AN ABSTRACT OF THE DISSERTATION OF

Kaitlin M. Bonner for the degree of Doctor of Philosophy in Zoology presented on September 6, 2013.
Title: Genetic and Phenotypic Variation Associated with the Resistance Gene SOD1 in *Biomphalaria glabrata*, an Important Intermediate Host of Schistosomiasis

Abstract approved:

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Michael S. Blouin

Schistosomiasis afflicts 200 million people and is responsible for 200,000 deaths per year. The infection is caused by a digenean trematode in the genus Schistosoma. The parasite must cycle through both a vertebrate (human) and invertebrate (snail) host to complete the life cycle. My dissertation focuses on the genetic mechanisms of resistance in the snail intermediate host *Biomphalaria glabrata* to the parasite *Schistosoma mansoni.* Understanding the underlying mechanisms controlling resistance in the intermediate host is essential for elucidating transmission dynamics and the development of appropriate biological control techniques. Much of what we know about the resistance of the intermediate host has been determined using established laboratory strains of both the snail and trematode species.

The *B. glabrata/S. mansoni* system serves as a model system for studying schistosomiasis. Resistance to parasite infection in the snail is highly heritable. While many genes have been implicated, allelic variation correlating with resistance has only been observed at a single gene, copper-zinc superoxide dismutase (SOD1). SOD1 is

functionally relevant because it catalyzes the conversion of the highly reactive superoxide into the less reactive hydrogen peroxide and water. In the snail, hydrogen peroxide is released as part of the immune response to the invading parasite.

The goal of my dissertation was to further evaluate SOD1 as a resistance locus. In the second chapter I examined allelic variation at SOD1 and looked for evidence of selection on SOD1 in natural populations of *B. glabrata*. To determine the utility of SOD1 as a resistance locus in natural populations, we needed to first determine if there was indeed allelic variation in natural population of *B. glabrata.* This is important because if the alleles do not co-occur in natural populations, then the relative selective advantage of one allele over another in terms of resistance would be of little ecological relevance in natural transmission zones. Variation at SOD1 in natural populations cannot be assumed because the initial study describing the correlation between resistance to infection and allelic variation used a hybrid laboratory strain of *B. glabrata* (13-16-R1). Therefore, we did not know if the co-occurrence of particular alleles at SOD1 in the 13-16-R1 lab strain was a natural phenomenon or is an artifact of the breeding history of the strain. In this study we were able to identify the likely geographic origins of the alleles in the lab strain 13-16-R1 and determine that different alleles do co-occur in some natural populations. Additionally, we found heterozygote excess at SOD1 in all the populations segregating for the allele that confers highest resistance in 13-16-R1. This result raises the possibility that overdominance is acting at SOD1 or some locus closely linked to it.

My third chapter evaluated the effects of genetic background on the ability of allelic variation at SOD1 to predict resistance and potential fitness costs associated with carrying the most resistant allele of SOD1. We found no cost of carrying the most resistant allele in terms of fecundity, and a possible advantage in terms of growth and mortality. These results suggest that it might be possible to drive resistant alleles of SOD1 into natural populations of the snail vector for the purpose of controlling transmission of *S. mansoni*. However, we also observed a strong effect of genetic background on the association between SOD1 genotype and resistance. Thus, epistatic interactions with other loci may be as important a consideration as costs of resistance in the use of SOD1 for vector manipulation.

Genetic and Phenotypic Variation Associated with the Resistance Gene SOD1 in *Biomphalaria glabrata*, an Important Intermediate Host of Schistosomiasis

by
Kaitlin M. Bonner

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Major Professor, representing Zoology

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Chair of the Department of Zoology

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Kaitlin M. Bonner, Author

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CONTRIBUTIONS OF AUTHORS

Chapter 2: Michael S. Blouin was involved in experiment conception and design, contributed reagents/materials/analysis tools, and helped with writing the final manuscript. Elizabeth A. Thiele provided tissue samples and microsatellite analysis for some populations in Brazil. Guilherme Oliveira, André Théron, Philippe Jarne, and Rodrigo R. Miranda provided tissue samples and DNA samples from natural populations.

Chapter 3: Michael S. Blouin was involved in experiment conception and design, contributed reagents/materials/analysis tools, and helped with writing the final manuscript. Christopher J. Bayne and Maureen K. Larson contributed reagents/materials/analysis tools.

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CHAPTER 1

INTRODUCTION

Vector-borne diseases account for approximately one-sixth of the global human disease burden (Townson et al. 2005, LaBeaud and Aksoy 2010). Schistosomiasis is the most important vector-borne water-based disease (Steinmann et al. 2006). Morbidity caused by schistosomiasis rivals malaria, in terms of disability adjusted life years lost to the illness (Hotez and Pecoul 2010, King 2010). It occurs in more than 70 countries across the tropics with nearly 800 million people at-risk and 200 million people suffering from chronic and acute infections. Globally, it causes approximately 200,000 deaths per year. (Crompton 1999, Ross 2002, Chitsulo 2004, Steinmann et al 2006) Schistosomiasis is caused by a digenean trematode in the genus Schistosoma, and the primary causative species include *S. mansoni*, *S. haemotobium*, and *S. japonicum* (Figure 1.1).

Currently, the most successful methods of transmission manipulation include recurrent use of both the chemotherapy drug praziquantel to treat active infections in humans and the application of mass heavy metal molluscicides (e.g. niclosamide) to remove intermediate host populations from standing water (Fenwick and Webster 2006, Kariuki et al. 2013). However, drug resistance to praziquantel has become a concern, and recurrent molluscicide treatments result in habitat degradation (Melman *et al.* 2009, Fenwick et al 2009). Thus, finding alternative means of transmission disruption are becoming increasingly important for eliminating schistosomiasis,

including driving resistance genes into susceptible vector populations (Marrelli et al. 2006, Marrelli et al. 2007, Coelho et al. 2008, Lambrechts et al. 2008, Cohuet et al. 2010, Wise de Valdez et al. 2011). Therefore, Identifying the mechanisms by which a snail is resistant or susceptible to infection by the pathogen can aid our ability to alter the transmission dynamics of schistosomiasis through disrupting the parasitic infection in the snail (see reviews Lockyer et al. 2004, Bayne 2009, Loker 2011, Mitta et al. 2012).

The overarching goal of my dissertation is to further investigate a previously identified resistance locus identified in *Biomphalaria glabrata*, a snail vector of the human pathogen *Schistosoma mansoni*. *B. glabrata* is a facultative, hermaphroditic freshwater pulmonate snail that occurs throughout much of the New World tropics (DeJong et al. 2001, Morgan et al. 2001, Mavarez et al. 2002). The *B. glabrata/S. mansoni* system is a well-established model for investigating host-parasite interactions in a controlled laboratory setting (Bayne 2009).

Resistance to *S. mansoni* infection in *B. glabrata* is highly heritable in many lab and field populations, and is almost certainly controlled by multiple loci (Richards and Merritt 1972, Shoukry 1997, Webster and Woolhouse 1999, Webster and Davies 2001, Theron et al. 2008, Zavodna et al. 2008). While a number of recent studies have demonstrated that expression patterns of known immune-relevant genes differ between resistant and susceptible snails (Lockyer et al. 2007, Hanelt et al. 2008, Roger et al. 2008, Ittprasert et al. 2009, Adema et al. 2010, Hannington et al. 2010, Ittiprasert et al. 2010, Lockyer et al. 2012, Ittiprasert et al 2013), only a handful of genes have

been identified as candidate resistance loci. The only known association between allelic variation at an immune-relevant locus and resistance to infection has been described for a single locus, copper-zinc superoxide dismutase. (SOD1: Goodall et al 2006, Bonner et al. 2012).

SOD1 is a promising candidate locus for use as both a resistance marker to evaluate host-parasite interactions and for driving resistance alleles into susceptible natural populations of snails. Among its many important cellular functions, it is also involved in cell signaling and immune response (Nappi and Ottaviani 2000, Fink and Scandalios 2002, Ramasarma 2007, Abreu and Cabelli 2010). It is immunologically relevant to this study system because SOD1 catalyzes the reduction of highly reactive superoxide (O_2^-) to hydrogen peroxide (H_2O_2). Hydrogen peroxide is a known cytotoxic component of the oxidative burst, which is the primary defense mechanism for parasite clearance in molluscs (Hahn et al. 2001, Bayne 2009, Loker 2010). Upon recognition of a parasite invasion, snail hemocytes surround the parasite and are thought to generate H_2O_2 as part of the killing mechanism (Hahn et al. 2001, Bender et al. 2005, Bayne 2009). Increased H_2O_2 production during the oxidative burst is correlated with resistance (Bender et al. 2005, Bayne 2009). Allelic variation at the fourth intron of SOD1 is correlated with resistance to infection (Goodall et al. 2006). Additionally, the ability to produce reactive oxygen species in some strains of *B. glabrata* is correlated with the anti-oxidant defenses of their respective compatible *S. mansoni* strains (Mone et al. 2011). Thus, loci involved in the oxidative burst, such as SOD1, may be very important in the evolution of schistosome-snail interactions.

In my dissertation I further investigate SOD1 as a resistance locus. Initially, the discovery of the association between allelic variation at SOD1 and resistance to parasite infection was completed in a laboratory setting using laboratory strains of *B. glabrata* (strain 13-16-R1) and *S. mansoni* (strain PR-1). The second chapter of my dissertation is a geographic survey of allelic variation at SOD1 in natural populations of *B. glabrata* in the New World Tropics. In this chapter I examine the ecological relevance of the allelic variation at SOD1 observed in the laboratory population. Because previous work was completed using a laboratory population created by crossing Brazilian and Caribbean snails we want to determine the geographic origins of the SOD1 alleles that are segregating in our laboratory population (Newton 1955, Richards and Merritt 1972, DeJong et al 2003). In particular, if the alleles we are studying never co-occur in natural populations, then any fitness variation associated with those alleles has little bearing on their evolution in nature (although they are still interesting for applied work in which one might want to manipulate vector populations). In this chapter I also present an analysis of potential balancing selection at SOD1. This work was motivated by unexpected observations of positive Tajima's D and an excess of heterozygotes at SOD1 in several samples from Brazil. Both of these patterns are consistent with balancing selection, although there are alternate explanations. I test whether those patterns are unique to SOD1 or are found genome-wide in those populations, which would point to alternate explanations. In the third chapter I examine the effects of genetic background on the association between allelic variation at SOD1 and the resistance to *S. mansoni* infection, as well as look for

evidence of a fitness cost associated with allelic variation at SOD1 in a laboratory setting. For a resistant locus to be a good candidate for use in vector mediation there must not be a fitness cost and the resistance conferred must be predictable such that it confers resistance regardless of genetic background or environment. The results of my dissertation contribute to our understanding of genetic determination of resistance to infection in *B. glabrata*, the vector for the human disease schistosomiasis. Vector control is important in mitigating schistosomiasis and this study lends insight into the utility of a previously identified resistance locus for vector mediation.

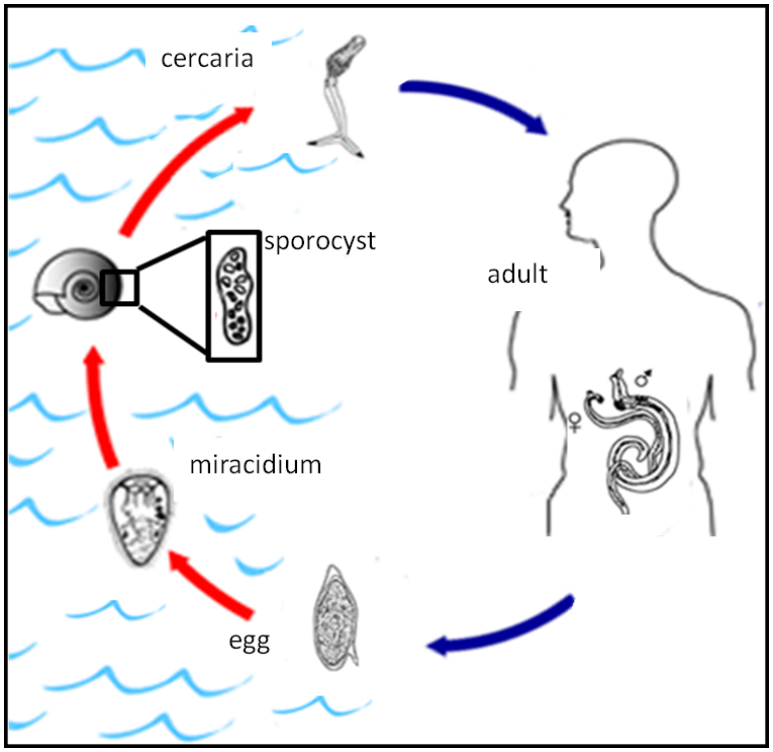

Figure 1.1

The life-cycle of *Schistosoma mansoni*, the causative agent of schistosomiasis in the New World Tropics. *S. mansoni* eggs are eliminated from the definitive host into freshwater. The eggs hatch and develop into the first larval stage, miricidia. The miricidium penetrates the headfoot of the snail and develops into a mother sporocyst, undergoing clonal reproduction. Daughter sporocysts migrate to the digestive and gonadal tissue of the snail for further clonal reproduction and development. Approximately 5 weeks post-penetration, the parasite emerges as the second larval stage, cercariae, from the snail directly through the headfoot. Shedding of the parasite out of the snail host can continue for months post penetration. Infection by a single miricidium can result in the release of tens of thousands of cercariae from a single snail. Cercariae directly abrade through the skin of the definitive host, humans. Within the human, the parasite first travels via the bloodstream to the lungs and eventually to the superior mesenteric veins, maturing 4 to 6 weeks post infection. Mated *S. mansoni* females lay eggs within the mesenteric veins, the eggs pass through the intestinal wall, and are eliminated in fecal matter. However, the eggs also become imbedded in the liver via the hepatic portal system. Much of the pathology and morbidity associated with schistosomiasis caused by *S. mansoni* occurs as a result of the aggregation of eggs in the liver due to the inflammatory immune response forming granulomas surrounding the eggs. The adult parasites can live in the host for up to 30 years, with an average of 3-5 years. This figure is adapted from CDC.

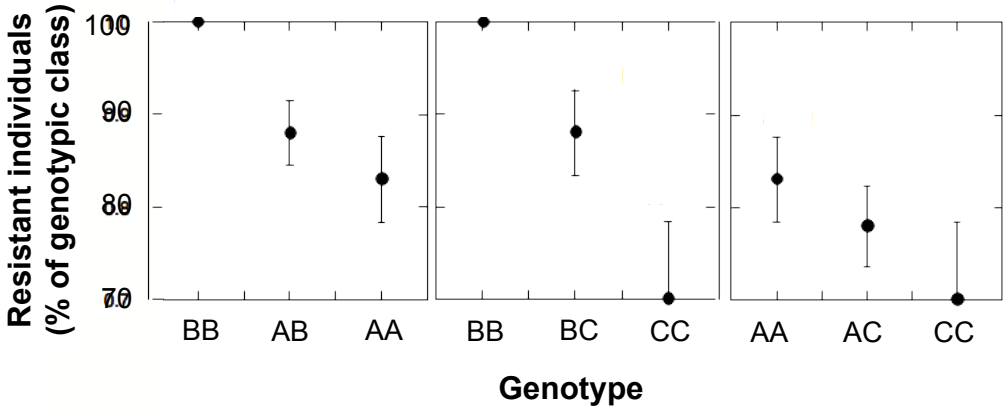

Figure 1.2

The association between allelic variation at SOD1 and resistance in the laboratory strain 13-16-R1 of *B. glabrata.* Allelic variation within the fourth intron of SOD1 in *B. glabrata* strain 13-16-R1 is associated with resistance to infection by *S. mansoni* strain PR-1, such that the B allele confers resistance and the C allele is associated with susceptibility. The three allele classification, A, B, and C, is determined by diagnostic polymorphisms in the fourth intron. Adapted from Goodall *et al.*, 2006.

ALLELIC VARIATION AND SIGNATURES OF SELECTION AT CU/ZN SUPEROXIDE DISMUTASE (SOD1) IN NATURAL POPULATIONS OF *BIOMPHALARIA GLABRATA*, AN IMPORTANT INTERMEDIATE HOST OF SCHISTOSOMIASIS.

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Infection, Genetics, and Evolution

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CHAPTER 2

ALLELIC VARIATION AND SIGNATURES OF SELECTION AT CU/ZN SUPEROXIDE DISMUTASE (SOD1) IN NATURAL POPULATIONS OF *BIOMPHALARIA GLABRATA*, AN IMPORTANT INTERMEDIATE HOST OF SCHISTOSOMIASIS.

2. 1 Abstract

Allelic variation at the fourth intron of SOD1 in *B. glabrata* (13-16-R1) correlates with resistance to *S. mansoni* (PR-1) infection. Due to the hybrid nature of the strain it is unknown if the co-occurrence of particular alleles in the 13-16-R1 lab strain is a natural phenomenon or is an artifact of the breeding history of the strain. This is important because if the alleles do not co-occur in natural populations, then the relative selective advantage of one allele over another in terms of resistance would be of little ecological relevance in natural transmission zones. We surveyed fourteen natural populations of *B. glabrata* for allelic variation at the fourth intron of SOD1. We identified sequence variants in natural populations that were similar to all four of the 13-16-R1 alleles. Furthermore, the variation observed in the laboratory strain reflects the complete range of sequence variation observed in natural populations. The alleles do co-occur in natural populations in Brazil. Additionally, we found heterozygote excess at SOD1 in all the populations segregating for the allele that confers highest resistance in 13-16-R1. This heterozygote excess was higher at SOD1 than at nine other, putatively neutral loci examined in the same populations. This

result raises the possibility that overdominance is acting at SOD1 or some locus closely linked to it. This hypothesis warrants further study.

2.2 Introduction

Schistosomiasis afflicts 200 million people in 74 countries across the tropics, and is responsible for 200,000 deaths per year (Crompton 1999, Chitsulo et al. 2000, Ross et al. 2002). The infection is caused by a digenean trematode in the genus *Schistosoma*. Prior to developing into the larval stage capable of infecting humans, the parasite must pass through the intermediate host, a snail (*Biomphalaria* sp., *Bulinus* sp., or *Oncomelania* sp.). Identifying the mechanisms by which the snail is resistant or susceptible to infection by the pathogen can aid our ability to alter the transmission dynamics of schistosomiasis through disrupting the parasitic infection in the snail (see reviews in Lockyer et al. 2004, Théron and Coustau 2005, Bayne 2009, Loker 2010, Moné et al. 2011, Mitta et al. 2012). The *Biomphalaria glabrata/Schistosoma mansoni* system has become the model for studying schistosomiasis. However, work on the genetics of resistance by *B. glabrata* to *S. mansoni* has been limited to laboratory populations. Establishing the utility and relevance of research on model organisms in controlled laboratory environments to natural systems is necessary to assess the translatability of laboratory research to the natural world (Boete 2005, Lambrechts et al. 2007, Steinauer and Bonner 2012).

In many laboratory and field populations of *B. glabrata* resistance to infection by *S. mansoni* is highly heritable (Richards and Merritt 1972, Shoukry 1997, Webster and Woolhouse 1999, Webster and Davies 2001, Théron et al. 2008, Zavodna et al. 2008). While a number of recent studies have demonstrated that expression patterns of known immune-relevant genes differ between resistant and susceptible snails either constitutively or in response to a pathogen challenge (Lockyer et al. 2007a, Lockyer et al. 2007b, Hanelt et al. 2008, Roger et al. 2008, Ittiprasert et al. 2009, Adema et al. 2010, Hanington et al. 2010, Ittiprasert et al. 2010, Lockyer et al. 2012, Ittiprasert et al. 2013), only a handful of genes have been identified as candidate resistance genes (FREPs (fibrinogen-related proteins): (Zhang and Loker 2003, Adema et al. 2010, Hanington and Zhang 2011), BgMIF (macrophage migration inhibitory factor): (Garcia et al. 2010), BgTEP (thioester-containing protein): (Moné et al. 2010), HSP70 and HSP90: (Ittiprasert and Knight 2012), and Biomphalysin: (Galinier et al. 2013)). To date, the only known association between *allelic* variation at an immune-relevant locus and resistance to infection has been described for a single locus, copper zinc superoxide dismutase (SOD1: (Goodall et al. 2004, Bonner et al. 2012)).

Among the many important cellular functions of SOD1, it is responsible for the production of hydrogen peroxide by mediating the reduction of superoxide to hydrogen peroxide (Nappi and Ottaviani 2000, Fink and Scandalios 2002, Ramasarma 2007, Abreu and Cabelli 2010). Hydrogen peroxide (H_2O_2) is a major component of the cytotoxic agents released during the respiratory (oxidative) burst, a known mechanism by which molluscs clear parasite infections (Bayne et al. 2001, Hahn et al.

2001, Bender et al. 2005, Bayne 2009, Loker 2010). *B. glabrata* strains differ in ability to produce H_2O_2, and increased production of H_2O_2 is associated with resistance to parasite infection in *B. glabrata* (Hahn et al. 2001, Bender et al. 2005, Moné et al. 2011).

Goodall et al. (2006) determined that there was an association between allelic variation at intron four of copper zinc superoxide dismutase (SOD1) and resistance to *S. mansoni* in *B. glabrata* laboratory strain 13-16-R1. Three major sequence variants (*A, B,* and *C* alleles), as well as a rare variant (allele *D* at ~1% allele frequency), were identified in the *B. glabrata* laboratory strain 13-16-R1. In this laboratory population there is a strong association between resistance to infection and snails that carry the *B* allele at SOD1 (Goodall et al. 2006, Bonner et al. 2012, Blouin et al. 2013). Additionally, relative overexpression of SOD1 is associated with the *B* allele at SOD1 compared to the *A* and *C* alleles present in the 13-16-R1 population (Bender et al. 2007). Interestingly, there appears to be no fitness cost (i.e. growth, fecundity, or mortality) to carrying the *B* allele relative to the *C* allele when maintained in standard laboratory conditions (Bonner et al. 2012). These intriguing results have only been demonstrated in the 13-16-R1 strain.

The 13-16-R1 *B. glabrata* strain has a hybrid history, such that it was created through two different breeding events using snails from three different populations, one Brazilian in origin and two different Puerto Rican populations (Newton 1955, Richards and Merritt 1972, DeJong et al. 2003). As a result, the geographic origins of each of the three major alleles (as identified by SNPs in the fourth intron of SOD1)

found in the laboratory population are unknown. The opportunity for differential selection among SOD1 alleles in natural populations depends on there actually being allelic variation *within* natural populations of *B. glabrata*. Thus, it is important to know if those alleles do in fact co-occur in the same natural populations. It could be that the geographically distinct populations used to create the strain are in fact fixed for different SOD1 alleles (Caribbean and South American populations of *B. glabrata* are known to be highly differentiated at other loci; Mavarez et al. 2002a,b; DeJong et al., 2003). In that case the co-occurrence of those alleles in the 13-16-R1 population would simply be an artifact of the hybrid breeding history. On the other hand, if the different SOD1 alleles in our laboratory population *do* co-occur in natural populations, then examining the ecological consequences of that variation and how that variation is maintained would be an important step in understanding the possible role allelic variation at SOD1 plays in determining resistance to infection in natural populations. Therefore, the two goals of this study were to: (1) identify the geographic origins of the three major alleles of SOD1 found in the 13-16-R1 strain and (2) determine if the SOD1 alleles we observe in the laboratory population actually co-occur in natural populations.

We found that natural populations are indeed polymorphic for intron four variants of SOD1. Intriguingly, there was substantial heterozygote excess relative to Hardy-Weinberg expectations (negative F_{IS}) and a large, positive Tajima's D (Tajima 1989) at SOD1 in many of the populations (positive Tajima's D means excess divergence among alleles relative to neutral expectations). These results raise the

possibility of overdominance or some other strong balancing selection acting on SOD1 in these populations. Signatures of balancing selection are commonly seen at disease-related loci. Examples include the vertebrate major histocompatibility complex (MHC) (Garrigan and Hedrick 2003, Piertney and Oliver 2006) (Garrigan and Hedrick 2003, Piertney and Oliver 2006), immunoglobulin genes (Su and Nei 1999), plant R-genes (Bakker et al. 2006), and antimicrobial peptides in amphibians (Tennessen and Blouin 2008). On the other hand, most of the above examples include proteins involved in recognition, whereas SOD1 is involved in killing pathogens once they have been recognized. Observing balancing selection on an effector molecule would be unusual (but, see Tennessen and Blouin 2008). Also, overdominance has rarely been demonstrated in natural populations, so documenting an example in snails would be quite interesting. However, it is possible that strong family structure or extremely small effective size in our samples caused the heterozygote excess (Robertson 1965, Rasmussen 1979, Pudovkin et al. 1996, Luikart and Cornuet 1999, Balloux 2004), and so it has nothing to do with SOD1 *per se*. Similarly, positive Tajima's D can result from non-selective causes such as recent mixture of diverged populations or shrinking populations (Hedrick 2005, Nielsen 2005). Therefore, in order to determine whether the heterozygote excess and positive Tajima's D were genome-wide phenomena or unique to SOD1, we compared genetic variation at SOD1 with that at two additional, putatively neutral sequence loci (intron sequence from actin and myoglobin) and at seven microsatellite loci for all our natural populations that were polymorphic at SOD1.

2.3 Methods

2.3.1 *Study populations*

Goodall et al. (2006) identified the association between allelic variation at the fourth intron of SOD1 in *B. glabrata* and resistance to *S. mansoni* using the 13-16-R1 laboratory strain of *B. glabrata* and *S. mansoni* PR-1 (Puerto Rico) laboratory strain. Both the snail and parasite strains are well-established laboratory strains and have been maintained in C. Bayne's laboratory at Oregon State University since the mid-1970s. We surveyed fourteen natural populations of *B. glabrata* from throughout the range of *B. glabrata* in the Caribbean and South America for allelic variation at SOD1 (Table 2.1). These included samples from a single population in Puerto Rico, four populations in Guadeloupe, two populations from Venezuela, and six populations in Brazil (Table 2.1, Figure 2.1A).

2.3.2 *Allelic variation at SOD1 in natural populations*

As in previous studies, we determined SOD1 genotype through sequence analysis of approximately 540 bp of the fourth intron of SOD1 (Goodall et al. 2006, Bonner et al. 2012, Blouin et al. 2013). We restricted our analysis to the fourth intron of SOD1 because there is little genetic variation in coding sequence among the alleles of SOD1 in 13-16-R1. See Appendix Table 1 for primers and amplification conditions. We aligned sequences using MEGAv5.10 (Tamura et al. 2011), and generated an unrooted

network tree of unique sequences across the region using TCS (Clement et al. 2000). For populations that were polymorphic for SOD1, we calculated allele frequencies and estimated deviation from Hardy-Weinberg equilibrium (F_{IS}; where negative F_{IS} suggests excess heterozygosity and positive F_{IS} indicates a deficiency) using Genepop (Raymond and Rousset 1995). We assessed Tajima's D using DnaSPv5 (Librado and Rozas 2009). Reference sequences for the laboratory population alleles A-D are available in GenBank under accession numbers (DQ239577-DQ239579).

2.3.3 *Assessing evidence for selection on SOD1 in natural populations*

As previously stated, our results showed that in populations that were polymorphic for SOD1 there was a heterozygote excess and a positive Tajima's D. However, there are other explanations for those patterns that have nothing to do with selection on SOD1 *per se*. Therefore, we compared Fis and Tajima's D for SOD1 with that for nine other loci in the same populations to determine if the non-neutral patterns observed at SOD1 were a genome-wide phenomenon or a unique signature of SOD1. Of the nine additional loci, we used two single copy sequence loci (a portion of the first intron of β-actin and the third intron of myoglobin) and seven microsatellite loci. See Appendix Table 1 for primers and amplification conditions.

2.4 Results

2.4.1 *Allelic variation at SOD1 in natural populations*

Of the four SOD1 intron 4 alleles (*A, B, C,* and *D*) that are segregating in our 13-16-R1 laboratory strain of *B. glabrata*, the *B* allele was the only allele for which we found identical sequence in natural populations. However, we did observe sequence variants that were very similar to the *A, C*, and *D* alleles in natural populations (Figure 2.1B). Furthermore, the A-D alleles are spread throughout the tree (Figure 2.1B), so the range of variation found in natural populations appears to be represented in the 13-16-R1 laboratory population, suggesting that there are no highly differentiated clusters of alleles found in nature that were not represented in the our laboratory population.

Alleles similar to the *A*, *B*, and *D* alleles were present only in populations from Brazil (Figure 2.1A). *C*-like alleles were found in the Caribbean (Puerto Rico and Guadeloupe) and in one population in Brazil (Figure 2.1A). However, the 13-16-R1 *C*-allele was more similar to the Brazilian *C*-like allele than to the *C*-like alleles from the Caribbean. A single nucleotide polymorphism differentiates the 13-16-R1 *C* allele from the sequence variant identified in the Puerto de Galinhas population in Brazil, while a 6bp insertion/deletion (indel) and a SNP differentiates the 13-16-R1 *C* allele from the Caribbean sequence variants. Thus, we have the interesting result that all four 13-16-R1 SOD1 alleles are more similar to sequence variants found in Brazil than to variants found in Puerto Rico.

Only five populations, all from Brazil, were polymorphic for two or more alleles. Puerto Rico or Guadeloupe each showed a single *C*-like allele, with these two alleles differing by a single SNP (Figures 2A, B). Similarly, the two populations

surveyed in Venezuela each had a single allele that differed by a single SNP and were most similar to the 13-16-R1 *A* allele (Figures 2A, B). Populations that were polymorphic for SOD1 had alleles present from throughout the tree (Figure 2B). More importantly, alleles identical to, or very similar to, the alleles in our laboratory population do co-occur in natural populations of *B. glabrata*.

Finally, the four populations that had *B* or an allele most similar to the *B* alleles segregating with other alleles showed an excess in heterozygosity (negative F_{IS}), with significant deviations from Hardy-Weinberg Equilibrium in two of them (Table 2.2). The fifth polymorphic Brazilian population (Virgem das Graca) was segregating for two alleles similar to the lab population *A* and *C* alleles, and showed no significant excess in heterozygosity. Tajima's D was positive and significant at SOD1 for the entire tree and for four of the five polymorphic populations examined individually (Table 2.3).

2.4.2 *Assessing evidence for selection on SOD1 in natural populations*

In order to determine if the negative Fis observed in four of the five polymorphic populations was unique to SOD1, we examined deviations from Hardy-Weinberg Equilibrium at seven microsatellite loci and two single copy intron loci (intronic sequence from β-actin and myoglobin). When compared to the nine other loci, SOD1 had the lowest F_{IS} in all four populations segregating for a *B* or *B*-like allele (Figure 2.2). Tajima's D was very positive and significant for β-actin and myoglobin in populations that were polymorphic at those loci (Table 2.3). Therefore,

the large, positive values observed at SOD1 appear to be a genome-wide phenomenon and not unique to SOD1.

2.5 Discussion

Given the association between allelic variation at the fourth intron of SOD1 and resistance to infection in the 13-16-R1laboratory strain of *B. glabrata* (Goodall et al. 2006), the goals of this study were to identify the geographic origins of the SOD1 alleles found in our laboratory strain and to determine if the alleles co-occur in natural populations. Due to the hybrid nature of the strain, it is possible that the co-occurrence of the alleles in this population is an artifact of the breeding history rather than reflective of natural populations (Newton 1955, Richards and Merritt 1972, DeJong et al. 2003). Given the opportunity for selection to act on SOD1 in natural populations depends on the presence of allelic variation in natural populations of *B. glabrata*, it was therefore necessary to determine if allelic variation at SOD1 did occur in natural populations.

2.5.1 *SOD1 allele variants do co-occur in natural populations*

We found alleles identical to the *B* allele and sequence variants very similar to the *A, C,* and *D* alleles in natural populations (Figure 2.1). Interestingly, it is the *B* allele that confers the highest resistance in our laboratory strain. The *A-D* alleles occur throughout the tree of alleles, so the sequence variation observed in our

laboratory population reflects the range of variation found in natural populations (Figure 2.1). Importantly, sequence variants identical to, or similar to, the laboratory alleles were found to co-occur in natural populations (Figure 2.1).

The most similar sequence variants to the 13-16-R1 alleles were all identified in Brazilian populations. The *B* allele and similar sequence variants were only observed in Brazil. It is interesting that the *B* allele, a Brazilian allele, confers resistance in 13-16-R1 lab strain when challenged with a Puerto Rican parasite (PR-1). Additionally, only the Brazilian populations were polymorphic for SOD1. The higher allelic diversity at SOD1 observed in natural populations in Brazil compared to populations in the Caribbean is consistent with previous phylogeography and population genetics studies that utilized neutral markers (Mavarez et al. 2002a, Mavarez et al. 2002b, DeJong et al. 2003, Wethlngton et al. 2007).

2.5.2 *Weak evidence for patterns of overdominance and balancing selection*

We initially observed a heterozygote excess (negative F_{IS}) at SOD1 in four of the five populations that had multiple alleles. We then evaluated the pattern observed at SOD1 by comparing F_{IS} at nine additional, putatively neutrally evolving loci. Because we chose to examine F_{IS} at the nine additional loci *after* observing low F_{IS} at SOD1, we did not have a truly unbiased test of whether SOD1 shows unusually low F_{IS}. It is also possible that microsatellites are more prone to null alleles leading to a bias upwards compared to intron sequence. On the other hand, the myoglobin and actin intron alleles do not show consistently lower Fis than the microsatellites (Figure

2.1). Nevertheless, that SOD1 did show the lowest F_{IS} of all loci in all populations that were segregating for *B*-like alleles makes the overdominance hypothesis very intriguing and worthy of follow up. Typing the same loci in additional, independent samples from nature would provide an independent test of the hypothesis.

In addition to negative F_{IS}, we also found that the four populations segregating for the *B* or *B*-like allele had a positive Tajima's D for SOD1, indicating balancing selection might be acting on SOD1 (Tajima 1989). However, we found similar patterns of Tajima's D at introns of β-actin and myoglobin. It is conceivable that balancing selection is acting on all three regions of genome where these loci are located. However, it is far more likely the deviations from neutral expectation are the result population demographics (i.e. population mixing or recent population shrinking) (Hedrick 2005, Nielsen 2005).

2.5.3 *Conclusions*

The goal of this study was to determine the origins of the alleles in the 13-16-R1 laboratory population, and if they co-occur in natural populations. If these alleles did not co-occur, then the relative selective advantage of one allele over another in terms of resistance would not have been relevant in natural populations. We found alleles identical to, or very similar to, our laboratory alleles in natural populations, and that these alleles do indeed co-occur in natural populations (at least in Brazil). Furthermore, the four laboratory alleles are spread throughout the tree of alleles. Thus, they represent the range of sequence variation observed at the fourth intron of

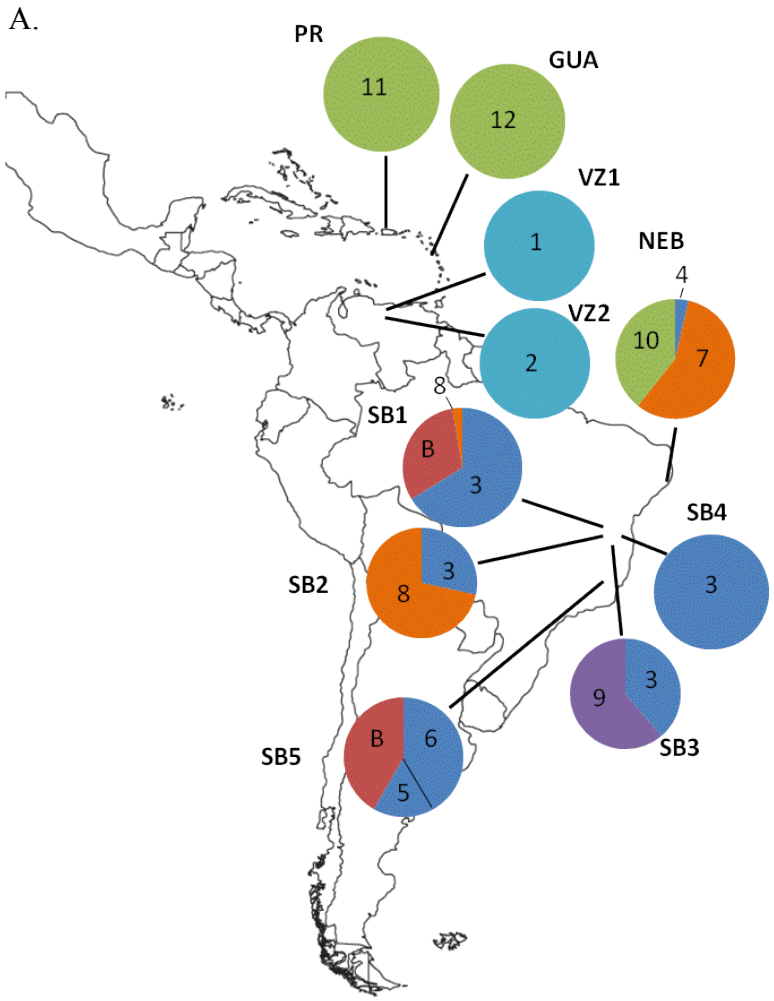
SOD1 in nature. Whether the associations between allelic variation and resistance observed in the laboratory population also occur in nature, still needs to be tested. Finally, we noticed an intriguing excess heterozygosity at SOD1 in the four populations that were segregating for the *B* allele, and that this excess was greater than that observed at any of the nine putatively neutral loci. How widespread this pattern is remains to be seen, but it suggests the intriguing hypothesis that overdominance might be acting on SOD1 (or some locus closely linked to it; Bonner et al. 2012; Blouin et al. 2013).

Table 2.1

Populations of *B. glabrata* surveyed for allelic variation at the fourth intron of SOD1. The population ID corresponds to the map of alleles (Figure 2.1A) and the SOD allele corresponds to the network tree (Figure 2.1B).

Population ID	Country	Location	Coordinates	N	SOD N_a	SOD allele
PR	Puerto Rico	unknown	unknown	11	1	11
GUA	Guadeloupe	Jacquot (JAC)	N. 16°16.241' W. 061°31.999'	24	1	12
	Guadeloupe	Belle Plaine (BLP)	N. 16°17.405' W. 061°31.444'	24	1	12
	Guadeloupe	Dans Fond (DFO)	N. 16°18.500' W. 061°30.720'	24	1	12
	Guadeloupe	Geffrier (GEF)	N. 16°19.907' W. 061°29.952'	24	1	12
	Guadeloupe	Dubelloy (DUB)	N. 16°19.660' W. 061°28.524'	24	1	12
VZ1	Venezuela	Haras tamanaco (HTA)	N. 10°08.26' W. 67°36.60'	32	1	1
VZ2	Venezuela	Puerta Negra (MPN)	N. 10°07.16' W. 67°36.38'	24	1	2
NEB	Brazil	Porto de Galinhas, Pernambucco	S. 8°32' W. 35°02'	14	3	4, 7, 10
SB1	Brazil	Caju, Minas Gerais	S. 16°21'08.95" W. 41°17'28.18"	37	3	3, 8, B
SB2	Brazil	Sao Pedro do Jequitinhonha, Minas Gerais	S. 16°29'44.14" W. 41°20'59.38"	37	2	3, 8
SB3	Brazil	Virgem das Graca, Minas Gerais	S. 16°56'37.58" W. 41°21'56.33"	54	2	3, 9
SB4	Brazil	Virgem das Graca, Minas Gerais	S. 16°56'37.58" W. 41°21'56.33"	52	1	3
SB5	Brazil	Belo Horizonte, Minas Gerais	unknown	18	3	5, 6, B

N= sample size, N_a = number of alleles


A.
PR
GUA
11
12
VZ1
1
NEB
4
VZ2
2
10
7
8
SB1
B
3
SB4
3
SB2
3
8
9
3
SB3
B
6
5
SB5

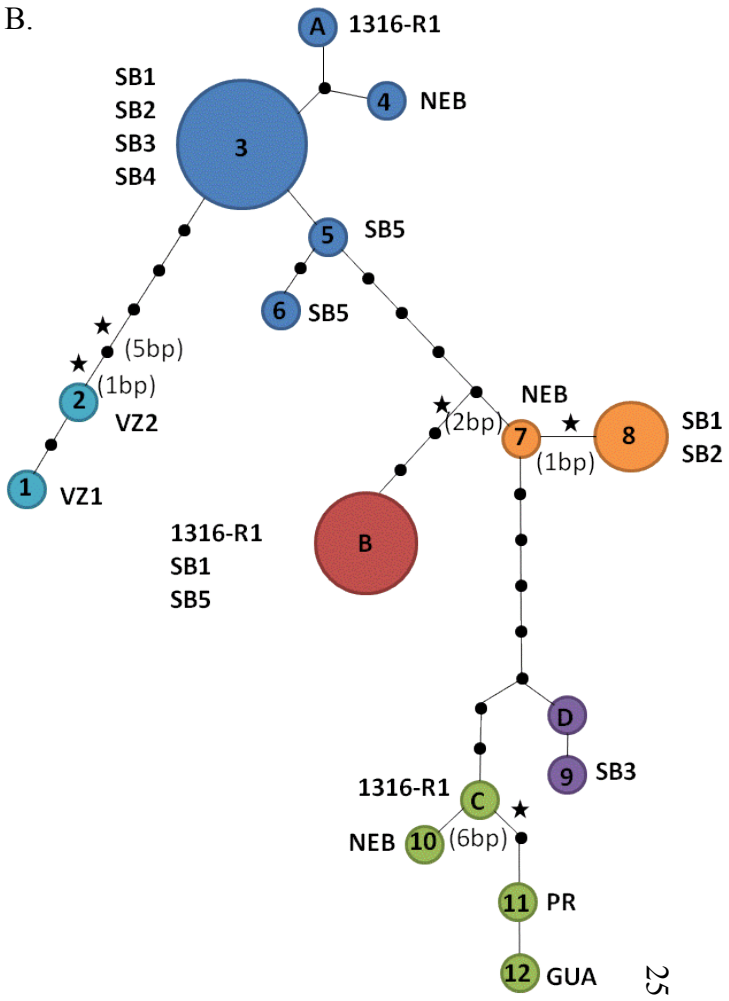

B.
A
1316-R1
SB1
SB2
SB3
SB4
3
4
NEB
5
SB5
6
SB5
(5bp)
(1bp)
2
VZ2
1
VZ1
NEB
(2bp)
7
(1bp)
8
SB1
SB2
1316-R1
SB1
SB5
B
D
9
SB3
1316-R1
C
NEB
10
(6bp)
11
PR
12
GUA

Figure 2.1

Geographic distribution and unrooted network tree of allelic variation in the fourth intron of SOD1 in natural populations of *B. glabrata*. A. Allele types corresponding are indicated within each circle. Population identifiers are located adjacent to the circle. For the populations that were polymorphic for SOD1, the circle represents the allele frequency distribution in the population. Populations that were segregating for more than one allele at SOD1 were exclusively found in Brazil. These results indicate that different SOD1 alleles do co-occur in natural populations. **B.** The size of the circle represents the number of populations that have that specific allele variant (haplotype). Population identifiers are located adjacent to the haplotypes to indicate the populations that have each allele type (e.g. four populations have allele type 3, and they are SB1, SB2, SB3, and SB4). Each small black circle represents a node of transition and the lines between the nodes represent SNPs or indels. Indels are indicated by stars and the size of the indel identified next to the star. The alleles in the 13-16-R1 lab population are identified with letters. Note that these fall throughout the network tree, each having highly similar sequences observed in natural populations. The colors of clusters of alleles in the network were assigned arbitrarily to visually clarify allelic variation in accordance with the geographic distribution map.

Table 2.2

Deviations from Hardy-Weinberg Equilibrium (F_{IS}) at SOD1 in natural populations.

ID	Brazil Populations	SOD alleles[1]	F_{IS}	*P*
NEB	Porto de Galinhas, Pernambucco	4, ***7***, 10	-0.3472	0.267
SB1	Caju, Minas Gerais	3, ***8***, ***B***	-0.3184	0.0553
SB2	Sao Pedro do Jequitinhonha, Minas Gerais	3, ***8***	-0.3846	0.0356
SB3	Virgem das Graça, Minas Gerais	3, 9	-0.0036	1
SB5	Belo Horizonte, Minas Gerais	5, 6, ***B***	-0.1571	0.1902

[1]Alleles present in each population. *B* or most similar to the 13-16-R1 *B* allele in bold

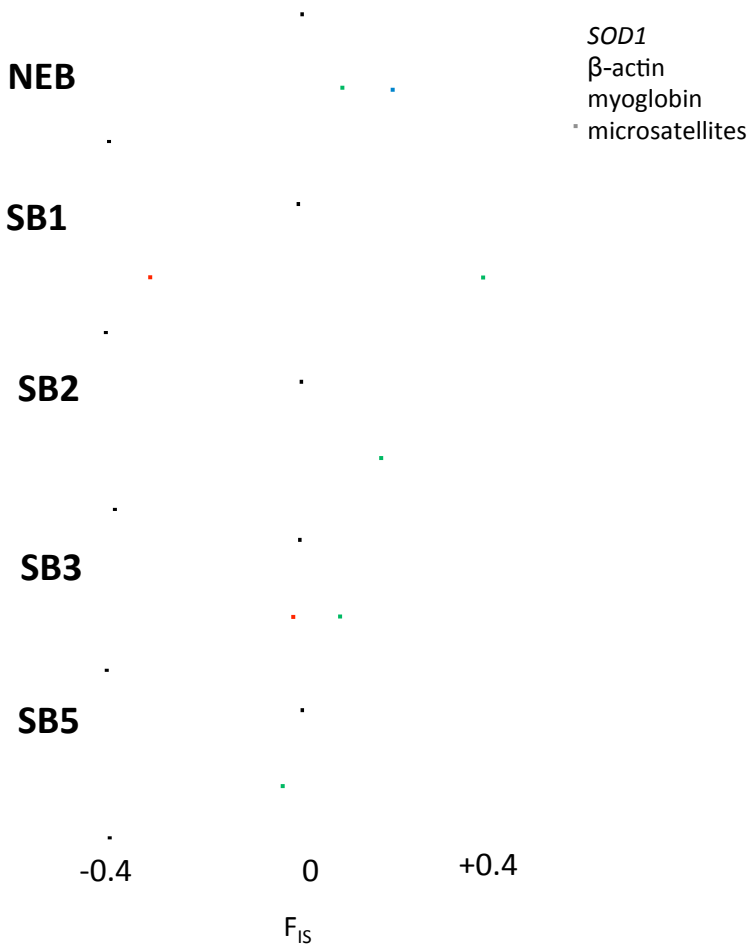


Figure 2.2

Deviations from Hardy-Weinberg Equilibrium (F_{IS}) at SOD1, β-actin, myoglobin, and microsatellite loci. For each population that was polymorphic for the fourth intron of SOD1 we surveyed genetic variation at two additional sequence loci (the first intron of β-actin and the fourth intron of myoglobin) and seven microsatellites (BgE4, BgE5, BgC7, BgC8, uBg1, Bgu15, and Bgu16). We found the strongest deviations from Hardy-Weinberg equilibrium were indeed at SOD1 compared to all other loci, but this was true only for the four populations that were segregating for a *B* or *B*-like allele (NEB, SB1, SB2, and SB5). Population SB3 is segregating for *A*-like and *C*-like alleles. Not all of the "neutral" loci were polymorphic in every population.

Table 2.3

Tajima's D at SOD1, β-actin, and myoglobin.

ID	Brazil Populations	Tajima's D		
		SOD1	β-actin	myoglobin
NEB	Porto de Galinhas, Pernambucco	0.862	0.6422	n/a
SB1	Caju, Minas Gerais	2.878*	2.372*	3.640*
SB2	Sao Pedro do Jequitinhonha, Minas Gerais	2.484*	n/a	3.709*
SB3	Virgem das Graça, Minas Gerais	3.845*	2.123*	2.103*
SB5	Belo Horizonte, Minas Gerais	2.963*	2.699*	3.968*

* $P<0.05$

n/a indicates populations that were monomorphic at that locus

EFFECTS OF CU/ZN SUPEROXIDE DISMUTASE (SOD1) GENOTYPE AND GENETIC BACKGROUND ON GROWTH, REPRODUCTION AND DEFENSE IN *BIOMPHALARIA GLABRATA*

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CHAPTER 3

EFFECTS OF CU/ZN SUPEROXIDE DISMUTASE (SOD1) GENOTYPE AND GENETIC BACKGROUND ON GROWTH, REPRODUCTION AND DEFENSE IN *BIOMPHALARIA GLABRATA*

3.1 Abstract

Resistance of the snail *Biomphalaria glabrata* to the trematode *Schistosoma mansoni* is correlated with allelic variation at copper-zinc superoxide dismutase (SOD1). We tested whether there is a fitness cost associated with carrying the most resistant allele in three outbred laboratory populations of snails. These three populations were derived from the same base population, but differed in average resistance. Under controlled laboratory conditions we found no cost of carrying the most resistant allele in terms of fecundity, and a possible advantage in terms of growth and mortality. These results suggest that it might be possible to drive resistant alleles of SOD1 into natural populations of the snail vector for the purpose of controlling transmission of *S. mansoni*. However, we did observe a strong effect of genetic background on the association between SOD1 genotype and resistance. SOD1 genotype explained substantial variance in resistance among individuals in the most resistant genetic background, but had little effect in the least resistant genetic

background. Thus, epistatic interactions with other loci may be as important a consideration as costs of resistance in the use of SOD1 for vector manipulation.

3.2 Author summary

Driving resistance genes into vector populations remains a promising but underused method for reducing transmission of vector-borne diseases. Understanding the genetic mechanisms governing resistance and how resistance is maintained in vector populations is essential for the development of resistant vectors as a means of eradicating vector-borne diseases. We investigated the utility of one gene (cytosolic copper-zinc superoxide dismutase - SOD1) for driving resistance associated alleles into populations of the snail *Biomphalaria glabrata,* a vector of the trematode parasite of humans, *Schistosoma mansoni*. Under controlled laboratory conditions we found no evidence for costs of resistance associated with carrying the most resistant allele at SOD1 (in terms of growth, fecundity, or mortality). However, we did find a strong effect of genetic background on how strongly SOD1 genotype influences resistance. Thus, epistatic interactions with other loci may be as important a consideration as costs of resistance in the use of SOD1 for vector manipulation in the field.

3.3 Introduction

Although vector-borne diseases account for approximately one-sixth of the global human disease burden (Townson et al. 2005, LaBeaud and Aksoy 2010), we still lack effective drugs and vaccines for many of these diseases. Even when effective drugs are available, high-risk populations often cannot be adequately treated due to a lack of funding and infrastructure in the heavily impacted countries (Townson et al. 2005, Hotez and Pecoul 2010). Therefore, in the absence of vaccines, eradication efforts that include both drug therapy and vector control can be the most effective approach (Fenwick 2006). Vector control methods most often utilize chemicals for eradication (Townson et al. 2005, Fenwick 2006). This approach has obvious drawbacks because it results in habitat degradation and risk of human exposure to pesticides. Also, recurrent pesticide application is often necessary because it is nearly impossible, with a single treatment, to completely remove all possible vector individuals from an epidemiologically relevant site (Fenwick and Webster 2006).

Recent advances in understanding the genetics of host-parasite interactions have led to increased interest in driving resistance genes into susceptible vector populations (Marrelli et al. 2006, Marrelli et al. 2007, Coelho et al. 2008, Lambrechts et al. 2008, Cohuet et al. 2010, Wise de Valdez et al. 2011). In this context, the term "resistance" describes a continuously varying trait we define as the probability of becoming infected after being challenged by a parasite, rather than to mean the absolute inability to become infected (i.e. a population or genotype can have high or low average resistance). Making vector populations more resistant to infection could be a better long-term solution and an ecologically safer way of breaking transmission

cycles. Unfortunately, this approach faces major population-genetic hurdles. A non-exhaustive list includes: (1) genotype-by-environment (GxE), where the performance of a gene or gene(s) of interest depends on environmental conditions such that interactions can affect how a resistance gene performs in the field versus in the lab (de Roode et al. 2009, Gandon and Nuismer 2009, Lazzaro and Little 2009, Wolinska and King 2009, Seppala and Jokela 2010), (2) parasites and hosts are genetically more variable in the field, and there can be interactions between host genotypes and parasite genotypes (genotype-by-genotype (GxG) interactions; (Lively 1999, Lively and Dybdahl 2000, Lambrechts et al. 2006b, Gandon and Nuismer 2009)), (3) genetic background can influence how a resistance gene performs in a natural versus a lab population. In other words, the gene of interest may perform differently depending on the genomic context in which it is interacting (epistasis), and (4), there may be a cost of resistance such that natural selection in the absence of parasites favors the "wild-type" alleles that we wish to replace.

Cost of resistance may be a particularly vexing problem for resistance-gene introduction programs. Such costs have been demonstrated in many host-parasite systems (reviewed in (Antonovics and Thrall 1994, Sheldon and Verhulst 1996, Lochmiller and Deerenberg 2000, Zuk and Stoehr 2002, Schmid-Hempel 2003, Schulenburg et al. 2004, Sadd and Schmid-Hempel 2009)). Nevertheless, costs of resistance are not universal (Schmid-Hempel and Schmid-Hempel 1998, Svensson et al. 1998, Rigby et al. 2002, Lewis et al. 2003, Rosa et al. 2006, Lambrechts et al. 2008), and they may be context dependent (e.g. revealed only in stressful

environments; (Sandland and Minchella 2003, Lambrechts et al. 2006a, Rashed et al. 2008, Lazzaro and Little 2009, Huvet et al. 2010, Salice et al. 2010)). Costs of resistance presumably involve a reallocation of metabolic resources between one or more of the following life-history components: reproduction, growth, and somatic maintenance/immune function (Reznick 1992, Roff 1992, Schmid-Hempel 2003, Sadd and Schmid-Hempel 2009). Also, the severity of the cost should depend on the particular mechanism of resistance (Coustau et al. 2000, Rigby et al. 2002). For example, it was predicted that mechanisms involving over-expression of particular genes might be among the most costly (Coustau et al. 2000).

This study was designed to measure costs of resistance and epistatic effects of genetic background associated with a single locus in *Biomphalaria glabrata*, a snail vector of the human pathogen *Schistosoma mansoni*. Schistosomiasis is responsible for approximately 200,000 deaths yearly, with 200 million people infected worldwide (Crompton 1999, Chitsulo et al. 2000, Ross et al. 2002). *B. glabrata* is a facultative, hermaphroditic freshwater pulmonate snail that occurs throughout much of the New World tropics (DeJong et al. 2001, Morgan et al. 2001, Mavarez et al. 2002). The *B. glabrata/S. mansoni* system is a well-established model for investigating host-parasite interactions in a controlled laboratory setting (Bayne 2009).

Resistance to *S. mansoni* infection in *B. glabrata* is highly heritable in many lab and field populations, and is almost certainly controlled by multiple loci (Richards and Merritt 1972, Shoukry 1997, Webster and Woolhouse 1999, Webster and Davies 2001, Théron et al. 2008, Zavodna et al. 2008). The expression patterns of known

immune-related genes have been found to differ between individuals from more resistant and less resistant strains when each is challenged with the same strain of parasite (Lockyer et al. 2007, Hanelt et al. 2008, Roger et al. 2008, Ittiprasert et al. 2009, Adema et al. 2010, Hanington et al. 2010, Ittiprasert et al. 2010). However, to date only a single locus has been identified at which allelic variation clearly associates with resistance to the parasite: copper-zinc superoxide dismutase (SOD1) (Goodall et al. 2004, Goodall et al. 2006). SOD1 is a ubiquitous protein involved in several cellular functions including signaling and immune response (Nappi and Ottaviani 2000, Fink and Scandalios 2002, Ramasarma 2007, Abreu and Cabelli 2010). Among the various functions of SOD1, it catalyzes the reduction of highly reactive superoxide (O_2^-) to hydrogen peroxide (H_2O_2). Hydrogen peroxide is a known cytotoxic component of the oxidative burst, which is the primary defense mechanism for parasite clearance in molluscs (Hahn et al. 2001, Bayne 2009, Loker 2010). When a schistosome invades a snail, hemocytes surround the invading parasite and are thought to generate H_2O_2 as part of the killing mechanism (Hahn et al. 2001, Bender et al. 2005, Bayne 2009). Consistent with this hypothesis, increased H_2O_2 production was correlated with the difference in resistance between snails from the M-line strain and the more resistant 13-16-R1 strain (Bender et al. 2005, Bayne 2009). An SOD1 allele present in the 13-16-R1 strain was over-expressed relative to the other alleles, and correlated with a more effective defense against parasite infection (Goodall et al. 2006, Bender et al. 2007, Bayne 2009). More recently, Moné et al. (Moné et al. 2011) demonstrated a correlation between the ability of certain strains of *B. glabrata* to

produce reactive oxygen species and the anti-oxidant defenses of their respective compatible *S. mansoni* strains. Thus, loci involved in the oxidative burst, such as SOD1, may be very important in the evolution of schistosome-snail interactions. Therefore, SOD1 is a promising candidate locus for driving resistance alleles into susceptible natural populations of snails.

Although SOD1 seems a favorable candidate for genetic manipulation of snail populations, there are two reasons why one might expect a cost of resistance associated with the allelic polymorphism at SOD1. First, increased expression of any gene is likely to be costly (Coustau et al. 2000). Second, increased expression of SOD1 might incur a cost due to increased oxidative stress on the host (Dowling and Simmons 2009, Monaghan et al. 2009). Therefore, investigating the fitness costs associated with allelic variation at SOD1 is an important first step in evaluating the potential use of SOD1 for creating highly resistant vector populations in the field.

3.4 Methods

3.4.1 *Study population*

We used a population of the 13-16-R1 strain of *B. glabrata* that has been maintained as a large population (hundreds) in C.J. Bayne's lab at Oregon State University since the mid-1970s. The 13-16-R1 strain was reportedly created by crossing highly resistant strains of snails isolated from Brazil and Puerto Rico (Richards and Merritt 1972) but it has been in culture for so long in so many

laboratories that its history is not entirely clear. Our population has been maintained in the absence of parasite exposure, and therefore under relaxed selective pressure in regards to resistance to *S. mansoni*. *B. glabrata* is a facultative self-fertilizing hermaphrodite such that snails will preferentially outcross when given access to a mate, but when isolated will usually reproduce through self-fertilization (e.g. (Vianey-Liaud 1992, 1995, Vianey-Liaud and Dussart 2002); our laboratory population is in Hardy-Weinberg Equilibrium for SOD1 and microsatellite loci: (Goodall et al. 2006, Bender et al. 2007); unpub. data). We recently created 52 inbred lines: we started with haphazardly picked juvenile snails and completed three generations of selfing using a single offspring from each self-fertilization event to begin the next generation. The inbred lines are mostly fixed for one of three alleles of SOD1 *A*, *B* and *C*, as described in (Goodall et al. 2006). These lines also vary substantially for resistance within each SOD1 genotypic class (*AA, BB*, and *CC*). That there are highly resistant and highly susceptible lines within each SOD1 class suggests that other loci besides SOD1 have a large effect in determining resistance. These inbred lines can be used to compare directly the fitness effects of carrying a specific genotype at SOD1 and the effects of genetic background on the association between resistance and SOD1 genotype.

3.4.2 *Breeding scheme*

Several inbred lines were used to create three outbred F2 populations, each of which was segregating for the *B* and *C* allele (Figure 3.1). We hereafter refer to these three F2 populations as "genetic backgrounds" because we wanted to know if the

phenotypic effects of variation at SOD1 depend on the genomic context in which those alleles are expressed. These F2 individuals were then used to evaluate the effects of SOD1 allele on life history traits and resistance. Inbred lines were chosen so that the three populations differed in average resistance. *BB* and *CC* fixed lines were chosen because the B allele confers the highest resistance and the *C* allele the lowest (Goodall et al. 2006). Additionally, in hemocytes (the defense cells) the *B* allele is constitutively over-expressed relative to the other two alleles (Bender et al. 2007). To create the three F2 populations, we paired an individual from an inbred line fixed for the *B* allele with an individual from an inbred line fixed for the *C* allele (*BB* x *CC*), which resulted in offspring that were heterozygous at SOD1 (*BC*). Three unique *BB* and *CC* inbred lines were used, and each cross was completed in triplicate with unique individuals (n=9 crosses). To compare directly the effects of carrying the *BB* and *CC* genotypes within a family and among different backgrounds, we paired heterozygous offspring from each initial cross with a heterozygous individual from a different initial cross using a factorial design. This resulted in three different F2 populations of outbred individuals that had the same SOD1 genotypes, but in different genetic backgrounds (Figure 3.1). The F2 individuals in each of the three populations carried the *BB*, *BC* and *CC* genotypes in the expected (1:2:1) Mendelian ratios (SOD1 genotypes were verified by sequencing). We used these F2 individuals to test the effects of SOD1 genotype on fecundity, growth and resistance in each of the three genetic backgrounds. Our three populations (genetic backgrounds) differed in overall

resistance (77.8%, 63.8%, and 38.9%), which strongly correlated with the resistance of their grandparents (the original inbred lines) (Figure 3.2).

3.4.3 *Resistance*

For each F2 population (genetic background), a total of 72 individuals were haphazardly chosen from a pool of offspring from the final set of crosses. We exposed single juvenile snails (4-5mm diameter) to five *S. mansoni* strain PR-1 miracidia in 3mL of artificial spring water (ASW; (Ulmer 1970)) for two hours at 26°C, in 12-well culture plates. The PR-1 strain has been maintained in Syrian hamsters and the M-line (Oregon) strain of *B. glabrata* snails by the Bayne lab for 36 years. Challenged individuals were then reared in moderately dark tubs in groups of 24, with three replicate tubs for each background (n=72). We examined the snails for infection at six, nine, and eleven weeks (we rarely see shedding after 11 weeks). Each examination week we induced cercarial shedding (parasite emergence) by exposing snails individually in 3mL of ASW to direct fluorescent light for two hours at 26°C in 12-well culture plates. The presence of cercarial shedding indicated a positive infection. Infected snails were preserved in 95% ethanol (EtOH), and non-infected snails were returned to rearing tubs after each assay. After the final cercarial shedding attempt (eleven weeks) we preserved the remaining snails, and all tissue samples were processed for SOD1 genotyping (described below in 'Molecular Methods' section). Resistance to parasite infection was scored in each tub group as the percentage of snails that did not shed cercariae by eleven weeks post-challenge. Snails that died

prior to shedding assays were excluded from the experiment. Average mortality observed from the parasite challenge ranged from 8-12% among tubs, and did not differ among genetic backgrounds (One-way ANOVA, p=0.442).

3.4.4 *Growth*

We collected single egg masses (n=58) from Styrofoam substrate within 48-hours of egg mass deposition from individual pairs of the final set of crosses (i.e. embryos in the eggs are F2s). The single egg masses were reared individually and allowed to hatch. We measured offspring size (diameter of the shell) twelve weeks after egg mass deposition. All snails were then preserved in 95% ethanol for subsequent SOD1 genotyping. Clutch sizes (the numbers of eggs/embryos in single egg masses) ranged from 2 to 34 (n=58). Initial analysis revealed that average offspring size was correlated with clutch size, (adjusted R^2=0.363, P<0.001) suggesting a strong density-dependent effect of number of snails per bowl on growth (same effect across all genetic backgrounds). Therefore, we restricted our analysis of effects of SOD1 genotype to the offspring of clutch sizes between 13-17 eggs/embryos (there was no association between clutch size and snail size within that limited range of clutch sizes; adjusted R^2 0.001, P=0.28). We compared snail growth from 3-4 clutches in each genetic background (background 1: n=45, background 2: n=57, background 3: n=58).

We also measured growth (shell diameter) in snails that were raised individually for 32 weeks as part of the egg production and hatch success experiments

described below (hereafter referred to as "late growth" compared to the "early growth" measures described in the above experiment).

3.4.5 *Fecundity*

As in the growth study, we collected egg masses from individual pairs of the final crosses (i.e. the F2 offspring). From each population, we haphazardly chose 50 sexually immature offspring (4-5 mm shell diameter). Each snail was reared singly and a portion of a tentacle was excised to determine its SOD1 genotype. We then randomly chose ten juveniles of each genotype (*BB*, *BC*, and *CC*) from each set of 50 genotyped snails, and reared them individually for subsequent fecundity comparisons (i.e. n = 30 per genetic background). Because *B. glabrata* is a facultative self-fertilizing hermaphrodite, we provided a mate to each snail prior to measuring egg production and hatch success to ensure offspring were not the result of selfing (because inbreeding depression is expected to affect egg survival). We chose to mate the genotyped individuals with snails from an isogenic inbred population to keep consistent the relative contribution of the "male-acting" snail to egg production. The isogenic inbred individuals were from a population of inbred M-line strain of *B. glabrata* established at the University of New Mexico through 32 generations of selfing (Si-Ming Zhang *pers comm.*). Because the M-line and F2 offspring look morphologically similar, we marked the M-line snails with a white dot using nail polish 24 hours prior to mating.

All snails were individually reared until reproductively active, as determined by the presence of well-formed egg masses containing developing embryos. *B. glabrata* preferentially use allosperm for fertilization and store sperm for up to 10 days (Vianey-Liaud 1995). Consequently, each snail was paired with a size-matched, painted, inbred M-line individual for one week, then separated and allowed to lay eggs for one week in a new cup. These eggs were thus presumably fertilized by allosperm, even though layed in the absence of a partner (Vianey-Liaud 1992, 1995, Vianey-Liaud and Dussart 2002). Egg numbers were counted at the end of each 1-week laying period, after which snails were re-paired with a different mate. We continued the mating/laying schedule for ten weeks, resulting in five one-week accumulated egg production measurements from each snail. We present the sum of the five one-week egg accumulation measures as the total egg production for each snail over five weeks.

3.4.6 *Hatch success*

We examined egg hatch success in the same set of genotyped individuals in which we surveyed egg production. Each snail was paired with a size-matched painted inbred M-line individual for 48 hours, and then isolated in a new cup. Two egg masses from each snail were carefully collected 72 hours post-transfer and reared individually (n=180). Egg masses were surveyed for total egg count upon collection, and final hatch counts were conducted six weeks later. Hatch success (percent of eggs hatched at six weeks) from the two egg masses was averaged for each snail.

3.4.7 *Mortality*

In addition to measuring egg production and egg hatch, we also monitored mortality at eight and twelve months in the same set of F2 snails used for the egg production and hatch success experiments. Mortality was measured as percent of individuals from each SOD1 genotype alive at the time of census for each genetic background.

3.4.8 *Snail rearing conditions*

All snails were reared in an environmentally controlled room kept at 26°C and on a 12hr day/12hr night light cycle with full spectrum light. Snails were fed green leaf lettuce *ad libitum* throughout all experiments. In experiments other than those in which we measured resistance, egg masses and snails were reared, mated, and maintained in 500 mL cups with 300 mL of ASW. Complete water changes were carried out weekly. When generating the three different populations (i.e. the three different genetic backgrounds) for the fecundity experiments, the egg masses (and offspring) were reared in 2L of ASW in aerated, lidded 1-gallon, clear plastic boxes (IRIS, USA). The egg masses monitored in the hatch success experiment were reared in petri-dishes (100 x 15mm) with 5mL of ASW. Finally, in the resistance assay we reared exposed snails in moderately dark, lidded 3-gallon plastic tubs (Dark Indigo Rubbermaid Roughneck boxes). Each contained 7.5L of aerated dechlorinated water supplemented with 10 mL of calcium carbonate shell hardening solution (30mg

Ca^{++}/L). Half of the water was changed with dechlorinated water between each infection assay.

3.4.9 *Molecular methods*

We extracted genomic DNA from snail head foot tissue following the CTAB protocol (Winnepenninckx et al. 1993), and used chelex extraction methods for tentacle tissue. SOD1 genotype was determined using fragment analysis on an ABI 3730 capillary sequencer following amplification with AmpliTaq (Applied Biosystems, Inc.) (F-(VIC) - TCA TTG GTC GCA GCT TAG TG, R - GTC CTG TCA TGT AGC CAC CA). The *B* and *C* alleles are differentiated by a two base-pair (bp) insertion/deletion in the fourth intron that is fully resolved by the capillary system (the full sequences for the fourth intron are available for the *B* and *C* allele on NCBI GenBank from Goodall *et al.* 2006). Sequence analysis of a subset of samples corroborated fragment analysis methods. Fragment analysis peaks were visualized using GENOTYPER (Applied Biosystems, Inc.), and sequence data were analyzed using SEQUENCHER (GeneCodes, Inc.).

3.4.10 *Statistical methods*

Data were assessed for normality (Shapiro-Wilk) and equal variance. To examine the effects of genetic background on the association between carrying the B allele and resistance to parasite infection we used generalized linear models (logit function) to compare resistance (coded as a binomial response for each snail,

infected=1, not infected=0) among genetic backgrounds and SOD1 genotypes. We used regression coefficients from individual logistic regressions to quantify the relative effect sizes of substituting one allele for another in each of the genetic backgrounds. We compared fitness measures (growth rate, egg production, and hatch success) among genetic backgrounds and genotypes using two-way ANOVAs and Tukey post-hoc tests. For mortality we used generalized linear models (logit function, surviving snail at time of census=1, dead snail=0). No transformations were needed to normalize any of these data. We defined significance at the level of alpha = 0.05. For data analyses, we used the statistical packages SPlus version 8.1 for Windows (TIBCO Software, Inc) and SigmaPlot for Windows version 11.0 (Systat Software, Inc).

3.5 Results

3.5.1 *Resistance*

We found main effects of genotype and genetic background, and a background-by-genotype interaction (logit GLM; background: P=0.09, genotype: P=0.003, background x genotype: P=0.022). As expected, the *B* allele was most protective. However, the strength of the association between SOD1 genotype and resistance to infection depended on genetic background. The association was strongest in genetic background 1 and there was a similar but non-significant trend in background 2. In contrast, allelic variation at SOD1 explained little of the variance in resistance in background 3 (Figure 3.3). Substituting a *B* allele for a *C* allele

decreased the odds of infection by 6.2 in genetic background 1, and by 2.5 in genetic background 2 (logit GLM; $P = 0.0027$ and 0.0477, respectively). In genetic background 3 there was no significant additive effect. Thus, the effect of allelic variation at SOD1 on resistance to infection was most important in predicting infection in the genetic background having high average resistance, and was largely irrelevant in the low-resistance genetic background.

3.5.2 *Growth*

With regard to early growth (size at 12 weeks), we found significant main effects of genetic background and SOD1 genotype, but no interaction effect. Surprisingly, individuals with the *CC* genotype were smaller, on average, than those with *BB* and *BC* genotypes (two-way ANOVA; background: $F_{2,151}$=11.07,P<0.001; genotype: $F_{2,151}$=8.11,P<0.001; background x genotype: $F_{4,151}$=0.68, P=0.991) (Figure 3.4A). Thus the *B* allele was associated with faster growth and appeared almost completely dominant to the *C* allele for this trait (Figure 3.4A).

For late growth (size at 32 weeks), we again found significant main effects of genetic background and genotype, and no interaction (two-way ANOVA; background: $F_{2,75}$=39.8, P<0.001; genotype: $F_{2,75}$=3.68, P=0.030; background x genotype: $F_{4,75}$=1.54, P=0.20). The *CC* individuals were still smaller than the *BC* and *BB* individuals, and the *B* allele appeared to act dominantly (Figure 3.4B).

3.5.3 *Fecundity and hatch success*

In regard to egg production, we found a main effect of genetic background, but no main effect of SOD1 genotype and no significant interaction (two-way ANOVA; background: $F_{2,73}$=6.11, P=0.0035; genotype: $F_{2,73}$=0.533, P=0.59; background x genotype: $F_{4,73}$=0.472, P=0.756). The *BB* genotype had the lowest estimated fecundity in genetic backgrounds 1 and 2, but the *CC* genotype had the lowest in background 3 (Figure 3.4C). However, we examined only 10 individuals per genotype within each genetic background, and thus had low power to detect all but strong main or interaction effects, as evidenced from a post-hoc power analysis. Our calculated effect size for the main effect of genetic background was 0.432, while effect sizes for the main effect of genotype and interaction were only 0.15 and 0.17, respectively. Additionally, our calculated power was 0.95 for the main effect of genetic background but only 0.22 and 0.27 for the main effect of genotype and for the interaction, respectively. Thus, an effect of SOD1 genotype on fecundity would have had to be much stronger than observed to be detected with our sample sizes.

Average hatch success across all genetic backgrounds was 49%, and varied from 35% to 62% among genotypes (Appendix Figure 1). We did not find a significant main effect of genetic background or genotype on hatch success (two-way ANOVA; background: $F_{2,60}$=0.47, P=0.62; genotype: $F_{2,60}$=1.52, P=0.23; background x genotype: $F_{4,60}$=0.99, P=0.42). Thus, the *B* allele does not incur an obvious fitness cost associated with egg production (Figure 3.4C) or offspring hatch success. We note that although our average hatch rate of 49% is on the low side of rates reported in the literature, it is not unusually low (e.g. Rey, 1956; Costa et al., 2004).

3.5.4 *Mortality*

At the 8-month census we found significant main effects of both genetic background and genotype on mortality (logit GLM, background: P=0.002, genotype: P=0.04), but no interaction (drop-in-deviance test, P=0.19). *CC* individuals exhibited greater mortality, averaging 37% across genetic backgrounds, whereas *BB* and *BC* average 17% and 13% respectively (Figure 3.4D).

At 12 months we again found a significant main effect of genetic background, but the genotype effect was no longer significant (logit GLM, background: P=0.02, genotype: P=0.18), and there was no interaction (drop-in-deviance test, P=0.39). These results suggest there is no cost to having the *B* allele in terms of increased mortality, and a possible advantage in early survival (Figure 3.4E).

3.6 Discussion

In this study we considered the utility of a resistance-associated locus, cytosolic copper-zinc superoxide dismutase (SOD1) in *Biomphalaria glabrata*, for vector-mediated control of *Schistosoma mansoni*. We looked for evidence of fitness costs in growth rate and reproduction. We also tested for epistatic effects of genetic background by assessing influence of the *B* and *C* alleles on resistance and on life history traits.

3.6.1 *The effect of* SOD1 *on resistance depends on other loci in the genome*

The association between allelic variation at SOD1 and resistance to infection varied substantially among genetic backgrounds. The three genetic backgrounds differed in average resistance (78%, 64%, and 39%; Figure 3.2). SOD1 genotype was most predictive in the genetic background having the highest average resistance, and had a negligible effect in the genetic background having the lowest average resistance (Figure 3.3). Thus, SOD1 appears to interact epistatically with other genes that influence resistance, a result that might help us identify those other loci. That there are other resistance loci segregating in the 13-16-R1 population is evident because inbred lines having identical SOD1 genotypes vary substantially in resistance (Bender and Larson, unpublished observations). Through gene expression studies, several other loci have been identified in *B. glabrata* as being potentially immune relevant (Lockyer et al. 2007, Hanelt et al. 2008, Roger et al. 2008, Ittiprasert et al. 2009, Adema et al. 2010, Hanington et al. 2010, Ittiprasert et al. 2010), and various physiological differences have been noted between snail strains having high or low resistance to trematode parasites (reviewed in (Loker 2010)). However, candidates that seem particularly likely to interact with SOD1 as observed here include loci encoding proteins involved in non-self recognition and loci that control other steps in the oxidative burst pathways. Recognition loci are suggested because, as part of the effector mechanism used by the host to attack the parasite, SOD1 would come into play only after the parasite has been recognized. Thus, SOD1 genotype would be irrelevant in a low-recognition background, but very important in a high-recognition

background. Possible recognition loci include lectin-like molecules such as FREPs (Hanington and Zhang 2011). Loci affecting numbers or some other property of hemocytes might also behave epistatically with SOD1 in a similar manner such that if hemocytes were incompetent (or insufficient in number) to encapsulate the parasite, their ability to produce H_2O_2 would be irrelevant.

3.6.2 *No evidence for a cost of resistance at* SOD1

Costs of resistance have been demonstrated in many systems (Sheldon and Verhulst 1996, Lochmiller and Deerenberg 2000, Zuk and Stoehr 2002, Schmid-Hempel 2003, Schulenburg et al. 2004, Sadd and Schmid-Hempel 2009). Even in *B. glabrata*, there is some evidence that strains with higher resistance to schistosomes differ from strains with lower resistance in components of fitness (Minchella and Loverde 1981, 1983, Langand et al. 1998, Webster and Woolhouse 1999, Webster and Davies 2001, Webster et al. 2003, Webster and Gower 2006),(Salice and Roesijadi 2002). Furthermore, relative to the *A* and *C* alleles, the *B* allele of SOD1 is over-expressed. The SOD1 protein produces H_2O_2, a highly reactive species with the potential to damage host tissue as well as the parasite (Bender et al. 2007). Thus, it would be no surprise to see a cost of resistance associated with the *B* allele at SOD1. Nevertheless, here we failed to detect any disadvantage due to the *B* allele in terms of reproduction, and observed an advantage over the *C* allele in terms of growth rate and survival to 8 months post-hatch (Figure 3.4). Furthermore, there were no significant interactions between SOD1 genotype and genetic background with regard to life

history traits. It is also interesting that the *B* allele acted dominantly to the *C* allele for growth rate (Figure 3.3), a result that might be expected if the difference really results from over-expression of the *B* allele.

Given our data suggest that the *B* allele may confer a slight advantage in terms of growth and early survival, one might wonder why our population has not become fixed for the *B* allele. Possible explanations include: (1) this laboratory maintained population is not in equilibrium and the selection pressure is not strong enough to have driven the allele to higher frequency yet (we have no data on allele frequencies of SOD1 at the founding of this laboratory population); (2) there may be costs to having the *B* allele in other components of fitness that we did not measure; (3) perhaps there are complex interactions among the three major alleles in the population (*A*, *B*, and *C*) that prevent the *B* allele from increasing in frequency (e.g. Hartl and Clark,2007, p. 223-225).

3.6.3 *Potential use of* SOD1 *for vector manipulation: caveats and additional questions*

We showed the promising result of no obvious cost, and perhaps a life history trait advantage for the more-resistant allele at SOD1. Obvious caveats include that our experiments were conducted in a (presumably benign) laboratory setting, and would need to be replicated under field conditions. Other studies have found that costs of resistance are more likely to manifest under specific environmental conditions, such as low food and temperature stress (Sandland and Minchella 2003, Lambrechts et al.

2006a, Lazzaro and Little 2009),(Salice et al. 2010). Of perhaps greater concern is the strong epistatic effect on resistance between SOD1 and other loci in the genome. Defeating an attempted infection is a complex process that involves many steps including recognition, signaling and implementing the effector (killing) mechanisms. SOD1 can participate in both signaling and effector mechanisms, and the products of many loci may need to interact properly to sufficiently clear an infection. Thus, it will be essential to assess the performance of SOD1 in the field and in a variety of other genetic backgrounds.

There are also a number of basic questions, unrelated to those addressed here, about SOD1 and resistance to *S. mansoni* that need to be answered before one could seriously consider using SOD1 for vector manipulation in the field. We still need to prove that the association between resistance and SOD1 alleles is actually causal, and if so, if the protective effect of allele *B* is really owing to its overexpression. It is theoretically possible that SOD1 is not the actual causal locus, but is just in strong linkage disequilibrium with a closely-linked locus that actually controls resistance. This seems unlikely given the association between SOD1 genotype and resistance was discovered using a functional approach (e.g. knocking down H_2O_2 production in *B. glabrata* hemocytes increases their susceptibility to infection (Hahn et al. 2001)), but the functional basis of the association still needs to be proven. Additional work to test the causality of the association is underway. In the unlikely event it turns out that another locus is actually causal, then the results of this study are still quite relevant, but for the new locus of interest.

We also do not know yet if the effect of SOD1 we observed is generalizable to other populations/strains of *S. mansoni*. We have only studied the PR-1 strain of *S. mansoni* in interaction with the 13-16-R1 population of *B. glabrata*. It is possible that the protective effect of SOD1 alleles depends on the strain of parasite in addition to the strain of snail. In a similar vein, we also have no data on if, or how, SOD1 genotype affects resistance to other pathogens. A field population of snails interacts with many pathogens in addition to *S. mansoni*, and there could be fitness tradeoffs associated with other pathogens that render the use of SOD1 for vector manipulation ineffective in some environments.

In summary, we have here shown that, in a laboratory setting, there was no obvious cost to having the most protective allele at SOD1, and perhaps a slight advantage. The generality of this result will need to be verified in other environments, and for other components of fitness. We also demonstrated an effect of genetic background on the association between SOD1 genotype and resistance, a result that points to strong epistatic interactions with other loci in the genome. Clearly SOD1 is not the only locus in the genome that influences resistance. So perhaps vector manipulation will require changes at several interacting loci to insure success. Further work of this sort on SOD1 and other resistance-associated loci will be essential for evaluating the prospects for vector manipulation as a way to control transmission of *S. mansoni.*

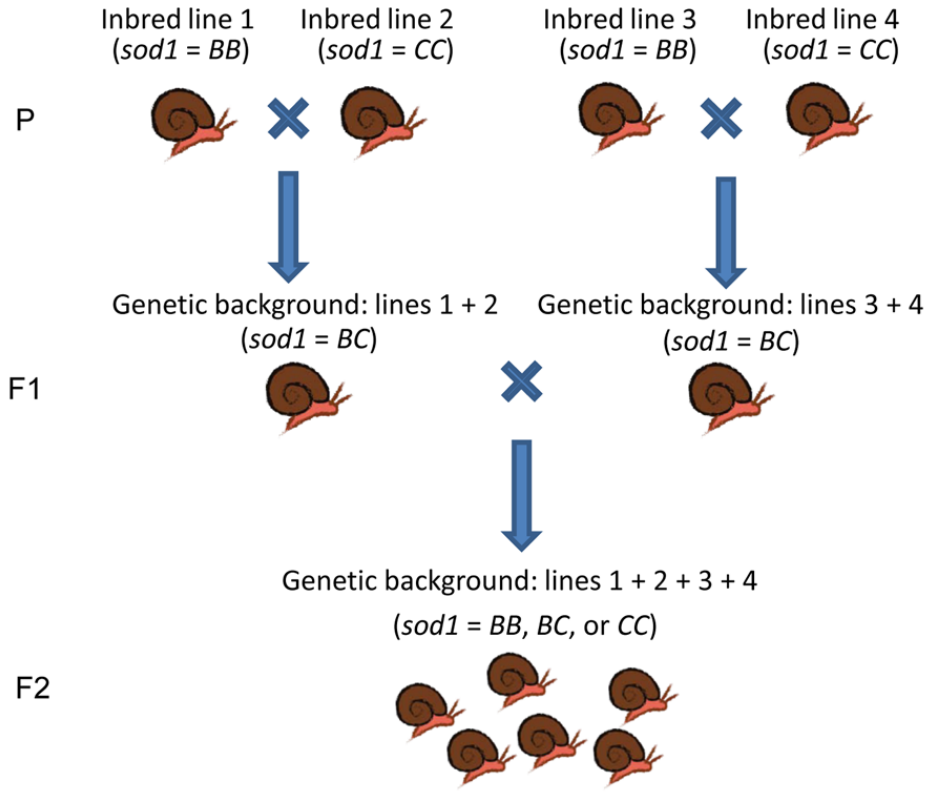


Figure 3.1

Breeding scheme for generating F2 populations with different genetic backgrounds. We created F2 populations by crossing inbred lines (3 generations of selfing) that were fixed for *BB* or *CC* genotypes. F1 offspring from unique inbred line crosses (fixed for the *BC* genotype) were then bred to generate outbred F2 populations that were segregating for *BB*, *BC*, *CC* genotypes in the expected Mendelian ratio. This was done three times to generate three different genetic backgrounds that differ in average resistance.

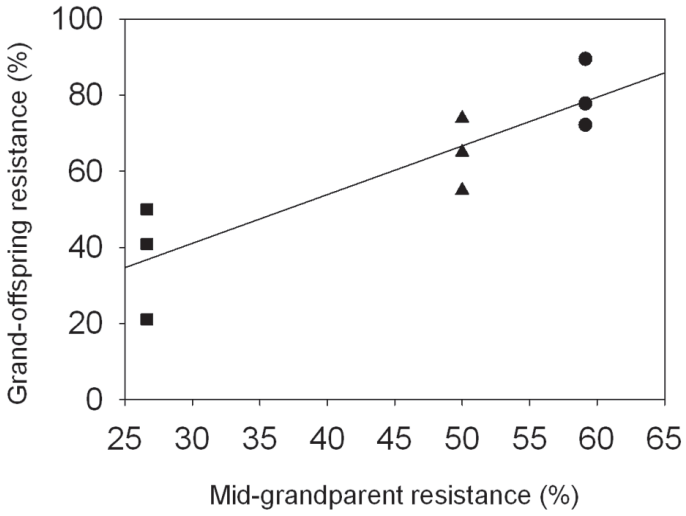


Figure 3.2

Resistance of genetic background as a function of average resistance of grandparental inbred lines. Mid-grandparent resistance was estimated by averaging the resistance of the four inbred, grandparental lines (determined previously). The resistance of each genetic background (Grand-offspring resistance) was estimated by parasite challenges done in triplicate (n=24 x 3) for each background (● genetic background 1, ▲ genetic background 2, and ■ genetic background 3).

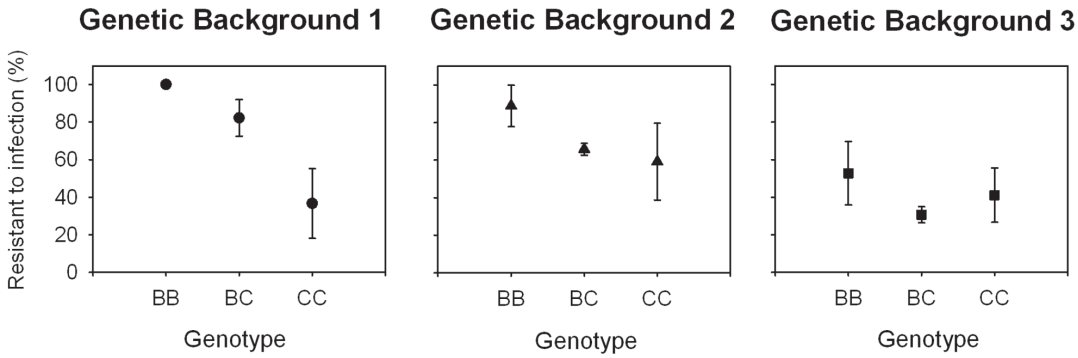


Figure 3.3

Effects of genetic background and SOD1 genotype on resistance to infection. Graphs illustrate the average resistance of each genotypic class within each background after a challenge with five PR-1 *S. mansoni* miracidia. Resistance means are the averages of three replicates (tubs starting with n = 24 snails each). Error bars represent 1±SE (background 1: n=55 (*BB*=10, *BC*=36, *CC*=9), background 2: n=63 (*BB*=8, *BC*=39, *CC*=16), background 3: n=63 (*BB*=14, *BC*=35, *CC*=14)). There were significant main effects of genetic background, genotype, and interaction between genetic background and SOD1 genotype. (See text for statistical analyses.)

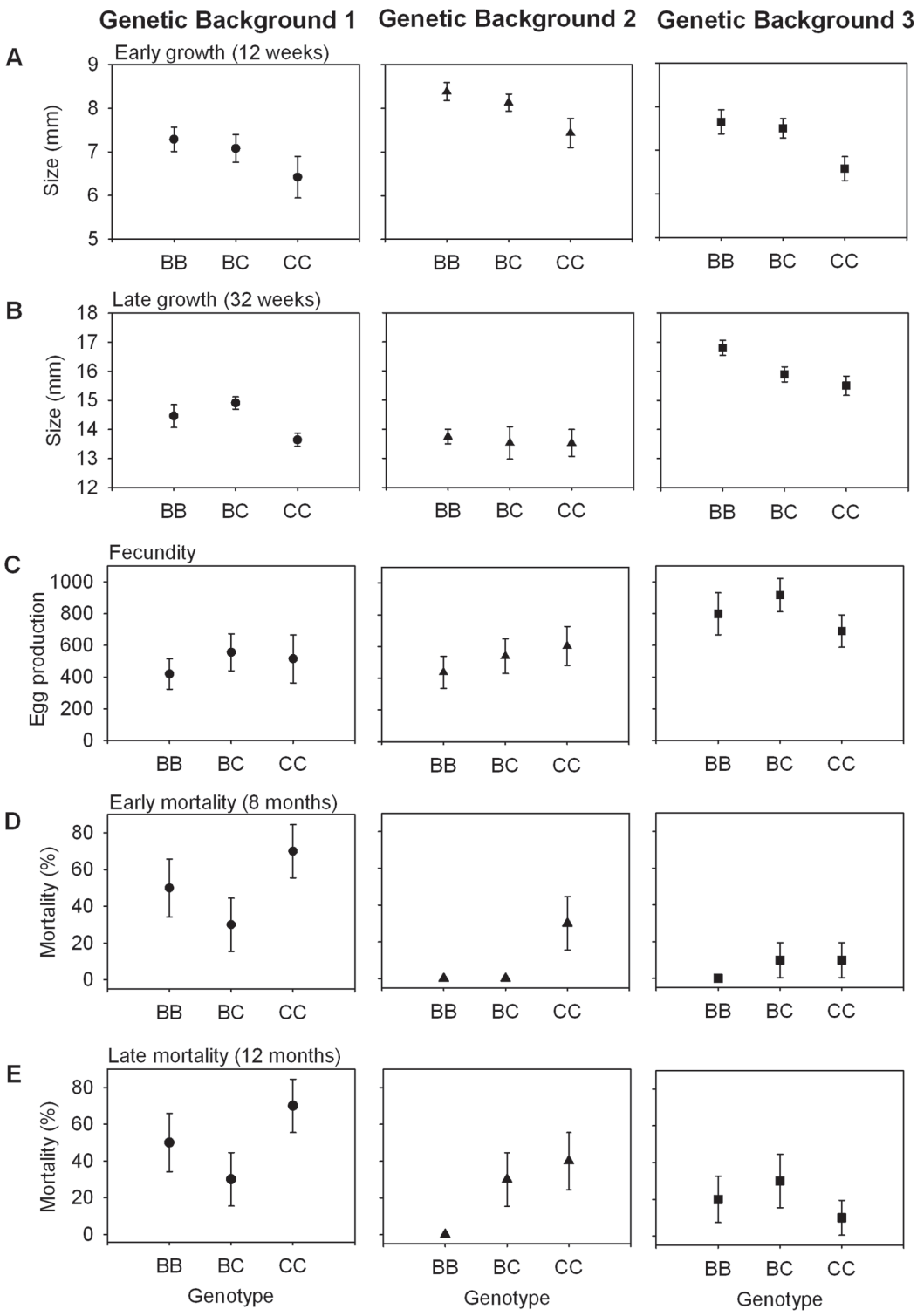

Genetic Background 1
Genetic Background 2
Genetic Background 3
A
Early growth (12 weeks)
Size (mm)
9
8
7
6
5
BB
BC
CC
B
Late growth (32 weeks)
Size (mm)
18
17
16
15
14
13
12
C
Fecundity
Egg production
1000
800
600
400
200
0
D
Early mortality (8 months)
Mortality (%)
80
60
40
20
0
E
Late mortality (12 months)
Mortality (%)
Genotype

Figure 3.4

Effect of genetic background and SOD1 genotype on life-history traits. (A) Average size by genotypic class within each lineage at 12 weeks after egg masses were deposited. The points represent the averages of the mean size of individuals of each genotype within each of 3-4 cups (containing 13-17 F2 snails per cup). Error bars represent 1±SE (background 1: n=45 (*BB*=16, *BC*=20, *CC*=9), background 2: n=57 (*BB*=16, *BC*=28, *CC*=13) background 3: n=58 (*BB*= 16, *BC*=28, *CC*=14)). Snails with the *CC* genotype grew significantly more slowly than those with *BC* and *BB* genotypes. (B) Average size at 32 weeks of each genotypic class within each lineage. Means are the average of all snails within the genotypic class, and error bars represent 1±SE (background 1: n=27 (*BB*=9, *BC*=10, *CC*=8), background 2: n=27 (*BB*=9, *BC*=10, *CC*=8), background 3: n=30 (*BB*= 10, *BC*=10, *CC*=10)). Again, snails with the *CC* genotype grew significantly more slowly than those with *BC* and *BB* genotypes. (C) Average total egg production for five weeks per snail (each raised individually) by genotypic class within each lineage. Means are the average of all snails within the genotypic class, and error bars represent 1±SE (background 1: n= 25 (*BB*=9, *BC*=9,*CC*=7), background 2: n= 27 (*BB*=9, *BC*=10, *CC*=8), background 3: n= 30 (*BB*=10, *BC*=10,*CC*=10)). (D) Mortality at 8-month census of each genotypic class within each lineage. Data points are estimates of the percent mortality in each genotypic class and error bars represent 1±SE on the proportion (for all backgrounds n=30 (*BB*=10, *BC*=10, *CC*=10)). Snails with the *CC* genotype exhibited significantly greater mortality than those with the *BB* or *BC* genotype. (E) Mortality at 12-month census of each genotypic class within each lineage. Data points are estimates of percent mortality in each genotypic class and error bars represent 1±SE on the proportion (for all backgrounds n=30 (*BB*=10, *BC*=10, *CC*=10)).

CHAPTER 4

CONCLUSIONS

The overarching goal of my dissertation was to further evaluate the utility of SOD1 as a resistance locus in *B. glabrata*. The *B. glabrata*/*S. mansoni* study system remains the premier model for studying schistosomiasis. Establishing the utility and relevance of research on model organisms in controlled laboratory environments to natural systems is necessary when attempting to determine how well the laboratory model predicts natural world phenomena (Boete 2005, Lambrechts et al. 2007, Steinauer and Bonner 2012). Thus, it is imperative that we are able to apply knowledge gained about the genetic mechanisms that contribute to snail resistance in the lab to natural populations. The interest in SOD1 as a potential resistance locus in *B. glabrata* was introduced by Goodall et al (2006), where allelic variation at the fourth intron of SOD1 was associated with resistance to infection. Additionally, elevated gene expression of the most resistant allele was observed relative to the other allelic variants (Bender et al 2007). However, both of these studies used a hybrid strain of *B. glabrata*, the 13-16-R1 strain. Due to the hybrid nature of the strain, it is possible that the co-occurrence of the alleles in the lab population is an artifact of the breeding history rather than reflective of natural populations (Newton 1955, Richards and Merritt 1972, DeJong et al. 2003). Determining if allelic variation occurs in natural populations is essential because the opportunity for selection to act on SOD1 in natural populations depends on the presence of allelic variation in natural populations.

The focus of the second chapter of my dissertation was to investigate the ecological relevance of allelic variation at SOD1 in natural populations. The results of this study suggest that SOD1 could play a role in determining resistance in natural populations. Since different alleles do co-occur in natural populations, the relative selective advantage of specific alleles at SOD1 observed in the lab could potentially occur in natural populations. Additionally, I identified sequence variants in natural populations similar to those observed in the 13-16-R1 lab strain. The most resistant allele in the 13-16-R1 population was the only identical sequence variant observed in natural populations. Interestingly, the allele that confers resistance in the 13-16-R1 lab strain when challenged with a Puerto Rican parasite (PR-1) was only found in Brazilian populations. Also interesting is that although the strain history suggests breeding events between Caribbean and Brazilian populations, the sequence variants most similar to the lab strain were all located in Brazil.

The heterozygote excess (negative F_{IS}) I observed at SOD1 in the majority of the populations that were polymorphic is consistent with overdominance. Additionally, the populations that were segregating for the most resistant allele had a positive Tajima's D for SOD1, indicating balancing selection might be acting on SOD1 (Tajima 1989). To determine if these patterns were the result of a genome-wide phenomenon or the result of selection acting at SOD1 I looked for evidence of overdominance at nine additional putatively neutral loci and two additional sequence loci for balancing selection. I found similar patterns of Tajima's D at the other two neutral sequence loci suggesting that the deviations from neutral expectation observed

at SOD1 initially were likely the result of population demographics. However, SOD1 did have the lowest F_{IS} of all loci in all populations that were segregating for resistant alleles, which supports the overdominance hypothesis.

Allelic variation at SOD1 correlates with resistance in a laboratory setting, and allelic variation is known to occur in natural populations. Additionally, heterozygote excess was observed in those populations carrying the allele that confers resistance in the lab. These results suggest that SOD1 could be playing a role in determining resistance in natural populations of *B. glabrata*. As with other vector-borne diseases, there is interest in identifying and utilizing resistance genes for vector manipulation because this approach offers advantages over current control methods. However, there are many problems that can plague the successful introduction of a resistant allele into a population, including: protection from resistance genes may vary in the field compared to the lab (GxE interactions), genetic variability in both parasite and host populations might be greater in the field making it difficult to predict the interactions between host genotypes and parasite genotypes in natural populations (GxG interactions), the different genetic backgrounds in natural populations may influence how a resistance gene performs in a natural versus a lab population (epistasis), and there may be a cost of resistance such that natural selection in the absence of parasite pressure favors the non-resistant allele. All of these factors must be assessed prior to the introduction of resistant vectors into natural populations.

In my third chapter I evaluated the potential for a cost of resistance associated with carrying the resistant allele at SOD1 on fitness (fecundity, growth, and survival)

and the effects of genetic background. I found that the association between allelic variation at SOD1 and resistance to infection varied substantially among genetic backgrounds. Thus, SOD1 appears to interact epistatically with other genes that influence resistance. Additionally, I failed to detect any disadvantage due to carrying the resistant allele in terms of reproduction, and observed an advantage in terms of growth rate and survival. Therefore, we found no obvious cost, and perhaps a life history trait advantage, for the more-resistant allele at SOD1. However, due to the genetic background effect observed in this study, the use of SOD1 for vector manipulation must be evaluated in each population prior to introduction because in some genetic backgrounds the protective effect of the resistant allele is either reduced or not protective at all, while in others it confers high resistance.

My research on the role of allelic variation at SOD1 on resistance in *B. glabrata* reveals other interesting research questions that deserve study. While I have demonstrated that natural populations show allelic variation similar to that in our laboratory population, we still do not know if that allelic variation correlates with resistance to *S. mansoni* in natural populations of *B. glabrata*. Also, these snails are host to many other pathogens and parasites. Therefore, understanding how allelic variation at SOD1 correlates with resistance to other, possibly more common, pathogens and parasites is another interesting research avenue. Also, while I found no fitness cost associated with carrying the resistant allele in the lab population under controlled laboratory conditions, determining whether there is a cost in a more variable environment (which natural populations are more likely to encounter) is of

importance when considering SOD1 for use in vector manipulation. I found that there was a genetic background effect. This result could lead to the identification of other loci involved in determining resistance. Finally, the molecular mechanism by which allelic variation at SOD1 affects resistance still needs to be proven. It needs to be verified that SOD1 is not simply acting as a marker for another causal locus that is closely linked to SOD1. Therefore, while my research has contributed to our understanding of the genetic mechanisms of resistance in *Biomphalaria glabrata*, there are ample opportunities for further investigations on role of allelic variation at SOD1 and other genetic mechanisms of resistance.

BIBLIOGRAPHY

Abreu, I. A. and D. E. Cabelli. 2010. Superoxide dismutases-a review of the metal-associated mechanistic variations. Biochimica Et Biophysica Acta-Proteins and Proteomics 1804:263-274.

Adema, C. M., P. C. Hanington, C. M. Lun, G. H. Rosenberg, A. D. Aragon, B. A. Stout, M. L. L. Richard, P. S. Gross, and E. S. Loker. 2010. Differential transcriptomic responses of Biomphalaria glabrata (Gastropoda, Mollusca) to bacteria and metazoan parasites, Schistosoma mansoni and Echinostoma paraensei (Digenea, Platyhelminthes). Molecular Immunology 47:849-860.

Antonovics, J. and P. H. Thrall. 1994. Cost of Resistance and the Maintenance of Genetic-Polymorphism in Host-Pathogen Systems. Proceedings of the Royal Society of London Series B-Biological Sciences 257:105-110.

Bakker, E. G., C. Toomajian, M. Kreitman, and J. Bergelson. 2006. A genome-wide survey of R gene polymorphisms in Arabidopsis. Plant Cell 18:1803-1818.

Balloux, F. 2004. Heterozygote excess in small populations and the heterozygote-excess effective population size. Evolution 58:1891-1900.

Bayne, C. J. 2009. Successful parasitism of vector snail Biomphalaria glabrata by the human blood fluke (trematode) Schistosoma mansoni: A 2009 assessment. Molecular and Biochemical Parasitology 165:8-18.

Bayne, C. J., U. K. Hahn, and R. C. Bender. 2001. Mechanisms of molluscan host resistance and of parasite strategies for survival. Parasitology 123:S159-S167.

Bender, R. C., E. J. Broderick, C. P. Goodall, and C. J. Bayne. 2005. Respiratory burst of Biomphalaria glabrata hemocytes: Schistosoma mansoni-resistant snails produce more extracellular H2O2 than susceptible snails. Journal of Parasitology 91:275-279.

Bender, R. C., C. P. Goodall, M. S. Blouin, and C. J. Bayne. 2007. Variation in expression of Biomphalaria glabrata SOD1: A potential controlling factor in susceptibility/resistance to Schistosoma mansoni. Developmental and Comparative Immunology 31:874-878.

Blouin, M. S., K. M. Bonner, B. Cooper, V. Amarasinghe, R. P. O'Donnell, and C. J. Bayne. 2013. Three genes involved in the oxidative burst are closely linked in the genome of the snail, Biomphalaria glabrata. International Journal for Parasitology 43:51-55.

Boete, C. 2005. Malaria parasites in mosquitoes: laboratory models, evolutionary temptation and the real world. Trends in Parasitology 21:445-447.

Bonner, K. M., C. J. Bayne, M. K. Larson, and M. S. Blouin. 2012. Effects of Cu/Zn Superoxide Dismutase (sod1) Genotype and Genetic Background on Growth, Reproduction and Defense in Biomphalaria glabrata. Plos Neglected Tropical Diseases 6.

Chitsulo, L., D. Engels, A. Montresor, and L. Savioli. 2000. The global status of schistosomiasis and its control. Acta Tropica 77:41-51.

Clement, M., D. Posada, and K. A. Crandall. 2000. TCS: a computer program to estimate gene genealogies. Molecular Ecology 9:1657-1659.

Coelho, P. M. Z., F. M. Rosa, E. Maciel, D. A. Negrao-Correa, O. S. Carvalho, R. L. Caldeira, L. K. Jannotti-Passos, L. A. Moreira, G. C. Oliveira, and H. M. S. Teles. 2008. Transmission control of schistosomiasis mansoni by introduction of a resistant strain of Biomphalaria tenagophila in areas where transmission is maintained by this species. Acta Tropica 108:245-248.

Cohuet, A., C. Harris, V. Robert, and D. Fontenille. 2010. Evolutionary forces on Anopheles: what makes a malaria vector? Trends in Parasitology 26:130-136.

Coustau, C., C. Chevillon, and R. ffrench-Constant. 2000. Resistance to xenobiotics and parasites: can we count the cost? Trends in Ecology & Evolution 15:378-383.

Crompton, D. W. T. 1999. How much human helminthiasis is there in the world? Journal of Parasitology 85:397-403.

de Roode, J. C., J. Chi, R. M. Rarick, and S. Altizer. 2009. Strength in numbers: high parasite burdens increase transmission of a protozoan parasite of monarch butterflies (Danaus plexippus). Oecologia 161:67-75.

DeJong, R. J., J. A. T. Morgan, W. L. Paraense, J. P. Pointier, M. Amarista, P. F. K. Ayeh-Kumi, A. Babiker, C. S. Barbosa, P. Bremond, A. P. Canese, C. P. de Souza, C. Dominguez, S. File, A. Gutierrez, R. N. Incani, T. Kawano, F. Kazibwe, J. Kpikpi, N. J. S. Lwambo, R. Mimpfoundi, F. Njiokou, J. N. Poda, M. Sene, L. E. Velasquez, M. Yong, C. M. Adema, B. V. Hofkin, G. M. Mkoji, and E. S. Loker. 2001. Evolutionary relationships and biogeography of Biomphalaria (Gastropoda : Planorbidae) with implications regarding its role as host of the human bloodfluke, Schistosoma mansoni. Molecular Biology and Evolution 18:2225-2239.

DeJong, R. J., J. A. T. Morgan, W. D. Wilson, M. H. Al-Jaser, C. C. Appleton, G. Coulibaly, P. S. D'Andrea, M. J. Doenhoff, W. Haas, M. A. Idris, L. A. Magalhaes, H. Mone, G. Mouahid, L. Mubila, J. P. Pointier, J. P. Webster, E. M. Zanotti-Magalhaes, W. L. Paraense, G. M. Mkoji, and E. S. Loker. 2003. Phylogeography of Biomphalaria glabrata and B-pfeifferi, important intermediate hosts of Schistosoma mansoni in the New and Old World tropics. Molecular Ecology 12:3041-3056.

Dowling, D. K. and L. W. Simmons. 2009. Reactive oxygen species as universal constraints in life-history evolution. Proceedings of the Royal Society B-Biological Sciences 276:1737-1745.

Fenwick, A. 2006. Towards the control of schistosomiasis, intestinal helminths and other neglected tropical diseases in Africa. American Journal of Tropical Medicine and Hygiene 75:273-273.

Fenwick, A. and J. P. Webster. 2006. Schistosomiasis: challenges for control, treatment and drug resistance. Current Opinion in Infectious Diseases 19:577-582.

Fink, R. C. and J. G. Scandalios. 2002. Molecular evolution and structure-function relationships of the superoxide dismutase gene families in angiosperms and their relationship to other eukaryotic and prokaryotic superoxide dismutases. Archives of Biochemistry and Biophysics 399:19-36.

Galinier, R., J. Portela, Y. Mone, J. F. Allienne, H. Henri, S. Delbecq, G. Mitta, B. Gourbal, and D. Duval. 2013. Biomphalysin, a New beta Pore-forming Toxin Involved in Biomphalaria glabrata Immune Defense against Schistosoma mansoni. Plos Pathogens 9.

Gandon, S. and S. L. Nuismer. 2009. Interactions between Genetic Drift, Gene Flow, and Selection Mosaics Drive Parasite Local Adaptation. American Naturalist 173:212-224.

Garcia, A. B., R. J. Pierce, B. Gourbal, E. Werkmeister, D. Colinet, J. M. Reichhart, C. Dissous, and C. Coustau. 2010. Involvement of the Cytokine MIF in the Snail Host Immune Response to the Parasite Schistosoma mansoni. Plos Pathogens 6.

Garrigan, D. and P. W. Hedrick. 2003. Perspective: Detecting adaptive molecular polymorphism: Lessons from the MHC. Evolution 57:1707-1722.

Goodall, C. P., R. C. Bender, E. J. Broderick, and C. J. Bayne. 2004. Constitutive differences in Cu/Zn superoxide dismutase mRNA levels and activity in hernocytes of Biomphalaria glabrata (Mollusca) that are either susceptible or resistant to Schistosoma mansoni (Trematoda). Molecular and Biochemical Parasitology 137:321-328.

Goodall, C. P., R. C. Bender, J. K. Brooks, and C. J. Bayne. 2006. Biomphalaria glabrata cytosolic copper/zinc superoxide dismutase (SOD1) gene: Association of SOD1 alleles with resistance/susceptibility to Schistosoma mansoni. Molecular and Biochemical Parasitology 147:207-210.

Hahn, U. K., R. C. Bender, and C. J. Bayne. 2001. Killing of Schistosoma mansoni sporocysts by hemocytes from resistant Biomphalaria glabrata: Role of reactive oxygen species. Journal of Parasitology 87:292-299.

Hanelt, B., C. M. Lun, and C. M. Adema. 2008. Comparative ORESTES-sampling of transcriptomes of immune-challenged Biomphalaria glabrata snails. Journal of Invertebrate Pathology 99:192-203.

Hanington, P. C., C. M. Lun, C. M. Adema, and E. S. Loker. 2010. Time series analysis of the transcriptional responses of Biomphalaria glabrata throughout the course of intramolluscan development of Schistosoma mansoni and Echinostoma paraensei. International Journal for Parasitology 40:819-831.

Hanington, P. C. and S. M. Zhang. 2011. The Primary Role of Fibrinogen-Related Proteins in Invertebrates Is Defense, Not Coagulation. Journal of Innate Immunity 3:17-27.

Hedrick, P. W. 2005. Genetics of Populations. 3rd edition. Jones and Bartlett Publishers, Sudbury, Massachusetts.

Hotez, P. J. and B. Pecoul. 2010. "Manifesto" for Advancing the Control and Elimination of Neglected Tropical Diseases. Plos Neglected Tropical Diseases 4.

Huvet, A., J. Normand, E. Fleury, V. Quillien, C. Fabioux, and P. Boudry. 2010. Reproductive effort of Pacific oysters: A trait associated with susceptibility to summer mortality. Aquaculture 304:95-99.

Ittiprasert, W. and M. Knight. 2012. Reversing the Resistance Phenotype of the Biomphalaria glabrata Snail Host Schistosoma mansoni Infection by Temperature Modulation. Plos Pathogens 8.

Ittiprasert, W., A. Miller, J. Myers, V. Nene, N. M. El-Sayed, and M. Knight. 2010. Identification of immediate response genes dominantly expressed in juvenile resistant and susceptible Biomphalaria glabrata snails upon exposure to Schistosoma mansoni. Molecular and Biochemical Parasitology 169:27-39.

Ittiprasert, W., A. Miller, X. Z. Su, J. B. Mu, G. Bhusudsawang, K. Ukoskit, and M. Knight. 2013. Identification and characterisation of functional expressed sequence tags-derived simple sequence repeat (eSSR) markers for genetic linkage mapping of Schistosoma mansoni juvenile resistance and susceptibility loci in Biomphalaria glabrata. International Journal for Parasitology 43:669-677.

Ittiprasert, W., R. Nene, A. Miller, N. Raghavan, F. Lewis, J. Hodgson, and M. Knight. 2009. Schistosoma mansoni infection of juvenile Biomphalaria glabrata induces a differential stress response between resistant and susceptible snails. Experimental Parasitology 123:203-211.

Kariuki, H. C., H. Madsen, J. H. Ouma, A. E. Butterworth, D. W. Dunne, M. Booth, G. Kimani, J. K. Mwatha, E. Muchiri, and B. J. Vennervald. 2013. Long term study on the effect of mollusciciding with niclosamide in stream habitats on the transmission of schistosomiasis mansoni after community-based chemotherapy in Makueni District, Kenya. Parasites & Vectors 6.

King, C. H. 2010. Parasites and poverty: The case of schistosomiasis. Acta Tropica 113:95-104.

LaBeaud, A. D. and S. Aksoy. 2010. Neglected Funding for Vector-Borne Diseases: A Near Miss This Time, a Possible Disaster the Next Time. Plos Neglected Tropical Diseases 4.

Lambrechts, L., J. M. Chavatte, G. Snounou, and J. C. Koella. 2006a. Environmental influence on the genetic basis of mosquito resistance to malaria parasites. Proceedings of the Royal Society B-Biological Sciences 273:1501-1506.

Lambrechts, L., S. Fellous, and J. C. Koella. 2006b. Coevolutionary interactions between host and parasite genotypes. Trends in Parasitology 22:12-16.

Lambrechts, L., I. Morlais, P. H. Awono-Ambene, A. Cohuet, F. Simard, J. C. Jacques, C. Bourgouin, and J. C. Koella. 2007. Effect of infection by Plasmodium falciparum on the melanization immune response of Anopheles gambial. American Journal of Tropical Medicine and Hygiene 76:475-480.

Lambrechts, L., J. C. Koella, and C. Boete. 2008. Can transgenic mosquitoes afford the fitness cost? Trends Parasitol 24:4-7.

Langand, J., J. Jourdane, C. Coustau, B. Delay, and S. Morand. 1998. Cost of resistance, expressed as a delayed maturity, detected in the host-parasite system Biomphalaria glabrata Echinostoma caproni. Heredity 80:320-325.

Lazzaro, B. P. and T. J. Little. 2009. Immunity in a variable world. Philosophical Transactions of the Royal Society B-Biological Sciences 364:15-26.

Lewis, F. A., C. N. Patterson, and C. Grzywacz. 2003. Parasite-susceptibility phenotypes of F-1 Biomphalaria glabrata progeny derived from interbreeding Schistosoma mansoni-resistant and -susceptible snails. Parasitology Research 89:98-101.

Librado, P. and J. Rozas. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451-1452.

Lively, C. M. 1999. Migration, virulence, and the geographic mosaic of adaptation by parasites. American Naturalist 153:S34-S47.

Lively, C. M. and M. F. Dybdahl. 2000. Parasite adaptation to locally common host genotypes. Nature 405:679-681.

Lochmiller, R. L. and C. Deerenberg. 2000. Trade-offs in evolutionary immunology: just what is the cost of immunity? Oikos 88:87-98.

Lockyer, A. E., A. M. Emery, R. A. Kane, A. J. Walker, C. D. Mayer, G. Mitta, C. Coustau, C. M. Adema, B. Hanelt, D. Rollinson, L. R. Noble, and C. S. Jones. 2012. Early Differential Gene Expression in Haemocytes from Resistant and Susceptible Biomphalaria glabrata Strains in Response to Schistosoma mansoni. Plos One 7.

Lockyer, A. E., C. S. Jones, L. R. Noble, and D. Rollinson. 2004. Trematodes and snails: an intimate association. Canadian Journal of Zoology-Revue Canadienne De Zoologie 82:251-269.

Lockyer, A. E., J. Spinks, L. R. Noble, D. Rollinson, and C. S. Jones. 2007a. Identification of genes involved in interactions between Biomphalaria glabrata and Schistosoma mansoni by suppression subtractive hybridization. Molecular and Biochemical Parasitology 151:18-27.

Lockyer, A. E., J. N. Spinks, A. J. Walker, R. A. Kane, L. R. Noble, D. Rollinson, E. Dias-Neto, and C. S. Jones. 2007b. Biomphalaria glabrata transcriptome: Identification of cell-signalling, transcriptional control and immune-related genes from open reading frame expressed sequence tags (ORESTES). Developmental and Comparative Immunology 31:763-782.

Loker, E. S. 2010. Gastropod Immunobiology. Invertebrate Immunity 708:17-43.

Luikart, G. and J. M. Cornuet. 1999. Estimating the effective number of breeders from heterozygote excess in progeny. Genetics 151:1211-1216.

Marrelli, M. T., C. Li, J. L. Rasgon, and M. Jacobs-Lorena. 2007. Transgenic malaria-resistant mosquitoes have a fitness advantage when feeding on Plasmodium-infected blood. Proc Natl Acad Sci U S A 104:5580-5583.

Marrelli, M. T., C. K. Moreira, D. Kelly, L. Alphey, and M. Jacobs-Lorena. 2006. Mosquito transgenesis: what is the fitness cost? Trends Parasitol 22:197-202.

Mavarez, I., C. Steiner, J. P. Pointier, and P. Jarne. 2002a. Evolutionary history and phylogeography of the schistosome-vector freshwater snail Biomphalaria glabrata based on nuclear and mitochondrial DNA sequences. Heredity 89:266-272.

Mavarez, J., M. Amarista, J. P. Pointier, and P. Jarne. 2002b. Fine-scale population structure and dispersal in Biomphalaria glabrata, the intermediate snail host of Schistosoma mansoni, in Venezuela. Molecular Ecology 11:879-889.

Melman, S. D., M. L. Steinauer, C. Cunningham, L. S. Kubatko, I. N. Mwangi, N. B. Wynn, M. W. Mutuku, D. M. S. Karanja, D. G. Colley, C. L. Black, W. E. Secor, G. M. Mkoji, and E. S. Loker. 2009. Reduced Susceptibility to Praziquantel among Naturally Occurring Kenyan Isolates of Schistosoma mansoni. Plos Neglected Tropical Diseases 3.

Minchella, D. J. and P. T. Loverde. 1981. A Cost of Increased Early Reproductive Effort in the Snail Biomphalaria-Glabrata. American Naturalist 118:876-881.

Minchella, D. J. and P. T. Loverde. 1983. Laboratory Comparison of the Relative Success of Biomphalaria-Glabrata Stocks Which Are Susceptible and Insusceptible to Infection with Schistosoma-Mansoni. Parasitology 86:335-344.

Mitta, G., C. M. Adema, B. Gourbal, E. S. Loker, and A. Theron. 2012. Compatibility polymorphism in snail/schistosome interactions: From field to theory to molecular mechanisms. Developmental and Comparative Immunology 37:1-8.

Monaghan, P., N. B. Metcalfe, and R. Torres. 2009. Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. Ecology Letters 12:75-92.

Moné, Y., B. Gourbal, D. Duval, L. Du Pasquier, S. Kieffer-Jaquinod, and G. Mitta. 2010. A large repertoire of parasite epitopes matched by a large repertoire of host immune receptors in an invertebrate host/parasite model. PLoS Negl Trop Dis 4.

Moné, Y., A. C. Ribou, C. Cosseau, D. Duval, A. Theron, G. Mitta, and B. Gourbal. 2011. An example of molecular co-evolution: Reactive oxygen species (ROS) and ROS scavenger levels in Schistosoma mansoni/Biomphalaria glabrata interactions. International Journal for Parasitology 41:721-730.

Morgan, J. A. T., R. J. Dejong, S. D. Snyder, G. M. Mkoji, and E. S. Loker. 2001. Schistosoma mansoni and Biomphalaria: past history and future trends. Parasitology 123:S211-S228.

Nappi, A. J. and E. Ottaviani. 2000. Cytotoxicity and cytotoxic molecules in invertebrates. Bioessays 22:469-480.

Newton, W. L. 1955. The establishment of a strain of *Australorbis glabratus* which combines albinism and high susceptibility to infection with *Schistosoma mansoni*. The Journal of Parasitology 41:526-528.

Nielsen, R. 2005. Molecular signatures of natural selection. Annual Review of Genetics 39:197-218.

Piertney, S. B. and M. K. Oliver. 2006. The evolutionary ecology of the major histocompatibility complex. Heredity 96:7-21.

Pudovkin, A. I., D. V. Zaykin, and D. Hedgecock. 1996. On the potential for estimating the effective number of breeders from heterozygote-excess in progeny. Genetics 144:383-387.

Ramasarma, T. 2007. Many faces of superoxide dismutase, originally known as erythrocuprein. Current Science 92:184-191.

Rasmussen, D. I. 1979. Sibling Clusters and Genotypic Frequencies. American Naturalist 113:948-951.

Rashed, A., B. Hamilton, and M. Polak. 2008. Ectoparasite Resistance Is Correlated with Reduced Host Egg Hatch Rate in the Drosophila-Macrocheles System. Environmental Entomology 37:1099-1104.

Raymond, M. and F. Rousset. 1995. Genepop (Version-1.2) - Population-Genetics Software for Exact Tests and Ecumenicism. Journal of Heredity 86:248-249.

Reznick, D. 1992. Measuring the Costs of Reproduction. Trends in Ecology & Evolution 7:42-45.

Richards, C. S. and J. W. Merritt. 1972. Genetic Factors in Susceptibility of Juvenile Biomphalaria-Glabrata to Schistosoma-Mansoni Infection. American Journal of Tropical Medicine and Hygiene 21:425-&.

Robertson, A. 1965. The interpretation of genotypic ratios in domestic animal populations. Animal Production 7:319-324.

Roff, D. A. 1992. The evolution of life histories : theory and analysis. Chapman & Hall, New York.

Roger, E., G. Mitta, Y. Mone, A. Bouchut, A. Rognon, C. Grunau, J. Boissier, A. Theron, and B. E. F. Gourbal. 2008. Molecular determinants of compatibility polymorphism in the Biomphalaria glabrata/Schistosoma mansoni model: New candidates identified by a global comparative proteomics approach. Molecular and Biochemical Parasitology 157:205-216.

Rosa, F. M., A. L. B. Godard, D. Negrao-Correa, H. A. Rodrigues, O. D. Carvalho, R. L. Caldeira, H. M. S. Teles, E. Maciel, L. K. Jannotti-Passos, and P. M. Z. Coelho. 2006. Biomphalaria tenagophila: dynamics of populations of resistant and susceptible strains to Schistosoma mansoni, with or without pressure of the parasite. Memorias Do Instituto Oswaldo Cruz 101:247-251.

Ross, A. G. P., P. B. Bartley, A. C. Sleigh, G. R. Olds, Y. S. Li, G. M. Williams, and D. P. McManus. 2002. Current concepts - Schistosomiasis. New England Journal of Medicine 346:1212-1220.

Shoukry, N., El-Assal, FM, Soliman, GN, Mansour NS. 1997. Susceptibility of three successive snail generations from positive and negative laboratory bred *Biomphalaria alexandrina* from different localities in Egypt to infection with

Schistosoma mansoni from Giza. Journal of the Egyptian Socieity of Parasitology 27:317-329.

Sadd, B. M. and P. Schmid-Hempel. 2009. Principles of ecological immunology. Evolutionary Applications 2:113-121.

Salice, C. J., T. A. Anderson, and G. Roesijadi. 2010. Adaptive responses and latent costs of multigeneration cadmium exposure in parasite resistant and susceptible strains of a freshwater snail. Ecotoxicology 19:1466-1475.

Salice, C. J. and G. Roesijadi. 2002. Resistance to cadmium and parasite infection are inversely related in two strains of a freshwater gastropod. Environmental Toxicology and Chemistry 21:1398-1403.

Sandland, G. J. and D. J. Minchella. 2003. Costs of immune defense: an enigma wrapped in an environmental cloak? Trends in Parasitology 19:571-574.

Schmid-Hempel, P. 2003. Variation in immune defence as a question of evolutionary ecology. Proceedings of the Royal Society of London Series B-Biological Sciences 270:357-366.

Schmid-Hempel, R. and P. Schmid-Hempel. 1998. Colony performance and immunocompetence of a social insect, Bombus terrestris, in poor and variable environments. Functional Ecology 12:22-30.

Schulenburg, H., C. L. Kurz, and J. J. Ewbank. 2004. Evolution of the innate immune system: the worm perspective. Immunological Reviews 198:36-58.

Seppala, O. and J. Jokela. 2010. Maintenance of Genetic Variation in Immune Defense of a Freshwater Snail: Role of Environmental Heterogeneity. Evolution 64:2397-2407.

Sheldon, B. C. and S. Verhulst. 1996. Ecological immunology: Costly parasite defences and trade-offs in evolutionary ecology. Trends in Ecology & Evolution 11:317-321.

Steinauer, M. L. and K. M. Bonner. 2012. Host Susceptibility Is Altered by Light Intensity After Exposure to Parasites. Journal of Parasitology 98:1052-1054.

Steinmann, P., J. Keiser, R. Bos, M. Tanner, and J. Utzinger. 2006. Schistosomiasis and water resources development: systematic review, meta-analysis, and estimates of people at risk. Lancet Infectious Diseases 6:411-425.

Su, C. and M. Nei. 1999. Fifty-million-year-old polymorphism at an immunoglobulin variable region gene locus in the rabbit evolutionary lineage. Proceedings of the National Academy of Sciences of the United States of America 96:9710-9715.

Svensson, E., L. Raberg, C. Koch, and D. Hasselquist. 1998. Energetic stress, immunosuppression and the costs of an antibody response. Functional Ecology 12:912-919.

Tajima, F. 1989. Statistical-Method for Testing the Neutral Mutation Hypothesis by DNA Polymorphism. Genetics 123:585-595.

Tennessen, J. A. and M. S. Blouin. 2008. Balancing Selection at a Frog Antimicrobial Peptide Locus: Fluctuating Immune Effector Alleles? Molecular Biology and Evolution 25:2669-2680.

Théron, A. and C. Coustau. 2005. Are Biomphalaria snails resistant to Schistosoma mansoni? Journal of Helminthology 79:187-191.

Théron, A., C. Coustau, A. Rognon, S. Gourbiere, and M. S. Blouin. 2008. Effects of laboratory culture on compatibility between snails and schistosomes. Parasitology 135:1179-1188.

Townson, H., M. B. Nathan, M. Zaim, P. Guillet, L. Manga, R. Bos, and M. Kindhauser. 2005. Exploiting the potential of vector control for disease prevention. Bulletin of the World Health Organization 83:942-947.

Ulmer, M. J. 1970. Notes on rearing of snails in the laboratory. Pages 143-144 *in* A. J. MacInnis, Voge, M., editor. Experiments and techniques in parasitology. W. H. Freeman and Co., San Francisco, California.

Vianey-Liaud, M. 1992. Sperm storage time in *Biomphalaria glabrata* (Gastropoda: Planorbidae): fertilization of albino snails by surgically castrated pigmented snails. Journal of Medical and Applied Malacology 4:99-101.

Vianey-Liaud, M. 1995. Bias in the production of heterozygous pigmented embros from succively mated *Biomphalaria glabrata* (Gastropoda: Planorbidae) albino snails. Malacological Review 28:97-106.

Vianey-Liaud, M. and G. Dussart. 2002. Aspects of pairing and reproduction in the hermaphrodite freshwater snail Biomphalaria glabrata (Gastropoda : Pulmonata). Journal of Molluscan Studies 68:243-248.

Webster, J. P. and C. M. Davies. 2001. Coevolution and compatibility in the snail-schistosome system. Parasitology 123:S41-S56.

Webster, J. P. and M. E. J. Woolhouse. 1999. Cost of resistance: relationship between reduced fertility and increased resistance in a snail-schistosome host-parasite system. Proceedings of the Royal Society of London Series B-Biological Sciences 266:391-396.

Webster, J. P. and C. M. Gower. 2006. Mate choice, frequency dependence, and the maintenance of resistance to parasitism in a simultaneous hermaphrodite. Integrative and Comparative Biology 46:407-418.

Webster, J. P., J. I. Hoffman, and M. Berdoy. 2003. Parasite infection, host resistance and mate choice: battle of the genders in a simultaneous hermaphrodite. Proceedings of the Royal Society of London Series B-Biological Sciences 270:1481-1485.

Wethington, A. R., M. Zavodna, M. K. Smith, G. Oliveira, F. Lewis, and D. J. Minchella. 2007. Population genetic structure of Biomphalaria glabrata in a schistosomiasis-endemic region in Brazil. Journal of Molluscan Studies 73:45-52.

Winnepenninckx, B., T. Backeljau, and R. Dewachter. 1993. Extraction of High-Molecular-Weight DNA from Mollusks. Trends in Genetics 9:407-407.

Wise de Valdez, M. R., D. Nimmo, J. Betz, H. F. Gong, A. A. James, L. Alphey, and W. C. t. Black. 2011. Genetic elimination of dengue vector mosquitoes. Proc Natl Acad Sci U S A 108:4772-4775.

Wolinska, J. and K. C. King. 2009. Environment can alter selection in host-parasite interactions. Trends in Parasitology 25:236-244.

Zavodna, M., G. J. Sandland, and D. J. Minchella. 2008. Effects of intermediate host genetic background on parasite transmission dynamics: A case study using Schistosoma mansoni. Experimental Parasitology 120:57-61.

Zhang, S. M. and E. S. Loker. 2003. The FREP gene family in the snail Biomphalaria glabrata: additional members, and evidence consistent with alternative splicing and FREP retrosequences. Developmental and Comparative Immunology 27:175-187.

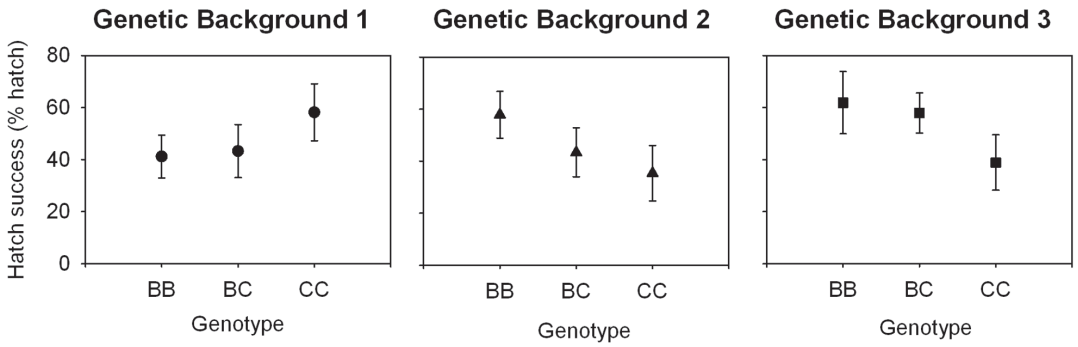
Zuk, M. and A. M. Stoehr. 2002. Immune defense and host life history. American Naturalist 160:S9-S22.

APPENDIX

Appendix Table 1

Primer sequences and PCR amplification conditions for three sequenced loci (SOD1, β-actin, myoglobin) and seven microsatellite loci (BgE4, BgE5, BgC7, BgC8, uBg1, Bgu15, Bgu16).

Locus	Primer Sequence	Annealing (°C)	Source
SOD1	F - TCA TTG GTC GCA GCT TAG TG R - GTC CTG TCA TGT AGC CAC CA	56	Bonner et al. 2012
Actin	F - CCT GCA CCA GAT TGG AA R - GGT GAG TGA ACT GAG AAT CAA	56	DeJong et al. 2003
Myoglobin	F - TTT CCT TAA CCC AAT CTG CAT R - TTG GCT GTT GCA TGA AAT AAA	50	
BgE4	F - GTCAGGACTGTGTGTAAAAGGAAG R - AGAGGGCAGATGATGCAAAG	52	Mavarez et al. 2000
BgE5	F - CAGCCTTAGCACCTCTAGTCG R - TCTCATGGAAGTGAAGCTGTG	57	Mavarez et al. 2000
BgC7	F - AAACGGGATTGTGTGAATGG R - GCCCAGCAGCAGAGATTG	57	Mavarez et al. 2000
BgC8	F - AGCCAGGACACCATGTTAGG R - GAAGCGAGCGTTTTGTTTTG	57	Mavarez et al. 2000
uBg1	F - TTAATTCTACTGGACTCACATGG R - CTGCCAATGTTTACATGCTG	57	Jones et al. 1999
Bgu15	F - AGGTTTGTATGTCTTGCTG R - GGTTCACTCAGATACATCC	57	Jones et al. 1999
Bgu16	F - CTGTTATTCATTATTTCATAGAGC R - GGGGATCTAACACATCAG	57	Jones et al. 1999



Appendix Figure 1

Average hatch success of each genotypic class within each lineage. Means are the average of percent hatch of two clutches per snail across genotypic class, and error bars represent 1±SE (background 1: n= 17 (*BB*=8, *BC*=6,*CC*=3), background 2: n= 24 (*BB*=9, *BC*=8,*CC*=7), background 3: n= 29 (*BB*=9, *BC*=10,*CC*=10)). No effects were significant.