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Research article

Title :

Female biased sex-ratio in *Schistosoma mansoni* after exposure to an allopatric intermediate host strain of *Biomphalaria glabrata*

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Abstract

For parasites that require multiple hosts to complete their development, the interaction with the intermediate host may have an impact on parasite transmission and development in the definitive host. The human parasite *Schistosoma mansoni* needs two different hosts to complete its life cycle:

the freshwater snail *Biomphalaria glabrata* (in South America) as intermediate host and a human or rodents as final host. To investigate the influence of the host environment on life history traits in the absence of selection, we performed experimental infections of two *B. glabrata* strains of different geographic origin with the same clonal population of *S. mansoni*. One *B. glabrata* strain is the sympatric host and the other one the allopatric host. We measured prevalence in the snail, the cercarial infectivity, sex-ratio, immunopathology in the final host and microsatellite frequencies of individual larvae in three successive generations.

We show that, even if the parasite population is clonal based on neutral markers, *S. mansoni* keeps the capacity of generating phenotypic plasticity and/or variability for different life history traits when confront to an unusual environment, in this study the intermediate host. The most dramatic change was observed in sex-ratio: in average 1.7 times more female cercariae were produced when the parasite developed in an allopatric intermediate host.

1. Introduction

Parasites with a complex life cycle require sequential hosts to complete their development. Each host represents an environment that can modulate infection success in the subsequent host (Zavodna *et al.* 2008). As proposed in the Red Queen hypothesis and according to local co-evolutionary interactions, parasites are assumed to be adapted to the most common local host phenotypes (*i.e.* sympatric association) (Lively & Dybdahl 2000). Host-parasite sympatric associations would thus result in higher parasite fitness than those in allopatric associations. Parasite fitness is generally measured as prevalence and/or infectivity, which reflects both the capacity of a parasite to develop and the incapacity of the host to resist to the parasite infection.

Schistosomes are parasitic platyhelminthes causing schistosomiasis. Schistosomiasis ranks second only to malaria in terms of parasite-induced human morbidity and mortality : more than 200 million people are infected and 600 million at risk (King 2010). The life cycle of *Schistosoma mansoni* is characterized by passage through two obligatory hosts: the fresh-water snail *Biomphalaria glabrata* (or other *Biomphalaria* species, depending on the geographical location) for the asexual multiplication, and Human or rodents as final host for the sexual reproduction at the adult stage. *S. mansoni* eggs are excreted with the host feces and free-swimming larvae (miracidia) are released when eggs get into contact with water. Eggs of the parasite also accumulate into the liver of the vertebrate host and cause the symptoms of the disease. Miracidia infect the intermediate snail host and transform into primary and secondary sporocysts. Finally, a third larval stage, the cercariae, capable of infecting the Human host, is released into the water.

The impact of allopatric *vs* sympatric associations on host-parasite compatibility has been extensively studied in schistosome-snail interactions. Many authors have observed differences in infectivity when the parasite is exposed to sympatric or allopatric snails (Woolhouse 1989; Webster & Woolhouse 1999; Sandland *et al.* 2009; Jones-Nelson *et al.* 2011). Geographic patterns of compatibility between schistosomes and snails suggest that there is a tendency for sympatric combinations to be more compatible than allopatric combinations (Sandland *et al.*, 2009). This

pattern is in agreement with the patterns reported for other parasite-host systems (Sandland *et al.*, 2009). However, reverse patterns can also occur (Morand *et al.* 1996). Variations in *B. glabrata* susceptibility and *S. mansoni* infectivity from various geographic areas were reported by Files and Cram (Files & Cram 1949), who suggested that these variations were genetically based. Susceptibility is now known to vary between geographic areas, between individuals in the same population, and in the same snail at different ages (Richards 1976). Genetic variations in snail susceptibility and parasite infectivity result in a variety of host-parasite relations (Sullivan & Richards 1981).

It has been shown that the genotype of the snail host influences the parasite infectivity for the vertebrate host and the adult sex-ratio (Zavodna *et al.* 2008). The authors have shown that the worm sex-ratio recovered in experimentally infected mice is male-biased when the parasite passes through a sympatric snail host and is female biased when the parasite passes through an allopatric snail host (Zavodna *et al.* 2008). However, because the sex-ratio was only measured at adult stage it was not possible to detect the origin of this bias. It could have occurred during cercariae development and shedding (*i.e.* inside the snail host) or during development of adult worms (*i.e.* inside the vertebrate host).

In the present study we used a parasite population originated from Brazil on two snail host populations from different geographic locations (Brazil and Guadeloupe). We measured life history traits (prevalence, infectivity and sex-ratio of the parasite) and population genetic structure of the parasite using 14 microsatellites markers. This experiment was, for the first time, maintained for 3 successive generations.

Our results show that *Schistosoma mansoni* can infect the two strains of *Biomphalaria glabrata* but with consequences for parasite life history traits. We show a female biased sex-ratio when the parasite is passes through the allopatric host (1.2 to 2 times higher compared to the sympatric association). This bias is maintained during three consecutive generations. Parasite infectivity and immunopathology of the definitive host stay unchanged for the two conditions and

during the first three generations. Prevalence in the snail host is lower in the allopatric association than in sympatric association. Genetic analyses show very few diversity based on 14 neutral markers suggesting that sex-ratio bias is not due to selection of rare sex-distorter alleles but based on the capacity to generate phenotypic plasticity and/or variability.

2. Material and Methods

2.1 Parasite and host strains

Two *Biomphalaria glabrata* snail populations, a Brazilian sympatric strain (*SmBre*), and a Guadeloupien allopatric strain (*BgGUA*) were infected using the same *Schistosoma mansoni* (*SmBre*) parasite strain (Figure 1).

2.2 Experimental scheme and measured parameters

SmBre was used to infect for three successive generations either a sympatric snail population *BgBRE* or an allopatric snail population *BgGUA*. The vertebrate host (Swiss OF1 mouse) was the same for the two lineages. For the first three generations we measured (i) prevalence (the number of infected snails divided by the total number of exposed snails), (ii) cercarial infectivity (the number of developing worms divided by the number of cercariae used for mouse infection), (iii) the host immunopathology assessed by the relative spleen weight (mouse spleen weight divided by body weight), (iv) the sex-ratio at each parasite stage: miracidia, cercariae and adult and (v) the genetic population structure (using 14 microsatellite markers) at miracidia and cercarial stages.

2.2.1 Snail infections and maintenance

SmBre eggs were axenically recovered from 60-day infected hamster (*Mesocricetus auratus*) livers and miracidia were hatched from eggs in 5 ml of spring water during 2-3 hours under light. For infection, 100 to 200 snails of each strain (*BgBRE* and *BgGUA*), 4-5 mm in diameter, were individually exposed to ten arbitrarily chosen *SmBre* miracidia of a mixed male/female population in 5 ml of springwater. To determine the mean sex ratio in this miracidia population used for infection, 24-30 individuals were arbitrarily sampled and sex was determined by PCR (see below).

Individual snails were then isolated and maintained in round, clear plastic containers for 24 hr. On the next day, the exposed *B. glabrata* were divided into two groups: the sympatric association (*SmBre/BgBRE*) and the allopatric association (*SmBre/BgGUA*) and maintained in a single water tank for each association. Snails were fed with fresh lettuce *ad libitum* and maintained at 25°C. Water was changed weekly. Photoperiod during the entire experiment was equilibrated to 12L:12D (Boissier *et al.* 2003). The prevalence was calculated, 35 days post-infection, by dividing the number of snails that presented sporocysts by the total number of snails.

2.2.2 Mice infections

At least 25 infected snails of the sympatric association (*SmBre/BgBRE*) and infected snails of the allopatric association (*SmBre/BgGUA*) were randomly chosen, pooled by sympatric or allopatric association and exposed to light to produce cercariae. These cercaria were collected to infest mice. Ten mice were anesthetized with a mixture of Rompun (20 mg/ml; Bayer) 0.50 ml and Imalgène (100 mg/ml; Rhône Mérieux) 1.00 ml in 8.5 ml of autoclaved NaCl 8.5 (%0) by injection of 0.1 ml/10g of body weight. Mice abdomens were shaved and exposed to 160 randomly selected cercariae for 1 hour (Boissier & Moné 2000)

After 60 days, mice were killed by a lethal intraperitoneal injection of sodium pentobarbital. Adults worms were recovered by retrograde perfusions of the hepatic portal system with citrate (7.5%) saline (8.5%) solution administrated through the left ventricle (Duvall & DeWitt 1967). Worms trapped in the liver or mesenteric system were collected after excising these organs. Sex of worms was determined by visual inspection based on morphology. The French Ministère de l'Agriculture et de la Pêche and French Ministère de l'Education Nationale de la Recherche et de la Technologie provided permit C66-136-01 to our laboratory for experiments on animals and certificate for animal experimentation (decree 2012-201-0008) for the experimenters. Housing, breeding and animal care followed the national ethical standards.

2.3 Sex identification and genotyping of larvae

Before DNA extraction, individual miracidia or cercariae were vacuum-dried for 15 min in a Speedvac evaporator. Next, 20 µl of NaOH (250 mM) was added to each tube. After a 15 min incubation period at 25°C, the tubes were heated at 99°C for 2 min. Then, 10 µl HCl (250 mM), 5 µl of Tris-HCl (500 mM) and 5 µl Triton X-100 (2%) were added and a second heat shock at 99°C for 2 min was performed (Beltran *et al.* 2008). The extracts were stored at -20°C.

2.3.1 Sex identification

Cercariae and miracidia were randomly samples from 84 to 143 snails and 6 to 9 mice livers, respectively. Polymerase Chain Reaction (PCR) amplifications were performed with female specific markers (scaff_002739) and housekeeping gene (rhodopsin 3) in multiplex on 24 to 32 cercariae and miracidia for the sympatric and the allopatric association on the 3 generations. The method was described before (Portela *et al.* 2010). Oligonucleotide primers were diluted to 0.1 µM for each female specific oligonucleotide primer and 0.2 µM for each housekeeping gene oligonucleotide primer (Table 1). The PCR reactions were carried out in a total volume of 10 µl containing 5 µl of 2x QIAGEN Multiplex PCR Master Mix (cat.nr. #206143) (providing a final concentration of 3 mM MgCl₂), 1 µl mix of primers previously diluted (providing a final concentration of 0.1 µM for each female specific oligonucleotide primer and 0.2 µM for each housekeeping gene oligonucleotide primer), 1 µl of diluted DNA (10⁻¹) and DNase-free water q.s.p. 10 µl. The PCR programme consisted in an initial denaturation phase at 95°C for 15 min, followed by 40 cycles at 94°C for 30 s, 57 °C for 90 s, 72°C for 60 s, and a final extension at 60°C for 30 min. The PCR products were separated by electrophoresis through a 2% TBE agarose gel. Presence of two bands indicate a female and presence of a single band indicate a male. The female ratio is calculated as number of females divided by the total number of studied individuals (females/(males + females)). Bias is calculated as female ratio in the infecting miracidia to female ratio in cercaria or adults produced from these miracidia.

2.3.2 Genotyping

All samples were genotyped using PCR spanning 14 microsatellite markers (Table 2). PCRs were performed in three multiplexes (Table 2) using the QIAGEN multiplex kit and a fluorescent labeled and a second non-labeled primer. Reactions were carried out according to the manufacturer's standard microsatellite amplification protocol in a final volume of 10 µl and with 57°C annealing temperature. The PCR programme consisted in an initial denaturation phase at 95°C for 15 min, followed by 40 cycles at 94°C for 30 s, 57 °C for 90 s, 72°C for 60 s, and a final extension at 60°C for 30 min. PCR products were diluted in Sample Loading Solution (Beckman Coulter) with red-labeled size standard (CEQTM DNA size standard kit, 400, Beckman Coulter, Fullerton, CA, USA) and size separated by electrophoresis on an automatic sequencer (CEQTM 8000, Beckman Coulter). Genotypes were determined using the fragment analyzer package from Beckman Coulter. (Bech *et al.* 2010)

2.4 Statistical and population genetics Analyses

Statistical analyses were performed using SPSS 18.0 (IBM). Fisher exact test was used to compare prevalences. The cercarial infectivity and relative spleen weight were compared using non-parametric Kruskal-Wallis or Mann-Whitney tests. Population genetic analyses (Hardy Weinberg equilibrium, Fst, genetic diversity and allelic richness) were calculated using Fstat 2.9.

3. Results

3.1 Prevalence is lower in the allopatric snail host

Prevalences in the sympatric (*SmBre/BgBRE*) and the allopatric association (*SmBre/BgGUA*) are shown in Figure 2. There are significant differences between the sympatric and the allopatric combination with a higher prevalence in the sympatric than in the allopatric condition. These differences were found for the three generations G (G1 : p = 0.04; G2: p = 0.0176; G3: p = 0.0021). Within each association, whatever sympatric or allopatric, the prevalence stays unchanged between the three generations at 95±1% in *SmBre/BgBRE* and 86±2% in *SmBre/BgGUA* (p>0.05, n=100 to 200).

3.2 *Cercaria sex-ratio becomes female-biased in the allopatric snail host*

The initial miracidia sex-ratio is slightly male biased (42% female in generation G1). Sympatric or allopatric snails were infected using this male biased miracidia population. Cercariae emitted by the sympatric snail have a sex-ratio around the equilibrium (50±6% females). The sex-ratio becomes male biased when penetrating into the definitive host (adult sex-ratio : 44±7% females). This pattern is the same for the three generations. In contrast, cercariae emitted by the allopatric snail have a strong female biased sex-ratio (66±14% females). This bias is also found at slightly lower level at the adult stage (61±6% female). The pattern is the same for the three generations (Table 3). Female bias in cercaria and adults is around 1 (*i.e.* no bias) in the sympatric interaction, and between 1.2 and 2 in the allopatric interaction (Table 3).

3.3 *No differences in genetic diversity between allopatric or sympatric condition*

Population genetic structure was measured at miracidia and cercarial stages using 14 microsatellites markers. No statistical difference has been observed between all F_{st} values whatever the host-parasite combination (allopatric *SmBre/BgBRE* or sympatric *SmBre/BgGUA*) or the generation considered (mean value of F_{st} : 1.1%). Heterozygosity and allelic richness were very low for both sympatric and allopatric associations (8.1% and 1.94, for heterozygosity and allelic richness respectively). For these two parameters, no statistical difference was detected for any host-parasite combination or generation ($p>0.05$).

3.4 *No differences in infectivity of cercariae or inflammatory response of the definitive host*

The cercarial infectivity and the inflammatory reaction was measured as the relative spleen weight. There was no difference between the host parasite combinations and/or generations ($p>0.05$).

4. Discussion

Our results show that when *S. mansoni* parasite is confronted to an allopatric snail host some life history parameters stay unchanged (cercarial infectivity and vertebrate host immunopathology) but others are modified. We show that in the allopatric condition compared to the sympatric one the prevalence in the intermediate host is reduced and the sex-ratio becomes female biased in cercariae and adults.

Prevalences show a significant decrease when the parasite is exposed to an allopatric intermediate host. The lower prevalence in the allopatric association is the same during 3 successive generations. This confirms earlier results that showed compatibility polymorphism between parasite and snail strains (Files & Cram, 1949, Morand *et al.*, 1996 (Richards & Shade 1987; Théron *et al.* 1997; Sandland *et al.* 2007; Roger *et al.* 2008; Sandland *et al.* 2009)

Our experiments show a significant change in sex-ratio when the parasite develops in an allopatric intermediate host compared to a sympatric one. Results are schematically represented in figure 3. In all three consecutive generations, there is a 1.7 ± 0.5 fold female enrichment in cercarial population and subsequently in the adult worm population in the allopatric association, whereas the sex-ratio stays male biased in the sympatric association. Schistosome sex-ratio has puzzled parasitologists for several years and factors determining its variations are still unclear (Beltran & Boissier 2009). The tertiary *S. mansoni* sex-ratio (male/female ratio of adults at maturity) is known to be male-biased in natural vertebrate host infrapopulations (Boissier & Moné 2001). The origin of such bias has been studied using both experimental and analytical approaches (see Beltran & Boissier, 2009 for synthesis). It is now established that the bias is initiated in the vertebrate host during the parasite development from cercariae to adult worms (Boissier *et al.* 2004) - the thin female being less able to resist to the blood flow than the muscled male. However, in the present study we show that the sex-ratio could change before penetration of the cercariae (*i.e.* secondary sex-ratio) and the resulting tertiary sex-ratio is a consequence of sex-ratio bias in the snail host. A modification in environmental conditions of the snail-parasite system is known to induce such sex-ratio distortion.

It has been shown that the presence of a non-vector snail (*Marisa cornuarietis*) in parallel with the vector snail induces a male biased sex-ratio. In this former study, the author showed that the presence of the non-vector snails stimulated the growth of sympatric vector snail and thus would increase the space available for the parasite (Moné 1997). In this environment (*i.e.* bigger snails) male parasites seem to benefit more than female parasite. In contrast, as we show in the current study, female parasites seem to have a selective advantage in a stressful environment such as an allopatric hosts. In host-parasite sympatric associations the sex-ratio is male-biased and becomes female biased in allopatric environment.

In the present work we did not address the question of the underlying mechanisms and the reasons behind the skewed sex ratio. It seems unlikely that the observed phenomenon is particular to our laboratory strains since a similar result, hence only for a single generation, was observed by Zavodna *et al.* 2008. We also show that, at least based on neutral markers, our parasite population is genetically homogeneous and our data suggest that no rare alleles are selected in the allopatric condition. However, the current low resolution study cannot formally rule out that selection of genetic sex-ratio distorters occurs.

We proposed earlier that male and female schistosomes are different in their life history performances and strategies to exploit their hosts (Boissier *et al.* 1999). In non-stressing conditions, male parasite would be more efficient than female, the opposite would occur in stressful environments. The globally lower prevalence in allopatric associations compared to sympatric ones indicates that the allopatric environment is indeed perceived as a stress by the parasite. Skewed sex ratios towards females under stressful environments is not particular to *S. mansoni*. In the 1960th Hamilton hypothesized that competition between siblings due to restrictive movement can lead to local mate competition (LMC) and predicted that females should produce a more female-biased sex ratio under conditions in which inbreeding increases and her sons compete with each other for mates (Hamilton 1967). There is a large empirical body of evidence for this theory, and recently the impact of LMC intensity on female-biased sex ratio was demonstrated in experimental evolution

of spiders (Macke *et al.* 2011). In a similar type of experimental evolution, it had been shown earlier that without LMC sex ratio evolves from female-biased towards the 1:1 (“Fisherian”) ratio (Carvalho *et al.* 1998). Trivers & Willard (Trivers and Willard, 1973) extended the Hamilton theory and proposed that in species with parental investment (PI), sex-ratio in offspring can change as function of parental ability to invest, *i.e.* sex-ratio could be distorted by environmental conditions. Female biasing distorters, apart from those due to infective agents, are for instance small effective population size (Pap *et al.* 2013; Teboh-Ewungkem and Wang, 2012; Al-Jahdali, 2012; Rishniw *et al.* 2012), socio-economic status of the mother (Chipman and Morrison, 2013), resource limitation in the host in which a parasite develops (Therese and Bashey, 2012), variation in fecundity (Teboh-Ewungkem and Wang, 2012), and competition of parasites in their hosts (Tormos *et al.* 2012; Boissier and Mone, 2001). *S. mansoni* cercaria multiply clonally in the snail, they are short living and as a consequence their dispersal is limited. Mating system of *S. mansoni* is known to be socially monogamous, but we have shown earlier that mate change occurs and offspring are produced, supporting the fact that schistosomes are not genetically monogamous (Beltran *et al.* 2008), *i.e.* mate competition could indeed be an issue. The intensity of mate competition between siblings in the adult stage will be higher following allopatric association when only a few miracidia succeed in snail infection. Nevertheless, adaptive significance of a female bias in Schistosomes remains difficult to be addressed. As outlined above, under field conditions adult schistosome sex ratio is male biased. We think that such bias is not adaptive and just occurs as a consequence of the sexual dimorphism between male and female (Beltran and Boissier, 2009). Females are thinner and they have a higher mortality rate than males, but their size is constrained by oviposition in small venules, thus preventing return to an equilibrated sex ratio (Beltran and Boissier, 2009). Increasing the number of female cercariae in the mollusc host might compensate for this up to a certain limit. However, more female than male (or the reverse) for a monogamous species is not optimal. Equilibrated sex ratio remains the better option. In addition, we have shown that in a female biased

sex-ratio situation mate-changes are unusual (Beltran *et al.* 2008b; Beltran and Boissier 2009b), making a LMC unlikely to be the only reason for the observed female bias.

One can only hypothesize about the mechanisms that lead to female bias. Our data show that female miracidia are more efficient in penetrating into, and/or developing in allopatric snail hosts. Monomiracidial infections could be done to elucidate the mechanism. But independent of the precise process that leads to more female than male cercariae it is clear that either females have a higher phenotypic plasticity compared to male miracidia/sporocysts, or the female population shows higher phenotypic variability.

The parasites sex is determined genetically with male individuals being homogametic (ZZ) whereas females are heterogametic (ZW). The reason for higher phenotypic plasticity/variability in females could be the presence of W-specific sequences. We and others have shown that sex chromosomes are almost entirely pseudoautosomal and no genes could be identified in the small W-specific part (Lepessant *et al.* 2012; Protasio *et al.* 2012). While there are probably no female-specific genes on the W chromosome, we found large blocks of W-specific satellite-type repeats. Chromatin structure changes occur around these repeats during development and we proposed that these chromatin structure changes could act in cis or trans and change the expression of genes, eventually leading to sex differentiation (Lepessant *et al.* 2012). A similar mechanism could be the basis for higher phenotypic plasticity/variability of females. This hypothesis could be tested if phenotypic markers for infection success in sporocysts were available, which is unfortunately not the case so far. If the phenomenon were indeed chromatin-structure based one would however also expect to see a footprint of the allopatric snail environment in the chromatin-structure of the resulting sporocysts or even cercariae. This hypothesis will be tested in the future.

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