Epigenetic and phenotypic variability in populaitons of Schistosoma mansoni - a possible kick-off for adaptative host/parasite evolution

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Abstract

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

Introduction

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

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

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

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

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

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

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

RT-PCR amplification: Duplicated genes are particularly prone to be under epigenetic control and we decided to use a family of genes that was recently discovered in our laboratory (*Sm*PoMuc, (Roger et al. 2008b)) as markers for gene expression variants. These genes are particularly useful because length polymorphism reflecting different transcripts can easily be detected using nested PCR with two primer pairs hybridizing to conserved regions in the cDNA (Roger et al. 2008a; Roger et al. 2008c). cDNA was PCR amplified using forward primer Exon1F12 GGAAGAATGAACAAGAAAATTATTCTC, reverse primer Exon1R TGACACAGAAAACTGTTAACGATCC and reaction conditions (95°C 1min; 40x 95°C 30 s, 65°C 30 s, 68°C 3 min; 68°C 10 min). In a subsequent nested PCR forward primer NestedExon1F TATNTTGCGCTGATAAG, reverse primer NestedExon15R ATCATAAACAAACACTGAGG and 46°C annealing temperature were used. These primers amplify polymorphic transcripts of the *Sm*PoMuc genes (Roger et al. 2008c). PCR was done with the Advantage® 2 PCR Enzyme System (Clontech). PCR products were separated by electrophoresis in 2% agarose gels, and visualized by staining with ethidiumbromide. Amplicons of different length were counted.

Infection of Biomphalaria glabrata and mice: Infection was performed as described before (Theron et al. 2008) by the same person. In brief, eggs were incubated in 0.9%

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

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

Results

Establishment of 20 µM TSA for 4 h as optimal experimental condition

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

Incubation with TSA changes phenotypic variability of explorative movement

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

Incubation with TSA changes phenotypic variability of morphology

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

Incubation with TSA changes phenotypic variability of gene transcription

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

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

Incubation with TSA influences infection success

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

TSA has an effect on the phenotype through several mitotic generations

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

(n=56), and 194±18 µm for cercariae derived from TSA-treated miracidia (n=50) (supplementary figure 5). Apparently, the effect of TSA is detectable on the morphology after three metamorphosis steps (from miracidia to primary sporocyst, secondary sporocyst and cercaria). Interestingly, body length variance did not change.

TSA has no effect on infection success after one meiotic generation

Finally we wondered whether phenotypic information that influences infection success would be transmitted to the next generation after meiosis. Miracidia were treated with TSA, used to infect snail and cercariae shed from these snails were used to infect 10 mice. After sexual reproduction of *S. mansoni*, eggs were extracted, miracidia were pooled and used to infect snails. In this case, no significant difference in infection success was observed (c_1^2=0.02, p=0.97). The F2 generation of TSA-treated F1 had 97% infection rate (n=30), and F2 of mock-treated F1 had 96% infection rate (n=57).

Discussion

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

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

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

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

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

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

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