotype is mitotically and  
40 to some degree meiotically heritable, but unlike in the genotype, changes in the  
41 epigenotype are reversible. It is now known that (i) individuals vary substantially in  
42 their epigenotypes (e.g. Kaminsky et al. 2009), (ii) that the epigenotype can change in  
43 response to environmental changes (e.g. Dolinoy et al. 2006), and consequently (iii)  
44 that epigenetic variation can result in phenotypic variation. We define here phenotypic  
45 variation as the change in a phenotypic character, and variability as the extent of  
46 change. Thus, epigenetic variation could be responsible for substantial phenotypic  
47 variation in natural populations that is both environmentally induced and responds to  
48 selection (at least for a few generations). While traditional models of evolution and  
49 heritability have been slow to incorporate epigenetics, some authors have suggested  
50 that epigenetics is crucial for adaptive evolution (e.g. Bossdorf et al. 2008; Jablonka  
51 and Lamb 1995). One way in which epigenetic variation could play a major role in  
52 evolution is by allowing phenotypic variability to increase, and populations to widely

53 explore the fitness (or adaptive) landscape. This could lead to a rapid adaptation to  
54 environmental changes or colonization of new environments. Changes in the  
55 epigenetic status of individuals in a population could result in random phenotypic  
56 changes that allow a population to explore new regions of the fitness landscape. Yet  
57 unlike in traditional models of adaptive phenotypic plasticity (Crispo 2008), the  
58 transient heritability of that induced variation allows favored phenotypes to spread  
59 among the descendants – even without continuous environmental induction - until the  
60 reversible variation can be converted into permanent genetic variation. Thus,  
61 epigenetics provides new mechanisms for old concepts such as genetic assimilation  
62 (see Pigliucci et al. (2006) for review). Epigenetics may also play a role in the rapid  
63 speciation and adaptation that sometimes follows hybridization between species  
64 (Barton 2001).

65 The physical carriers of epigenetic information are DNA methylation, modification of  
66 histones, non-coding RNA and the location of genes in the nucleus. These information  
67 carriers interact, and they form an Epigenetic Inheritance System (EIS) (Maynard  
68 Smith 1990). There are abundant examples for the modulation of gene activity and  
69 determination of phenotypes by epigenotypes (e.g. Esteller 2002, Weaver et al. 2004,  
70 Jacobsen and Meyerowitz 1997, Cubas et al. 1999). Since the phenotype is dependent  
71 on the epigenotype, epigenetic variability will have an effect on phenotypic  
72 variability. Theoretical models predict that phenotypic variability increases if (i)  
73 epigenetic variability is high, and (ii) epigenetic memory  $m$  is high (Pal and Miklos  
74 1999). Epigenetic memory  $m$  describes the capacity to transmit the epigenetic  
75 information from one generation to the other ( $0 \leq m \leq 1$ ). For DNA methylation in  
76 human lymphocytes for instance,  $m$  is close to 1 ( $m = 0.96$  (Laird et al. 2004)) during  
77 mitosis. If  $m = 0$  no epigenetic information transmission occurs, and phenotypic  
78 changes beyond the limits of phenotypic plasticity require changes of the genotype.  
79 Again, theoretical considerations predict  $m$  to be low if the effective population size  
80  $N_e$  is large (Rodin and Riggs 2003). Indeed, the only organisms in which epigenetic  
81 mechanisms appear to be the exception rather than the rule are bacteria (and viruses),  
82 i.e. those with very large  $N_e$ . The relation of fitness  $W$  to all phenotypic characters  $x$   
83 of the individuals in a population can be imagined as an  $n$ -dimensional fitness space  
84 or adaptive landscape (Wright 1932). If we consider a population that is located at a  
85 fitness maximum in an adaptive landscape, it is evident that every change in the  
86 phenotype will decrease the fitness of this population. In this situation, there will be

87 strong selection against a large phenotypic variability. Conversely, at a position where  
88 the adaptive landscape is concave, increasing phenotypic variability is advantageous  
89 for the population (Pal and Miklos 1999). In conclusion, the safest way for  
90 populations with low  $N_e$  to increase the phenotypic variability is to increase epigenetic  
91 variability but to keep the genotype constant. Given the impact of the epigenotype on  
92 the phenotype, the conclusion that increased epigenetic variability leads to increased  
93 phenotypic variability appears logical, however, an analysis of this relation is so far  
94 missing. Comparison of DNA methylation of 150 loci in 20 cultivars of cotton by  
95 msAFLP has shown that 67 % of the loci had differences in DNA methylation  
96 patterns (Keyte et al. 2006). In contrast, an earlier study of genetic polymorphism  
97 found only about 22 % of RFLP bands to be polymorphic (Brubaker and Wendel  
98 1994). Compared to genetic variability, epigenetic variability appears to be large. But  
99 so far no experiment relates unambiguously epigenetic and phenotypic variability.

100 Parasites are models of choice for this analysis of interrelation between epigenetics  
101 and adaptation because their evolution is fast and their effective population size is  
102 small (Poulin and Thomas 2008). Therefore, we decided to conduct such experiments  
103 using populations of larvae of the human blood-fluke *Schistosoma mansoni* as a  
104 model. *S. mansoni* is a parasitic helminth whose life-cycle is characterized by passage  
105 through two obligatory sequential hosts: the fresh-water snail *Biomphalaria glabrata*  
106 for the asexual part, and humans or rodent as hosts for the sexual part. An estimated  
107 200 million people in 74 countries suffer from schistosomiasis caused by *S.*  
108 *haematobium*, *S. japonicum*, and *S. mansoni*, and schistosomiasis is the most severe  
109 tropical diseases in terms of morbidity after malaria (Chitsulo et al. 2004). The eggs  
110 of the parasite are emitted with the feces but can also accumulate in the liver and  
111 cause the symptoms of the disease. When the eggs come into contact with water, free-  
112 swimming larvae (miracidia) hatch and actively seek *B. glabrata* snails as  
113 intermediate host. In natural populations less than 5% of snails are infected indicating  
114 the high selective pressure on the parasite at this point (Sire et al. 1999). After  
115 penetration into this host, the parasite develops via a primary (mother) sporocyst and a  
116 daughter sporocyst generation into the cercaria that infect the vertebrate host. This  
117 miracidia-sporocyst transition can easily be achieved *in-vitro* (Guillou et al. 2007).  
118 We wondered whether modulation of the epigenotypes in a population would increase  
119 the number of different phenotypes. Since DNA methylation is probably absent in *S.*  
120 *mansoni* (Fantappie et al. 2001), we decided to use Trichostatin A (TSA), a reversible

121 inhibitor of histone deacetylases (HDAC) (Yoshida et al. 1995), as epigenetic  
122 modulator. HDAC are present in *S. mansoni* (Oger et al. 2008) and TSA strongly  
123 reduces HDAC activity in this organism (Dubois et al. 2009). We show here that  
124 treatment of populations of *S. mansoni* larvae with TSA increases phenotypic  
125 variability on morphological and behavioral levels, and on the level of gene  
126 transcription, and leads to changes in snail infection success. Our data fit well with a  
127 theoretical model in which epigenetic generation of phenotypic variants is a first step  
128 in adaptive evolution.

## 129 **Material and Methods**

130 *Choice of parasite populations:* The ideal experimental design would be to use clonal  
131 populations derived from a single individual. Such clones are produced during one  
132 stage of the life cycle of *S. mansoni* (the daughter sporocyst produces clonal  
133 populations of cercariae). These are the larvae that infect the vertebrate host. For  
134 reasons of biological safety and because our laboratory focus on parasite/snail  
135 interactions we wanted to avoid using primarily cercariae. An alternative approach of  
136 generating genetically nearly identical individuals is the use of inbreeding. Therefore  
137 a *S. mansoni* strain (geographic origin Brazil) was used that had been inbred for more  
138 than 100 generations in our laboratory. Investigation of nine microsatellite markers  
139 revealed no genetic differences among individuals (details on request). We consider  
140 heritable genetic differences between individuals of this strain negligible.

141 *Parasite Culture:* Eggs were recovered from infected hamster (*Mesocricetus auratus*)  
142 8 weeks post-infection. Livers were collected and kept in sterile saline 0.85%  
143 containing an antibiotic / antimycotic mixture (penicillin 100 units/ml, streptomycin  
144 0.1 mg/ml, amphotericin 0.025 µg/ml; Sigma#057K2402). Livers were homogenized  
145 and eggs were filtered and washed. Miracidia were allowed to hatch and were  
146 concentrated by sedimentation on ice for 15 - 60 minutes. For initiation of *in-vitro*  
147 transformation into primary sporocysts miracidia were transferred into Chernin's  
148 balanced salt solution (CBSS) (Chernin 1963).

149 *Cytotoxicity test:* Cytotoxic effects of the drug were measured using the Roche  
150 Cytotoxicity Detection Kit (Roche #04744926001) that is based on the measurement  
151 of lactate dehydrogenase (LDH) activity released from dead and lysed cells into the  
152 supernatant. At least 320 larvae were used for each test.

153 *SDS-PAGE and Western Blot:* Western blots were used to evaluate the degree of  
154 histone acetylation. Four hundred sporocysts were re-suspended in denaturation buffer  
155 (0.2 % bromophenol, 10% sucrose, 3% SDS, and 0.2 M DTT, 62.5 mM Tris/Cl, pH  
156 6.8) treated by sonication (Vibra CellT.M. 75185) (60% intensity, 6 times 15 sec, with  
157 cooling intervals on ice) and boiled 10 min at 95°C. Proteins were separated by SDS-  
158 PAGE in 15% polyacrylamide and transferred to nitrocellulose membranes  
159 (Amersham RPN203D) by the semi-dry method (SEMI-PHOR Bio-Rad). The  
160 membrane was blocked overnight at 4°C in blocking buffer (150 mM NaCl, 0.05%  
161 v/v Tween 20, 5% w/v fat-free dry milk, 20 mM Tris/Cl, pH 7.5) and incubated with  
162 anti-histone H3 (Active Motif #39164 Lot#144, or abcam #ab1791-100 Lot#455351)  
163 and, after stripping in buffer ST (62 mM TRIS/Cl, 2% SDS, 0.8% beta-  
164 Mercaptoethanol, pH6.8) and exhaustive washing in water, with anti-hyperacetylated  
165 histone H4(Penta) (Upstate #06-946 Lot#29860) for 2 hours in blocking buffer.  
166 Membranes were washed, and incubated with peroxydase-coupled anti-rabbit (Pierce  
167 31460). Bands were revealed by Enhanced Chemical Luminescence (ECL Pierce) and  
168 direct exposure to x-ray film (Amersham EmNo.27304). Films were scanned (BioRad  
169 GS-800 Calibrated Densitometer) and band intensities were quantified with ImageJ  
170 (Abramoff et al. 2004).

171 *Trichostatin A solution:* Trichostatin A (TSA) (invivoGen met-tsa-5) was dissolved in  
172 ethanol to 20 mM and added either to the miracidia pool or the sporocyst culture, at  
173 appropriate concentration determined after preliminary trials (see Results). To the  
174 untreated control, an equal volume of ethanol was added (mock treatment).

175 *Tracking of larvae movement:* Larvae were kept in mineral water at 22-26°C and  
176 filmed with a conventional numerical camera (Nikon Coolpix 5000) adapted to a  
177 stereomicroscope for one minute at 15 frames per second (fps) and an image size of  
178 320 x 240 pixels. Background was removed, and larvae movement was tracked with  
179 ImageJ and a particle tracker plug-in (Sbalzarini and Koumoutsakos 2005). Positions  
180 were converted into x/y coordinates for each frame and each individual miracidium. A  
181 detailed description is available on our web-site ([http://methdb.univ-  
182 perp.fr/cgrunau/methods/Larvae\\_tracking.html](http://methdb.univ-perp.fr/cgrunau/methods/Larvae_tracking.html)). Points on the trajectory were  
183 considered different when they were at least two pixels apart. Straightness of the  
184 movement was calculated as rate of change of direction, i.e. the sum of all deviations  
185 in the trajectory as rad/sec (Ullyott 1936). Migration speed was converted into  
186 mm/sec.

187 *Measurements of larvae:* Individual sporocysts were photographed with a CCD  
188 camera under a microscope without coverslip, and length and largest width were  
189 measured using Visilog 6.3 (Noesis). Cercariae were fixed with hot 70% ethanol,  
190 stained with azocarmin (Touassem et al. 1992), photographed, and head length and  
191 width, body length and length of the bifurcated tail were measured as above.

192 *mRNA preparation from individual larvae and reverse transcription:* For RNA  
193 isolation, individual sporocysts were collected into 100 µl lysis buffer (Dynabeads  
194 mRNA DIRECT™ Micro kit, Dynal® Biotech) in Rnase-free tubes and stored at —  
195 80°C. Messenger RNA were extracted using the Dynabeads mRNA isolation Kit  
196 according the manufacturer's instructions. This protocol is based on base-pairing  
197 between the poly-A residues at the 3' end of the messenger RNA and the oligo-dT  
198 residues covalently coupled to the surface of the paramagnetic beads. For cDNA  
199 synthesis, reaction mixture was directly added to the bead-trapped mRNA. Reaction  
200 was carried out in a final volume of 20 µl (0.5 mM dNTPs, 0.01 mM DTT, 1x first-  
201 strand buffer, 2 U RNase out, 10 U SuperScript™ II RT (Invitrogen, lot#366592))  
202 50 min at 40°C.

203 *RT-PCR amplification:* Duplicated genes are particularly prone to be under epigenetic  
204 control and we decided to use a family of genes that was recently discovered in our  
205 laboratory (*SmPoMuc*, (Roger et al. 2008b)) as markers for gene expression variants.  
206 These genes are particularly useful because length polymorphism reflecting different  
207 transcripts can easily be detected using nested PCR with two primer pairs hybridizing  
208 to conserved regions in the cDNA (Roger et al. 2008a; Roger et al. 2008c). cDNA  
209 was PCR amplified using forward primer Exon1F12  
210 GGAAGAATGAACAAGAAAATTATTCTC, reverse primer Exon1R  
211 TGACACAGAAAAGTGTAAACGATCC and reaction conditions (95°C 1min; 40x  
212 95°C 30 s, 65°C 30 s, 68°C 3 min; 68°C 10 min). In a subsequent nested PCR forward  
213 primer NestedExon1F TATNTTGCCTGATAAG, reverse primer NestedExon15R  
214 ATCATAAACAAACACTGAGG and 46°C annealing temperature were used. These  
215 primers amplify polymorphic transcripts of the *SmPoMuc* genes (Roger et al. 2008c).  
216 PCR was done with the Advantage® 2 PCR Enzyme System (Clontech). PCR  
217 products were separated by electrophoresis in 2% agarose gels, and visualized by  
218 staining with ethidiumbromide. Amplicons of different length were counted.  
219 *Infection of Biomphalaria glabrata and mice:* Infection was performed as described  
220 before (Theron et al. 2008) by the same person. In brief, eggs were incubated in 0.9%



221 saline for 3 h in the dark, then mineral water was added and beakers were exposed to  
222 light for 1 h. Ten miracidia were brought into contact with individual *B. glabrata*  
223 (diameter 4-5 mm) for 24 h in 5 ml mineral water. Thirty to sixty snails were infected  
224 in parallel. For infection of female mice, 170 cercariae were used for each animal.

225 *Statistical methods:* Because analyzed variables are not normally distributed  
226 (Kolmogorov-Smirnov test,  $p < 0.05$ ), means were compared using non-parametric  
227 Mann-Whitney test and variances were compared using Brown-Forsythe test (Brown  
228 and Forsythe 1974). This test is an alternative formulation of the Levene test but is  
229 more robust under non-normal distribution (Conover et al. 1981). Infection success  
230 was tested using a  $\chi^2$ -test.

## 231 **Results**

### 232 *Establishment of 20 $\mu$ M TSA for 4 h as optimal experimental condition*

233 One hundred to four hundred sporocysts were incubated with increasing  
234 concentrations of TSA (2nM, 20nM, 200nM, 2 $\mu$ M, 20 $\mu$ M, 200 $\mu$ M and mock  
235 treatment) for 2, 4 and 16 hours and morphological changes were observed by light  
236 microscopy. Such changes became clearly visible after 4 hours. We decided to  
237 quantify the morphological alterations by measuring length and width of the  
238 sporocysts. A total of 580 sporocysts were photographed and measured after  
239 incubation for 4 hours in the above-mentioned conditions. TSA was replenished after  
240 2 hours. Most pronounced changes in morphological variability were observed with  
241 20 $\mu$ M TSA. Western blots (normalized to histone H3) indicate that acetylation of  
242 histone H4 increases by about 25% under these conditions, and the LDH test shows  
243 that cytotoxicity is at most 7% (supplementary figure 1 and supplementary table1).  
244 Consequently, all subsequent experiments were done with 20 $\mu$ M TSA for 4 hours.  
245 Since we had no *a priori* hypothesis about the phenotypic impact of the modulation of  
246 the epigenotype by TSA we decided to measure three very different phenotypic  
247 characters: (i) behavior (explorative movement of miracidia), (ii) morphology  
248 (length/width ratio of sporocysts) and (iii) gene transcription of a family of genes that  
249 code for polymorphic proteins: *SmPoMuc*. The *SmPoMuc* gene family and their  
250 polymorphism were earlier characterized and extensively studied by our laboratory  
251 (Roger et al. 2008a; Roger et al. 2008b; Roger et al. 2008c). When sporocysts were  
252 used, incubation with TSA was started after transfer into CBSS, for miracidia  
253 incubation was started with transfer into mineral water.

254 *Incubation with TSA changes phenotypic variability of explorative movement*

255 Incubation with TSA changes the distribution of swimming behavior in populations of  
256 miracidia. While in the mock-treated control almost all larvae move in straight lines  
257 with occasional U-turns, in TSA treated populations we observed a large variety of  
258 swimming behaviors ranging from straight lines to wave-like movements with a great  
259 frequency of changes of direction. To quantify this behavior we used a method  
260 developed by Ullyott (1936) that describes the straightness of the trajectory by a  
261 single numerical values: rate of change of direction (rdc). If this value is 0, the  
262 movement is perfectly straight, larger values indicate direction changes. For mock-  
263 treated controls, mean rdc is 0.086 rad/sec and variance is 0.003 rad/sec (n=43), while  
264 after TSA treatment mean rdc is 0.139 rad/sec and variance in rdc is 0.009 rad/sec  
265 (n=43) (figure 1 and supplementary figure 2). Differences in means and variances are  
266 statistically significant (U=609, p=0.006 and W=7.95, p=0.006, for means and  
267 variances respectively). In other words, after TSA treatment there are still miracidia  
268 that migrate in straight lines but in addition, new phenotypes with a different curve-  
269 rich movement occur. In contrast, mean and variance in velocity of the miracidia did  
270 not change (mock-treated: 6.04 points/sec±6.84 (1.61±0.70 mm/sec), with TSA: 5.95  
271 points/sec±5.64 (1.58±0.63 mm/sec), U=843, p=0.48 and W=0.73, p=0.39 for means  
272 and variances respectively).

273 *Incubation with TSA changes phenotypic variability of morphology*

274 Next, we analyzed the influence of TSA on the phenotypic variability on a  
275 morphological level. A simple way to describe morphology by a single numerical  
276 value is the ratio of length to width (L/W). A ratio of 1 describes a circular form, and  
277 higher values indicate lengthening of the shape. In mock-treated populations we  
278 observed round and moderately elongated forms with a L/W ratio from 1.01 to 4.85  
279 (n=63). In the TSA treated population, we still found round sporocysts but observed  
280 also very long forms with a L/W ratio up to 7.65 (n=65). The mean phenotype did  
281 only moderately change (mock-treated: m=2.04, TSA-treated: m=2.58, U=1141,  
282 p=0.17) but the TSA treatment led to the generation of additional long phenotypes  
283 and thus an increasing variance in morphology (mock-treated: V=0.78, TSA-treated  
284 V=2.37, W=7.95, p=0.006) (figure 1 and supplementary figure 3).

285 *Incubation with TSA changes phenotypic variability of gene transcription*

286 We then hypothesized that the observed changes in morphological and behavioral  
287 variability should be caused by and underlying change in gene expression. RNA was

288 extracted from individual sporocysts, cDNA was produced and *SmPoMuc* transcripts  
289 were amplified and separated by electrophoresis. The number of polymorphic bands  
290 was counted for each individual. We observed in the mock-treated control as well as  
291 in the TSA treated group, individuals with only 1 band, but with TSA we detected up  
292 to 6 bands, while without TSA we never found more than 5 bands. In the TSA treated  
293 population the average number of transcripts per individual increased from  $2.7 \pm 1.1$   
294 (mock: median=3, n=34) to  $3.6 \pm 1.1$  (with TSA: median=4, n=36) (figure 1 and  
295 supplementary figure 4). Significant difference is observed between means ( $U=346$ ,  
296  $p=0.001$ ). Clearly, TSA induces the generation of new combinations of transcripts in  
297 the population without losing the old combinations. Since *SmPoMuc* is a *bona fide*  
298 candidate for a key-element in the molecular interaction with the snail-host (Roger et  
299 al. 2008a; Roger et al. 2008c), the generation of new variants is expected to have an  
300 impact on infection success of the parasite larvae.

#### 301 *Incubation with TSA influences infection success*

302 Theoretical considerations predict that the increase of phenotypic variability in a  
303 population will have an influence on the fitness of the individuals of this population.  
304 We decided to use infection success as a measure for fitness and exposed mock-  
305 treated and TSA-treated miracidia to a compatible *B. glabrata* host. Four weeks post-  
306 infection the infected snails were counted. With TSA treatment, infection success  
307 decreased 1.6 fold from  $79 \pm 13\%$  infected snails for mock-treated larvae (n=90) to  
308  $48 \pm 22\%$  (n=90) with TSA. The experiment was repeated three times. The difference  
309 is significant ( $c_1^2=16.28$ ,  $p<0.0001$ ). Variance was not calculated since the number of  
310 experiments was too small.

#### 311 *TSA has an effect on the phenotype through several mitotic generations*

312 By definition, epigenetic information is heritable, but changes in the information are  
313 reversible. We wondered if phenotypic changes would be detectable after several  
314 mitotic generations. We therefore studied the phenotype of the second type of free-  
315 swimming larvae that are produced after infection by the miracidia by clonal  
316 reproduction in the snail-host: the cercariae. Cercariae were collected after 40 days of  
317 infection and length to width ratio of the head, length of the body and size of the  
318 bifurcated tail were measured. No differences between cercariae that were  
319 descendants of the TSA-treated miracidia and the control group were detected for  
320 head and tail. In contrast, body length differed significantly ( $U=575.5$ ,  $p<0.0001$ ;  
321  $W=2.915$ ,  $p=0.091$ ): body length of cercariae was  $210 \pm 14 \mu\text{m}$  in the control group

322 (n=56), and  $194 \pm 18 \mu\text{m}$  for cercariae derived from TSA-treated miracidia (n=50)  
323 (supplementary figure 5). Apparently, the effect of TSA is detectable on the  
324 morphology after three metamorphosis steps (from miracidia to primary sporocyst,  
325 secondary sporocyst and cercaria). Interestingly, body length variance did not change.

326

327 *TSA has no effect on infection success after one meiotic generation*

328 Finally we wondered whether phenotypic information that influences infection  
329 success would be transmitted to the next generation after meiosis. Miracidia were  
330 treated with TSA, used to infect snail and cercariae shed from these snails were used  
331 to infect 10 mice. After sexual reproduction of *S. mansoni*, eggs were extracted,  
332 miracidia were pooled and used to infect snails. In this case, no significant difference  
333 in infection success was observed ( $\chi^2=0.02$ ,  $p=0.97$ ). The F2 generation of TSA-  
334 treated F1 had 97% infection rate (n=30), and F2 of mock-treated F1 had 96%  
335 infection rate (n=57).

## 336 **Discussion**

337 Ten years ago Pal and Miklos (Pal and Miklos 1999) proposed that the reason for the  
338 existence of an alternative information transmitting system – the epigenetic  
339 inheritance system (EIS) (Maynard Smith 1990) – could reside in an evolutionary  
340 advantage that is conferred by such a system. With EIS, populations would dispose of  
341 a larger phenotypic variability that increases in response to changes in the  
342 environment, thus creating additional phenotypes on which selection would act. These  
343 phenotypes remain cryptic under normal conditions. There is ample evidence that the  
344 epigenotype influences the phenotype. The phenotype must therefore be considered as  
345 an expression of both genotype and epigenotype, under influence of the environment.  
346 However, in contrast to the genotype, the epigenotype can be influenced directly by  
347 the environment (reviewed for instance by Liu et al. (2008) and Pembrey (2002)).  
348 Nevertheless, the question whether changes in the environment increase epigenetic  
349 variability and this in turn increases phenotypic variability was never addressed. It is  
350 technically difficult, if not impossible for the moment, to prove that environmental  
351 changes induce changes in the epigenetic variability. Instead, we decided to influence  
352 directly epigenetic variability in a population and to measure the effect on phenotypic  
353 variability. TSA is a specific reversible inhibitor of HDAC. Treatment with this drug  
354 influences only histone acetylation and the equilibrium between heterochromatin and

355 euchromatin. Basically, euchromatin is the open, transcription permissive form of  
356 chromatin that is highly acetylated, while heterochromatin correspond to the de-  
357 acetylated condensed form where gene transcription is impaired. We show here that  
358 the treatment with TSA does not lead to strong change in the mean phenotype in a  
359 population, but increases phenotypic variability, i.e. reveals cryptic phenotypes. This  
360 is the first time that experimental proof for the hypothesis of Pal and Miklos is  
361 delivered (figures 1 and 2). Nevertheless, our findings can be related to earlier  
362 observations: in a now classical experiment C.H. Waddington treated pupae of  
363 *Drosophila melanogaster* with heat-shocks. He observed the induction of a cryptic  
364 phenotype (crossveinless) and could select for this phenotype (Waddington 1953).  
365 The molecular basis for this environmentally induced heritable change in phenotypic  
366 variability remains unclear. However, recently it was shown that heat-shock protein  
367 hsp90 interacts with chromatin modifying enzymes (Sollars et al. 2003) and this  
368 interaction could be the physical link between heat-shock and the release of epigenetic  
369 control of cryptic phenotypes. Waddington's original data are not anymore available  
370 but it is conceivable that he observed the same increase in phenotypic variability, but  
371 selected for a single, easily observable phenotype. In *S. mansoni*, the induction of a  
372 metastable phenotype (resistance to an antischistosomal drug hycanthone) by a single  
373 exposure to the drug was described (Jansma et al. 1977). Resistance was maintained  
374 through the first seven generations and became metastable in the subsequent  
375 generations. In these experiments the phenotypic variability of the studied population  
376 was not measured, so it is not clear if particular phenotypes were induced, and then  
377 selected for, or if a larger variety of additional phenotypes was induced and only those  
378 that were selected for were documented.

379 The hypothesis of Pal and Miklos predicts that: (i) environmental changes increase  
380 epigenetic variability, (ii) this increase in epigenetic variability allows for expression  
381 of cryptic phenotypes without losing old phenotypes. Our data support this second  
382 essential point. This increase in phenotypic variability permits to explore the adaptive  
383 landscape at a wide scale and allows for the colonization of new fitness maxima  
384 (figure 2). It was not a goal of our experiments to reach this fitness maximum, but to  
385 show that the mechanism that leads to increased fitness (increased phenotypic  
386 variability) can be based on an epigenetic mechanism. In the fitness landscape defined  
387 in our infection experiment, increasing epigenetic variability and generating novel  
388 phenotypes by TSA treatment decrease the proportion of mean phenotypes having the

389 optimal fitness. This explains why when we modified the epigenetic variability fitness  
390 of the larvae population, their infection success decreased by about 40%. We used the  
391 infection success as a measure for the fitness. One might argue that it is simply a toxic  
392 effect of the TSA that we observed. However, direct measurement of cytotoxicity of  
393 20 $\mu$ M TSA revealed low toxicity of  $\leq 7\%$  after 4h. After 4 h of treatment, no  
394 difference in H3 quantity could be detected. We also observed no difference in  
395 swimming speed of the miracidia. Their velocity (mock-treated:  $1.61 \pm 0.70$ , with TSA:  
396  $1.58 \pm 0.63$  mm/sec) corresponds to earlier published values (1.92 – 2.41 mm/sec  
397 (Mason and Fripp 1976)). If TSA would be highly toxic then we should find stronger  
398 impact on cell survival, protein synthesis and mobility. Our findings correspond well  
399 to a recent study on induction of apoptosis in schistosomules: after 24 h of treatment  
400 with TSA no caspase 3/7 activity or other signs of apoptosis could be detected  
401 (Dubois et al. 2009). Earlier we had shown that 20 $\mu$ M TSA does not influence the  
402 transformation of miracidia into sporocysts while higher concentrations do so (Azzi et  
403 al. 2009). Consequently, we conclude that the major part of the observed effect on  
404 fitness is in fact due to the increased phenotypic variability that produces phenotypes  
405 that are away from the fitness maximum. We detected such change in phenotypic  
406 variability in three arbitrarily chosen phenotypic characters (morphology, mobility  
407 and *SmPoMuc* expression) but it is likely that the same style of change had also  
408 occurred in characters that are directly related to infection success. In our  
409 experimental setting, where  $\sim 100\%$  matching between host and parasite is generally  
410 observed, the appearance of new phenotypes was of course disadvantageous for the  
411 parasites since new phenotypes have a high chance of not matching the host.  
412 However, in the natural environment where the host populations are probably  
413 fluctuating irregularly, phenotypic variability would be a way to assure that always at  
414 least a small proportion of phenotypes is produced that matches to whatever host is  
415 present. This idea is known as “risk-spreading” or “bet-hedging” (reviewed by  
416 Hopper (1999)). Theoretical models support the view that bet-hedging is a way to  
417 cope with fluctuating environments, but that this strategy is also costly for the  
418 population (Kussell and Leibler 2005). This increased cost should somehow be  
419 reflected in the descendants of the population that followed this strategy.  
420 Interestingly, we noticed a decrease in body length of cercariae (supplementary figure  
421 5) that was observed after exposure of the corresponding miracidia to TSA. We  
422 believe that this reduction of body length by about 10% in the cercariae that are

423 descendants of the TSA treated miracidia corresponds to the cost of bet-hedging.  
424 Environmentally-induced bet-hedging with positive feed back (i.e. epigenetic  
425 memory) would present a strategy that combines the advantages of constant  
426 phenotypes with high fitness in stable environments with phenotypic variations in  
427 fluctuating environments. Indeed, such a system was recently described for bacteria  
428 (Veening et al. 2008).

429 One might wonder why no effect of TSA was detectable after one meiotic generation.  
430 Experiments with *S. mansoni* larvae have the advantage that sufficiently large  
431 populations of genetically homogenous individuals can easily be produced and  
432 phenotypic characters can be measured. However, very little is known about  
433 epigenetic mechanisms in *S. mansoni*. Apparently, a reset of epigenetic information  
434 takes place during gametogenesis or early embryonic development. This was  
435 documented for a number of other species (e.g. (Rousseaux et al. 2008)). The  
436 biological reason for this reset is unknown. It was speculated that it serves to erase  
437 errors in the epigenetic information (Holliday 1984). The reset is however often  
438 incomplete and allows for transmission of a bit of information to the next generation.  
439 While it is clear that transgenerational epigenetic effects exist, the mechanism of  
440 transmission is still under discussion (reviewed by Youngson and Whitelaw (2008)).  
441 It is possible that the transgenerational effect of the TSA treatment in our system is  
442 too small to be detected after only one generation. It could also be that the amount of  
443 information that is passed to the next generation is a function of the environmental  
444 conditions. Investigation of such transgenerational epigenetic effects is of tremendous  
445 scientific interest. However, it was not in the focus of our present work. Further  
446 experiments are needed to identify the ways of transmission of epigenetic information  
447 through the germline of *S. mansoni* and its impact on adaptive evolution.

448 Our data provide evidence that in *S. mansoni* phenotypic variability is inducible by  
449 directly modulating the epigenotype of this parasite. However, we do not exclude that  
450 other mechanisms act as well. Increase of phenotypic variability could be the initial  
451 step for adaptive evolution towards a phenotype that is compatible with a new host.  
452 This could have occurred in the past during the recent colonization of the new world  
453 by *S. mansoni* (Morgan et al. 2005). While the resurgence of speciation is at the heart  
454 of many studies in evolutionary biology, the only attempt, to our knowledge, to  
455 anticipate how epigenetic inheritance could influence species diversification is  
456 theoretical (Pal and Miklos 1999). Pal and Miklos suggested that a peak shift could be

457 facilitated by epigenetic inheritance of the ecological trait under disruptive selection.  
458 This is directly relevant to adaptive speciation by host switch. However, host switch is  
459 not the only scenario allowing for parasite species adaptive diversification. Co-  
460 speciation has been documented for many years, and within-host duplication is  
461 currently being more and more widely acknowledged (Perez-Tris et al. 2007). In the  
462 last two cases, disruptive selection gradually appears as a result of host divergence or  
463 as a consequence of ecological interactions within hosts. It is not obvious that an  
464 epigenetically caused phenotypic variability would then favor adaptive diversification  
465 of the parasites. In conditions where disruptive selection arises as a result of  
466 ecological interactions, the evolution of phenotypic variability could actually be an  
467 alternative to (and thus impede) adaptive speciation (Rueffler et al. 2006). Expanding  
468 Pal and Miklos' theory to account for epigenetic inheritance of such plasticity in an  
469 ecological trait involved in co-speciation or within host duplication should  
470 definitively provide valuable theoretical insight contributing to the assessment of the  
471 potential impact of epigenetic mechanisms in the ecology and evolution of parasites.

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