AN ABSTRACT OF THE THESIS OF

Clint Sergi for the degree of Master of Science in Zoology presented on June 10, 2016.

Title: Cost to *Biomphalaria glabrata* of Resisting Infection by *Schistosoma mansoni*.

Abstract approved:

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Michael Blouin

Schistosomiasis is a neglected tropical disease caused by parasitic trematodes in the genus *Schistosoma*. 200 million people are infected with schistosomes. Schistosomiasis causes acute and chronic disease, and may lead to death in chronic infections. Schistosomes have a complex life cycle that requires passage through a snail intermediate host. Understanding the interactions between schistosomes and their snail hosts may lead to methods for breaking schistosome transmission through manipulation of their snail hosts. *Biomphalaria glabrata* that carry an allele at *SOD1* that causes them to produce more $H_2O_2$ (allele *B*) have increased resistance to infection by *S. mansoni*. ROS are thought to be important mediators of life history traits, and so it was hypothesized that there might be a cost to carrying the *B* allele. However, a recent study showed no constitutive costs in growth, survival or reproduction to carrying the *B* allele in the absence of challenge by parasites, relative to carrying the more susceptible *C* allele. We assessed whether inbred lines of *B.*

*glabrata* having the *BB* genotype incur greater fitness costs (measured by growth and survival) *after* successfully resisting a challenge by *S. mansoni* than lines having the *CC* genotype. Snails with either genotype did not bear any apparent costs to resisting infection, although we did find greater early mortality in snails with the *BB* genotype, regardless of exposure status. Furthermore, snail lines with greater percent resistance, regardless of *SOD1* genotype, grew less over the course of the study, regardless of parasite exposure status. Percent resistance *per se* had no effect on early snail mortality. We conclude laboratory populations of *B. glabrata* carrying the more resistant *SOD1* genotypes do not show greater costs in growth or mortality after resisting infection by *S. mansoni* than do more susceptible *SOD1* genotypes. However, in contrast to a previous study, we see some evidence of a slightly higher mortality among *BB* than *CC* lines, regardless of exposure, and evidence that resistance *per se* (regardless of *SOD1* genotype) was negatively correlated with growth.

# Cost to *Biomphalaria glabrata* of Resisting Infection by *Schistosoma mansoni*

by
Clint Sergi

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented June 10, 2016
Commencement June 2017

Master of Science thesis of Clint Sergi presented on June 10, 2016.

APPROVED:

Major Professor, representing Zoology

Chair of the Department of Integrative Biology

Dean of the Graduate School

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

Clint Sergi, Author

# ACKNOWLEDGEMENTS

I would like to express sincere gratitude to my advisor, Dr. Mike Blouin. Mike provided me the opportunity to complete a Masters in his lab at a time when I was struggling to decide where I wanted to start my graduate education. Mike's guidance was invaluable to me as a student and researcher. The insights about science and academia Mike shared will continue to help me as I progress in my scientific career.

I would also like to thank Dr. Michelle Steinauer. Michelle was an excellent mentor to me, both as an undergraduate volunteering in her lab and as a graduate student working on closely related projects. Michelle helped to smooth what would have been an otherwise bumpy transition from undergraduate to graduate student.

Other members of the Blouin lab have also been extremely helpful in various ways. Stephanie has been great at helping me trouble shoot and teaching me lab techniques. I can't thank her enough for the hours she's spent staring through a microscope pipetting miracidia. Neil and Jacob have provided valuable guidance on experimental design, data analysis, and advice for getting R to do what I intended without too much trouble. Euan has been a consistent source of help with lab work and helping me understand exactly where grad students fit in the academic world. Kate has been an enthusiastic and willing source of help since the day she joined the lab. I can't overstate how relieving it is to have her take over the parasite life cycle maintenance when I leave, knowing she'll be dedicated to making sure our various strains continue uninterrupted.

There are too many to name individually, but the other grad students in Integrative Biology all deserve thanks. They have been helpful at attempting to solve scientific, academic and general life problems. It's been great having such a friendly and relatable group of students to go through grad school with.

The Blouin lab has been lucky enough to have a great trio of undergrads who work mostly behind the scenes to keep our animals healthy and happy. Thomas, Leah, and Ryan all deserve a huge thank you for their help. Without Leah and Ryan maintaining our various snail lines, we would have much more work, or many fewer snails, on our hands. Thomas' willingness to spend hours measuring snails meant there were many days I did not have to.

I must also thank my family and friends for supporting me. My friends continue to be excellent friends, even when I am often too busy to leave home or the lab. My family has encouraged me to foster my interest in biology throughout my career as a student, and have been endlessly supportive and helpful along the way.

Finally, I'd like to thank my wonderful fiancée, Janelle. She has put up with long or awkward hours of lab work, teaching, and grading, and always welcomed me home with a smile and an excellent meal when I was done. Her unwavering support has helped me to become a better student, researcher, and significant other.

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# Cost to *Biomphalaria glabrata* of Resisting Infection by *Schistosoma mansoni*

## Chapter 1

## General Introduction

Schistosomiasis is a Neglected Tropical Disease caused by trematode parasites in the genus *Schistosoma*. In terms of number of people infected with parasites, Schistosomiasis is second only to malaria (Steinmann et al. 2006; Fenwick et al. 2009). Worldwide, more than 200 million people are infected with schistosomes, resulting in morbidity or death (Hatz 2005). Schistosomiasis causes acute and chronic disease, and is estimated to cause a loss of 13-15 million disability adjusted life years each year (King et al 2005; King 2010). Schistosomes are transmitted to humans through snail intermediate hosts, and have a complex life cycle that requires passage through the snail intermediate host and human, or other mammal, definitive hosts (see Figure 1).

To date, various control methods have been largely unsuccessful at eliminating Schistosomiasis (King et al 2006; Rollinson et al 2014; Ross et al 2014; King and Bertsch 2015). Before the creation of the anti-helminthic drug praziquantel, efforts to control Schistosomiasis were focused on reducing or eliminating populations of the snail hosts (Fenwick & Savioli 2011; King & Bertsch 2015). Use

of molluscicide to control snail populations was the most widely used control strategy through the 1980's (King & Bertsch 2015). However, mixed results and high labor costs contributed to molluscicide falling out of favor after the invention of praziquentel (King & Bertsch 2015). Since then, mass drug administration with praziquentel has become the main focus of control efforts (WHO 2006, Ross et al. 2014). Mass drug administration has had mixed results and is unlikely to be effective on its own as a method for eliminating Schistosomiasis (Rollinson et al. 2013; Ross et al. 2014). Recently, researchers have recognized the need for an integrative approach to Schistosomiasis control involving both mass drug administration and snail-based control efforts (Rollinson et al. 2013; Ross et al. 2014; Secor 2014; King and Bertsch 2015). Interactions between the common new world host snail species, *Biomphalaria glabrata*, and *Schistosoma mansoni* have been extensively studied in efforts to find potential snail-based Schistosomiasis control methods (Goodall et al. 2004; Bayne 2009).

*B. glabrata* naturally vary in resistance to infection by *S. mansoni* and resistance to *S. mansoni* can be selected for in snail lines (Webster and Woolhouse 1998; Webster and Woolhouse 1999; Larson et al. 2014). Snail resistance to infection is associated with genotype and gene expression at the *SOD1* locus, with snails possessing a B allele being more resistant to infection than snails carrying only A or C alleles (Bayne et al. 2001; Goodall et al. 2004; Bender et al. 2007). The *SOD1* locus codes the protein Cu/Zn Superoxide dismutase, which reduces superoxide (O2-) to $H_20_2$ (Ramasarma 2007; Bayne 2009; Bonner et al. 2012). H2O2 produced by snail hemocytes is important for killing invading *S. mansoni* miracidia (Hahn et al. 2001;

Bender et al. 2005; Bayne 2009). Scavenging of H202 with catalase leads to significantly decreased sporocyst killing, and resistant snails produce more H2O2 after stimulation with PMA (Hahn et al. 2001; Bender et al. 2005). $H_2O_2$ and other reactive oxygen species (ROS) are cytotoxic and may be important mediators of life history traits (Nappi & Ottaviani 2000; Monaghan et al. 2009; Bonner et al. 2012). Snails carrying B alleles produce greater amounts of $H_2O_2$ in response to immune challenge than snails carrying A or C alleles (Hahn et al. 2000). Resistant *B. glabrata* are thought to incur costs of resisting infection by *S. mansoni* due to increased energetic expense and ROS generation during immune activation (Bonner et al. 2012).

Maintenance and activation of an immune system is thought to be costly to organisms (Sheldon & Verhulst 1996; Lochmiller & Deerenberg 2000; Schmid-Hempel 2003; Schulenburg et al. 2016). These costs are assumed to be paid through reduced energy availability to other processes such as growth and reproduction (Lochmiller & Deerenberg 2000). However, recent studies have shown that costs of resistance may be context dependent or mediated by increased resource acquisition (Moret and Schmid-Hempel 2000; Brace et al. 2015). In some organisms, costs of resistance may occur rarely, or not at all (Labbé et al. 2010). Bonner et al (2012) showed no costs of carrying the *B* allele in unchallenged *B. glabrata* lines (Bonner et al. 2012). However, *B. glabrata* may only incur costs after exposure to *S. mansoni* miracidia and subsequent immune activation ( Schimd-Hempel 2003; Humphries & Yoshino 2008).

Modern efforts at vector-borne disease control through manipulation of the vector organism have been promising, most notably in mosquitoes (Gantz et al. 2015;

Alphey 2016; Champer et al. 2016). Research groups are already studying *S. mansoni* resistance in *B. glabrata* in efforts to use the snails as an effective method for reducing or eliminating Schistosomiasis transmission (Hanington et al. 2012; Tennessen et al. 2015; Tennessen et al. 2015; Pila et al. 2016). If we find no costs in *B. glabrata* of resisting infection by *S. mansoni*, we will provide further evidence that snail-based control methods using resistant snails are a potentially feasible method of Schistosomiasis reduction.

In Chapter 2, we describe a test to determine the cost of resistance in *B. glabrata* with *BB* genotypes at the *SOD1* locus compared to *B. glabrata* with *CC* genotypes, using snail growth as a proxy for overall fitness. We used twelve inbred lines of snails, six *BB* and six *CC* at the *SOD1* locus. We exposed snails from each line to five *S. mansoni* miracidia and compared the growth of snails that successfully resisted infection to snails that were not exposed to *S. mansoni* miracidia. If *B. glabrata* bear a cost of resisting infection by *S. mansoni*, we expect (1) growth of snails challenged with parasites to be decreased relative to unexposed snails, and (2) for challenged snails having the *BB SOD1* genotype to have a greater decrease in growth relative to unchallenged snails of the same line, than snails with the *CC SOD1* genotype. We also tested for differences in mortality between snail lines and genotypes. We expected greater mortality in both *CC* and *BB SOD1* genotype in challenged snails than in unchallenged snails, and greater mortality in *BB SOD1* genotype challenged snails than in *CC SOD1* genotype challenged snails. We chose to exclude snails that became infected from our study because the growth of infected snails has already been characterized, and infected snails have been shown to have

significantly decreased reproductive output and to produce non-viable eggs (Faro et al. 2013; Tavalire et al. 2016).

Figure 1:

Figure 1: Scistosomes have a complex life cycle requiring passage through a snail intermediate and mammal definitive host. Clockwise from top left: *i.* Adult worms form mating pairs in the mesentery of the small intestine. *ii.* Eggs produced by the adult worms are shed in feces or urine and miracdia hatch from shed eggs if eggs are shed into fresh water. *iii.* Miracidia infect snails intermediate hosts and develop into sporocysts. Sporocysts produce cercariae, which are shed from the snail into the water. *iv.* Cercariae infect mammalian hosts and migrate to the mesentery while developing into adult worms.

# Chapter 2

## Cost to *Biomphalaria glabrata* of Resisting Infection by *Schistosoma mansoni*

Clint Sergi

Target journal:

To be determined.

**Abstract:**

Schistosomiasis is a neglected tropical disease caused by parasitic trematodes in the genus *Schistosoma*. 200 million people are infected with schistosomes. Schistosomiasis causes acute and chronic disease, and may lead to death in chronic infections. Schistosomes have a complex life cycle that requires passage through a snail intermediate host. Understanding the interactions between schistosomes and their snail hosts may lead to methods for breaking schistosome transmission through manipulation of their snail hosts. *Biomphalaria glabrata* that carry an allele at *SOD1* that causes them to produce more $H_2O_2$ (allele *B*) have increased resistance to infection by *S. mansoni*. ROS are thought to be important mediators of life history traits, and so it was hypothesized that there might be a cost to carrying the *B* allele. However, a recent study showed no constitutive costs in growth, survival or reproduction to carrying the *B* allele in the absence of challenge by parasites, relative to carrying the more susceptible *C* allele. We assessed whether inbred lines of *B. glabrata* having the *BB* genotype incur greater fitness costs (measured by growth and survival) *after* successfully resisting a challenge by *S. mansoni* than lines having the *CC* genotype. Snails with either genotype did not bear any apparent costs to resisting infection, although we did find greater early mortality in snails with the *BB* genotype, regardless of exposure status. Furthermore, snail lines with greater percent resistance, regardless of *SOD1* genotype, grew less over the course of the study, regardless of parasite exposure status. Percent resistance *per se* had no effect on early snail mortality. We conclude laboratory populations of *B. glabrata* carrying the more resistant *SOD1* genotypes do not show greater costs in growth or mortality after

resisting infection by *S. mansoni* than do more susceptible *SOD1* genotypes. However, in contrast to a previous study, we see some evidence of a slightly higher mortality among *BB* than *CC* lines, regardless of exposure, and evidence that resistance *per se* (regardless of *SOD1* genotype), was negatively correlated with growth.

**Introduction:**

Schistosomiasis is a Neglected Tropical Disease caused by trematode parasites in the genus *Schistosoma*. In terms of number of people infected, Schistosomiasis is second only to malaria with more than 200 million people infected (Steinmann et al. 2006; Fenwick et al. 2009). Schistosomiasis causes acute and chronic disease, and is estimated to cause a loss of 13-15 million disability adjusted life years each year (King et al. 2005; King 2010). Schistosomes are transmitted to humans through snail intermediate hosts, and have a complex life cycle that requires passage through the snail intermediate host and human, or other mammal, definitive hosts (cdc.gov). Interactions between the common new world host snail species, *B. glabrata*, and *S. mansoni* have been extensively studied (Goodall et al. 2004; Bayne et al. 2009).

Previous studies have shown that in *B. glabrata*, genotype at the Cu/Zn superoxide dismutase (*SOD1*) locus is strongly associated with resistance to infection by *S. mansoni* miracidia (Goodall et al. 2006). In *B. glabrata*, the B allele at *SOD1* is associated with resistance, and the C allele at *SOD1* is associated with susceptibility (Goodall et al. 2006). The hemocytes of snails with B alleles produce more extracellular hydrogen peroxide ($H_2O_2$) than hemocytes from snails with no C alleles, through the reduction of the reactive oxygen species (ROS) superoxide ($O_2^-$) (Bender

et al. 2005; Bonner et al. 2012). $H_2O_2$ produced by hemocytes during an oxidative burst is important for the killing of *S. mansoni* primary sporocysts (Bayne 2009; Hahn et al. 2011).

It has been hypothesized that the production of reactive oxygen species may lead to immunopathology due to the oxidative stress placed on host tissues and thus induce costs in terms of growth, reproduction and mortality (Monaghan et al. 2009). Because resistance of *B. glabrata* is associated with greater production of ROS (Bender et al. 2005), there may be an effect of resistance on the fitness of resistant snails.

Bonner et al (2012) previously investigated the cost of resistance in unchallenged *B. glabrata* (Bonner et al. 2012). It was previously shown that the B allele at *SOD1* is overexpressed relative to the C and A alleles in the 13-16-R population of snails, and that individuals carrying the B allele are more resistant to infection by *S. mansoni* than individuals carrying the *C* or *A* alleles (Goodall et al. 2004; Bender et al. 2007; Tennessen et al., 2015). Interestingly, Bonner et al. (2012) found no significant costs in growth, survival or reproduction to *B. glabrata* of carrying one or two copies of the *B* allele versus the *C* allele at the *SOD1* locus (Bonner et al. 2012). Indeed, the pattern was opposite to expectations: snails with *CC SOD1* genotype grew less and had greater mortality on average than *BC* and *BB* individuals (Bonner et al. 2012). However, this work was done without the context of immune challenge. Thus it is possible that the costs are only induced after challenge with a pathogen.

Indeed, $H_2O_2$ and other ROS production via *SOD1* requires activation brought on by the presence of a pathogen within the snail (Babior 1999; Hahn et al. 2000; Humphries and Yoshino 2008; Bonner et al. 2012). Even though the *B* allele is constitutively more highly expressed than the *C* allele, generation of ROS in *B. glabrata* could be dependent on exposure to a pathogen. In that case, we would expect to see costs in life history traits due to immune activation only in snails that were exposed to *S. mansoni* miracidia. Therefore, here we test whether there is a cost to carrying the *B* allele in snails that have been challenged with parasites.

A cost to *B. glabrata* of resisting infection by *S. mansoni* could have implications for future methods of schistosomiasis control. Previous and ongoing programs to control Schistosomiasis have been largely unsuccessful (King et al. 2006; Lelo et al. 2014; Rollinson et al 2014; Ross et al 2014; King and Bertsch 2015). Historically, mass treatment with molluscicides has been used in attempts to control the transmission of schistosomes by removing the snail host from endemic areas of Schistosomiasis (Fenwick and Savioli 2011; King & Bertsch 2015). Although successful at reducing schistosoma transmission in some areas, mass molluscicide fell out of favor when oral drugs to treat active schistosoma infections became available (King & Bertsch 2015). Since praziquantel became widely available, mass drug administration has been the most widely used method for controlling Schistosomiasis (Tchuem Tchuenté et al. 2013). However, recent evidence suggests mass drug administration alone will be insufficient for effective control or elimination of Schistosomiasis (Ross et al. 2014). To successfully control or eliminate Schistosomiasis, an integrative approach, involving treatment of active schistosoma

infections with praziquantel and snail-based control methods, will be necessary (Ross et al. 2014; Secor 2014; King & Bertsch 2015).

If there if is no obvious cost to *B. glabrata* snails of carrying the *B* allele, then variants of *SOD1* could potentially be used in the creation of resistant snail populations via gene drive systems, as is being done with mosquitoes against the pathogens they carry (Bonner et al. 2012; Gantz et al. 2015; Alphey 2016; Champer et al. 2016). However, if there is a strong fitness cost to carrying a resistance allele (either constitutively or after parasite challenge), natural selection would counteract spread of the resistance allele. To determine whether the control of schistosomiasis is possible through the use of snails carrying resistant alleles, it is necessary to characterize costs of resistance in snail intermediate hosts under a variety of conditions (Bonner et al. 2012).

In this study, we tested whether there was a cost of resistance in *B. glabrata* with *BB* genotypes at the *SOD1* locus compared to *B. glabrata* with *CC* genotypes, using snail growth as a proxy for overall fitness. We used twelve inbred lines of snails, six *BB* and six *CC* at the *SOD1* locus. We exposed snails from each line to five *S. mansoni* miracidia and compared the growth of snails that successfully resisted infection to snails that were not exposed to *S. mansoni* miracidia. If *B. glabrata* bear a cost of resisting infection by *S. mansoni*, we expect (1) growth of snails challenged with parasites to be decreased relative to unexposed snails, and (2) for challenged snails having the *BB SOD1* genotype to have a greater decrease in growth relative to unchallenged snails of the same line, than snails with the *CC SOD1* genotype. We also tested for differences in mortality between snail lines and genotypes. We

expected greater mortality in both *CC* and *BB SOD1* genotype in challenged snails than in unchallenged snails, and greater mortality in *BB SOD1* genotype challenged snails than in *CC SOD1* genotype challenged snails due to immunopathology. We chose to exclude snails that became infected from our study because the growth of infected snails has already been characterized, and infected snails have been shown to have significantly decreased reproductive output and to produce non-viable eggs (Faro er al. 2013; Tavalire et al. 2016).

**Methods:**

***Snails:***

We used 12 populations of 1316-R *Biomphalaria glabrata* snails selected from the 52 inbred 1316-R lines created by Larson et al (2014) (Larson et al. 2014). These inbred lines were derived from the same outbred population (13-16-R), and randomly became homozygous for either the *B* or *C SOD1* alleles after three generations of selfing. Therefore, all six lines of each genotype should share a similar genetic background, randomly derived from the same gene pool. Both *BB* and *CC* lines varied in resistance to infection, so we selected more resistant lines, as measured by Larson et al. (2014), of each genotype to increase the number of exposed snails that did not become infected after challenge. Prior to parasite challenge, snail populations were expanded and 3-7mm snails were selected for parasite challenge. Post parasite exposure, snails were kept in individual plastic cups in 26$^{o}$C dechlorinated water, supplemented weekly with powdered $CaCO_3$ and fed green leaf lettuce *ad libitum*.

***Parasite exposure:***

3-7mm juvenile snails were selected from each of the 12 inbred lines. Using the previously estimated resistance of each line (Bonner et al. 2012), we selected enough snails from each line to expect ten challenged but uninfected snails from each line, and used between ten and 20 snails per line. We challenged 186 snails total, from the twelve snail lines. We used twelve control snails from 11 lines, and used six from the twelfth line for a total of 138 unchallenged snails. We only included 6 controls from the twelfth line because it was an unproductive line that only produced sixteen snails of the proper size. Snails were challenged with five PR-1 *S. mansoni* miracidia in 2mL of dechlorinated water in 24 well culture plates. We exposed two snails per line in each culture plate, and snails were randomly assigned wells using random numbers generated in R. Snails were kept in exposure wells for 24 hours. Unexposed control snails were also kept in randomly assigned culture plate wells in 2mL of dechlorinated water for the same 24 hour period. After 24 hours, snails were moved to individual cups for the remainder of the experiment.

***Snail growth:***

Snail cups were housed on shelves in a 26$^{o}$C laboratory after exposure to miracidia. To account for variation in snail growth due to conditions in the lab environment, snails were randomly assigned to shelves, and shelves were treated as blocks in subsequent statistical analyses. . We assigned each snail to a block using a random order generated by R. The temperature of water on each block was measured at 1 minute intervals using Hobo logger submersible temperature loggers for the duration of the experiment to use as a statistical covariate if we found a block effect.

Snail growth was measured weekly using digital calipers. Each snail's shell was measured at the widest point where the calipers were perpendicular to the snail's aperture. Snails were first measured when they were moved from the culture plate wells to their individual cups 24 hours after exposure to *S. mansoni* miracidia. Subsequently, we measured snails weekly, measuring two blocks of snails on each of four days, in a random order generated with R. After the first week of measurements, we measured snails in the same order so the measurements for each block were one week apart. The water in each cup was changed when snails were measured, and snails were given supplemental powdered $CaCO_3$. Each week, snails were checked for the presence of eggs and juveniles, but eggs and juveniles were not counted. Each week, from five weeks to fifteen weeks post-exposure, snails were checked for infection. To check for infection, snails were placed in dechlorinated water in wells in twelve-well culture plates. Plates were placed under direct light for 90 minutes, and then wells were checked for the presence of cercariae under a dissecting microscope. Infected snails were removed from the experiment.

***Statistical analysis: size***

We removed snails that became infected from the data set used for testing hypotheses about growth. We also removed snails that died prior to week eleven post-exposure because the infection status of these snails could not be reliably determined. We also removed inbred lines that had too few surviving and uninfected snails remaining. Figure 2 shows the size at weeks five and 26 for the lines used in this experiment. For data analysis, we removed the lines most susceptible to infection, as there were too few uninfected snails for a robust analysis. Five *BB* lines and four *CC*

lines that each had at least four surviving uninfected snails remained in the analysis, which included 177 snails that did not become infected and survived until at least eleven weeks post exposure. Table 1 contains a summary of each snail line and the number of dead, infected, and surviving snails. We used linear mixed effects models to test potential causes of variation in snail size at five weeks post exposure and at 26 weeks post exposure, when the experiment ended. We tested effects of the following variables on snail size at five and 26 weeks post exposure: (1) exposure state (control vs. exposed-but-uninfected), (2) genotype (*BB* vs. *CC* inbred lines), (3) inbred line nested within genotype, (4) block (shelf), and (5) percent resistant (estimates from this experiment). We tested for main effects of genotype, line, block, percent resistant, and exposure state, as well as interactions between genotype and exposure and between percent resistant and exposure. The genotype-by-exposure interaction term tests our hypothesis that there would be a greater difference in growth between unexposed and exposed-but-uninfected snails in *BB* lines than in *CC* lines. The percent resistant-by-exposure interaction term tests whether more highly resistant lines suffered more while resisting infection than less resistant lines, regardless of *SOD1* genotype. We conducted this test because percent resistant generally correlates with *SOD1* genotype, but not perfectly. We included size at time of exposure as a covariate to control for confounding effects of size at time of exposure.

We used Aikake's information criteria to perform model selection. Normality was assessed using quantile-quantile plots. We used the statistical computing software R and the lme4 package to perform these tests (Bates et al, 2015; R Core Team, 2016).

***Statistical analysis: mortality***

We used binomial regression to test whether genotype, snail line, parasite exposure or proportion resistant were associated with increased early mortality in *B. glabrata.* We also tested for a genotype-by-exposure status interaction, and for a percent resistant-by-exposure status interaction. Again, the first interaction term asked whether genotypes differ in their cost of resisting and the second interaction term tests whether more resistant lines suffer more or less while resisting.

Here we defined early mortality as any snail that died before 11 weeks without shedding cercariae (Allegretti et al. 2009). We found no snails shedding cercariae after ten weeks post miracidia exposure. So any snails that died before 11 weeks may or may not have been infected.

Two hundred fifty two total exposed and unexposed snails were used in our binomial regression model. We also calculated 95% confidence intervals for each snail line's percent resistance and mortality.

All statistical analyses and plotting were conducted using the free statistical computing software R (http://www.r-project.org) and R packages available on CRAN (https://cran.r-project.org/) (Wickham 2007, Wickham 2009, Wickham 2011, Dragulescu 2014, Bates et al 2015, Wilke 2016, R Core Team 2016).

***Power analyses***

We conducted a post-hoc power analysis for analyses on growth rate and on mortality using the G*power software to determine the achieved power for the five and 26 week growth models and the mortality model (Faul et al 2007, Faul et al 2009).

**Results:**

For size at five and 26 weeks, we found no significant genotype-by-exposure interaction (p=0.091, p=0.1145), which rejects our main hypothesis that *BB* snails would incur a larger cost of resisting infection than *CC* snails. We also found no main effects of exposure (p = 0.344, p=0.464), block (p=0.764, p=0.824), or genotype (p=0.14, p=0.63). Figures 3 and 4 show snail growth through five and 26 weeks, respectively. Growth differed significantly between inbred lines (p<0.0001, p<0.0001). At five and 26 weeks, we found a significant main effect of percent resistant on snail size (p<0.0001, p<0.0001). Our models estimate that at five weeks post exposure, snails from zero percent resistant lines were, on average, 1.89 mm larger than snails from 100 percent resistant lines, and at 26 weeks post exposure, snails from zero percent resistant lines were, on average, 4.28 mm larger than snails from 100 percent resistant lines. We tested for differences in average size at the time of exposure between exposed and unexposed snails for each line. Using ANOVA, we found suggestive evidence for differences in initial average size between exposed and unexposed snails (p = 0.072). We then used t-tests to test for differences in initial size between and found no significant differences after comparing p-values to a Bonferroni adjusted alpha level of 0.0056 (p-values ranged from 0.02 to 0.99). Our models use data from five *BB* and 4 *CC* lines ranging from 44 percent to 100 percent resistant. We did not characterize differences in growth between lines, because these differences are expected of inbred lines and are not of interest given our hypothesis. Figure 5 shows snail size at five and 26 weeks of measurement for each inbred line used in the statistical analysis.

Using a binomial regression model, we found no significant effect of line ($p = 0.983$), resistance ($p = 0.251$), or exposure ($p = 0.533$) on snail mortality.). Interestingly, *CC* snails were less likely to die than *BB* snails, where *CC* snails' odds of dying were 0.33 the odds of dying for *BB* genotype snails ($p=0.0473$). However, we found no significant interaction between genotype and exposure on snail mortality ($p=0.277$), so although *BB* lines suffered higher mortality than *CC* lines overall, the effect was not increased by exposure. (see also Fig. 6).

After rejecting our initial hypothesis that *BB* snails would incur a larger cost of resisting infection, we conducted post-hoc power analyses to determine the achieved power of our models, given our total sample sizes. We used estimates for the power of t-tests to detect differences in average size between exposed/uninfected and unexposed snails (the interaction term for our initial hypothesis). We found achieved power of only 0.15 at five weeks. However, we found achieved power of 0.76 at 26 weeks.

**Discussion:**

We found suggestive evidence that *Biomphalaria glabrata* bear a fitness cost of being resistant to infection by *Schistosoma mansoni*, however, this cost is unrelated to *SOD1* genotype. Thus, we reject our main hypothesis that *BB* snails would suffer a greater cost to resisting infection than *CC* snails. That challenged but uninfected snails grew just as well as unchallenged snails suggests that the overall cost of fighting off a challenge by five miracidia is not that great, so there was little opportunity to see a difference in cost between *BB* and *CC* genotypes. Perhaps we could have made the

challenge more stressful by challenging with more miriacida. Previous work has shown that over half of field collected snails were infected by multiple miracidia (Minchella et al 1995, Sire et al 1999). Other studies have found that even within highly compatible snail and parasite strain combinations, fewer than half of the miracidia snails are exposed to successfully develop into mother sporocysts (Théron et al. 1997; Theron et al. 2014). However, these studies do not provide evidence that *B. glabrata* are subject to challenge by multiple miracidia simultaneously. We expect simultaneous challenge by greater than 5 miracidia is probably rare in nature, so multi-miracidia challenges may not be that ecologically relevant.

We did find significant differences in snail growth between inbred lines. These differences in snail size between inbred lines are not surprising, as the effects of inbreeding on growth are well characterized (DeRose & Roff 1999; Keller & Waller 2002; Gerloff et al. 2003).

We found a difference in pre-patent mortality between *BB* and *CC* snails, in which *CC* snails had 0.33 relative odds of lower mortality compared to BB snails. Again, this difference was not associated with exposure to *S. mansoni* miracidia and so is not indicative of a fitness cost to resisting infection. Interestingly, this trend is not supported by Bonner et al, who found greater mortality in *CC* genotype snails. However, Bonner et al tested for differences in mortality at eight months, and we did not continue our experiment long enough to compare eight month snail mortality (Bonner et al. 2012). Although we found a difference in early mortality between *BB* and *CC* genotype snails, overall mortality was low. Fourteen percent of exposed snails that did not become infected died during the pre-patent period, and only 11% of

all snails (exposed plus controls) used in the experiment died during the pre-cercarial period. Of the 36 snails that died during the pre-patent period (both exposed and controls), 67% were *BB* genotype snails, and 33% were *CC* genotype snails. 38% of *BB* snails that died during the pre-cercarial period were exposed to miracidia, and 50% of *CC* snails that died during the pre-cercarial period were exposed. Although we found no apparent increase in mortality among snails exposed to miracidia and a greater percentage of dead *CC* snails had been exposed than *BB* snails, our sample size of dead snails was small. These results could be due to insufficient power to detect an effect of miracidia exposure, but the lack of apparent cost of resisting is consistent with our findings from snail growth measurement. Our study design did not allow us to distinguish mortality due to snail immune response from mortality due to injury caused by miracidia or cercariae. However, we would expect to see greater mortality in exposed *BB* snails or snails from highly resistant lines if differences in early mortality were due to a cost of resisting infection. We saw no evidence of that in our study.

We found a significant effect of percent resistance on snail size at five and 26 weeks post exposure. Because greater resistance was associated with smaller size at both time points, regardless of exposure status, this does not appear to be a cost of resisting in *B. glabrata*. This result may be an artefact of using only highly resistant lines in our statistical models. We used only five *BB* lines and four *CC* lines. Figure 2 shows snail size grouped by inbred line plotted against percent resistance for each inbred line. It appears that the significant difference in size between lines with different percent resistance is driven by two *BB* and one *CC* lines. With a greater

number of lines, including lines with near total susceptibility, we may not see a significant effect of resistance on snail size. Again, we did not find significant main or interaction effects of resistance on snail mortality, which supports our conclusion that we found no evidence of a cost of resisting *S. mansoni* infection in *B. glabrata*.

Our results showing no cost to *B. glabrata* of resisting infection by *S. mansoni* may not be generalizable outside of laboratory conditions. The *B. glabrata* used in this study are maintained in favorable conditions, with weekly water changes and fed lettuce *ad libitum*. Our *B. glabrata* were also kept individually, which may reduce or eliminate stress from competition. *B. glabrata* might also bear costs of resistance that are manifested in ways not apparent through measuring growth and survival. Sporocysts that are not killed mature in the tissue of the mantle and produce daughter sporocysts that migrate to the area around the digestive glands and ovotestes (Soomro et al. 2005). Soomro et al. (2005) noticed an increase in the number of amoebocytes in the areas around daughter sporocysts (Soomro et al. 2005). Although they reported no killing of daughter sporoscysts, it is possible that this late immune response, and presence of parasites near the digestive glands and ovotestes could restrict nutrient acquisition or reproductive ability (Soomro et al. 2005). Soomro et al. (2005) also noted degredation of cercariae around the digestive glands and ovotestes (Soomro et al. 2005). It seems possible that some snails might harbor daughter sporocysts that produce cercariae, but that these infections are missed by our method of checking for cercarial shedding, if the cercariae are being degraded by snail hemocytes. In this case, we might expect decreased reproduction in snails that we would call resistant.

Other studies have shown that costs of resistance may exist, but may not be apparent under all conditions. Moret and Schmid-Hempel (2000) showed costs of immune activation were apparent under starvation conditions in bumble bees, but not under conditions in which the bees were well fed (Moret and Schmid-Hempel 2000). Moret and Schmid-Hempel challenged bumble bees with non-pathogenic known immune response stimulators. They found no difference in survival between immune stimulated and non-immune stimulated bumble bees under favorable conditions. However, under starvation, they found a significant decrease in survival of immune stimulated bumble bees (Moret and Schmid-Hempel 2000). Sandland and Minchella (2003) found that snails infected with an echinostome parasite showed different growth patterns when fed a low-protein diet than snails fed a high-protein diet. These researchers found slower growth and smaller maximum size in infected snails fed a low-protein diet in one line of snails used in their study, but found slowed growth in both high- and low-protein fed snails, and smaller maximum size only in low-protein fed snails, in the second line of snails used in their study (Sandland & Minchella 2003). Further studies testing for costs of resistance under more stressful conditions will help determine whether *BB* genotype *B. glabrata* bear costs when resisting infection by *S. mansoni*. Bayne et al. (2001) also suggested that costs of resisting infection by *S. mansoni* may come as decreased resistance to other infections (Bayne et al. 2001). In this case, we would not expect to see a cost of resistance in lab populations of snails, but would expect to see costs in wild populations exposed to other pathogens.

If our results are shown to be generalizable, even under unfavorable conditions, then creating more resistant *B. glabrata* via changing *SOD1* genotype might be a viable biological control mechanism to reduce, or eliminate, transmission of *S. masnsoni*. Research groups are already working to identify other genes associated with resistance in *B. glabrata*, and are working to prove which locus or loci is responsible for resistance (Hanington et al. 2012; Tennessen et al. 2015; Tennessen et al. 2015; Pila et al. 2016). Other groups have successfully used gene editing and gene drive techniques to alter mosquitoes or reduce mosquito populations in an effort to control or eliminate mosquito-transmitted diseases (Gantz et al. 2015; Alphey 2016; Champer et al. 2016). These same gene editing and gene drive techniques may be able to be developed in snails and used to break the transmission cycle of *S. mansoni* and other schistosome species.

We have provided further evidence that *B. glabrata* do not bear costs of being genetically resistant to infection by *S. mansoni* via variation at the *SOD1* locus (Bonner et al. 2012). Further tests are needed to determine whether these results are generalizable, or only occur under the favorable growth conditions used in the laboratory. However, given our results and those of Bonner et al. (2012), *B. glabrata* are an increasingly attractive candidate for use as a biological mechanism for Schistosomiasis transmission control (Bonner et al. 2012; Tennessen et al. 2015).

**Figures:**

Figure 2:

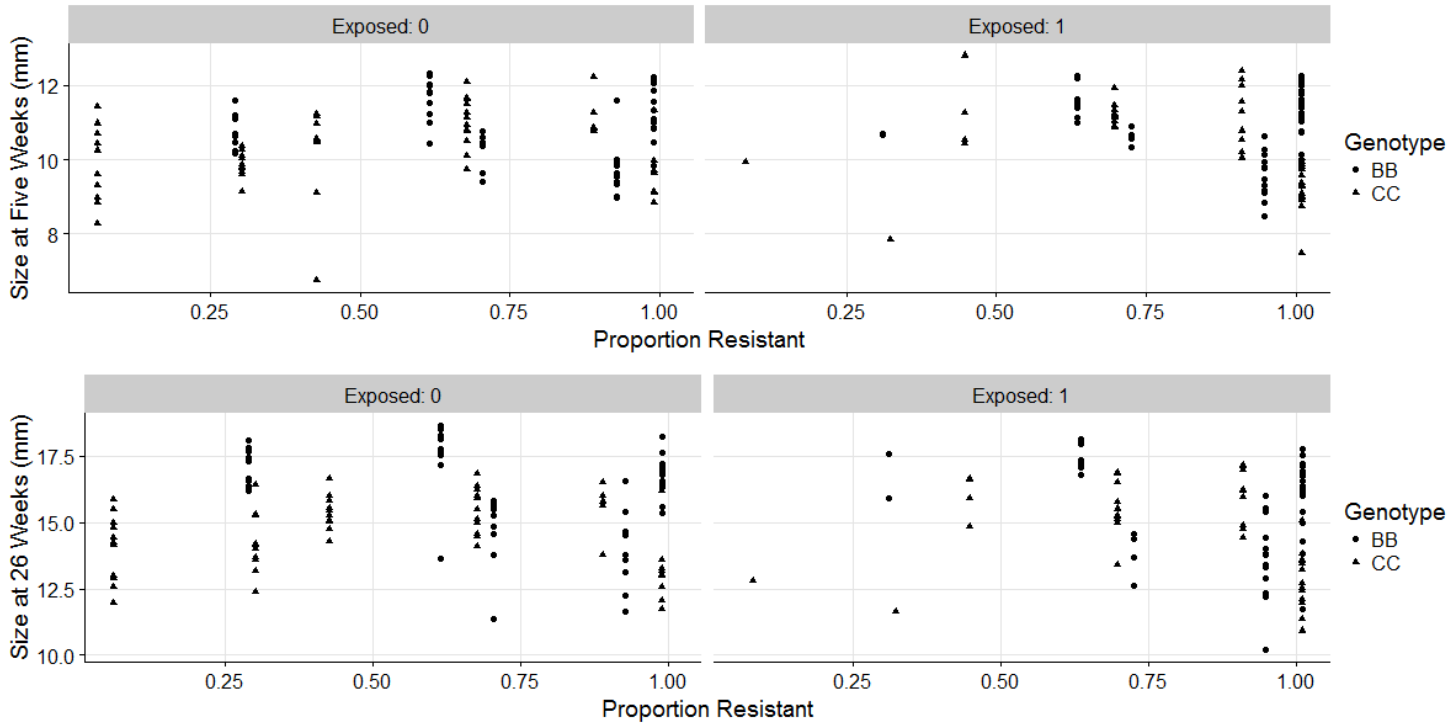


Figure 2: Snail size of 12 inbred lines of snails at five weeks (top panel) and 26 weeks (bottom panel) post exposure in relation to their relative resistance level (Proportion Resistant) and their genotypes at the *SODI* locus (BB or CC). Left panels show size of unexposed snails at five and 26 weeks, while right panels show the size of exposed snails.. Some lines had as few as one surviving and uninfected snail.

Figure 3:

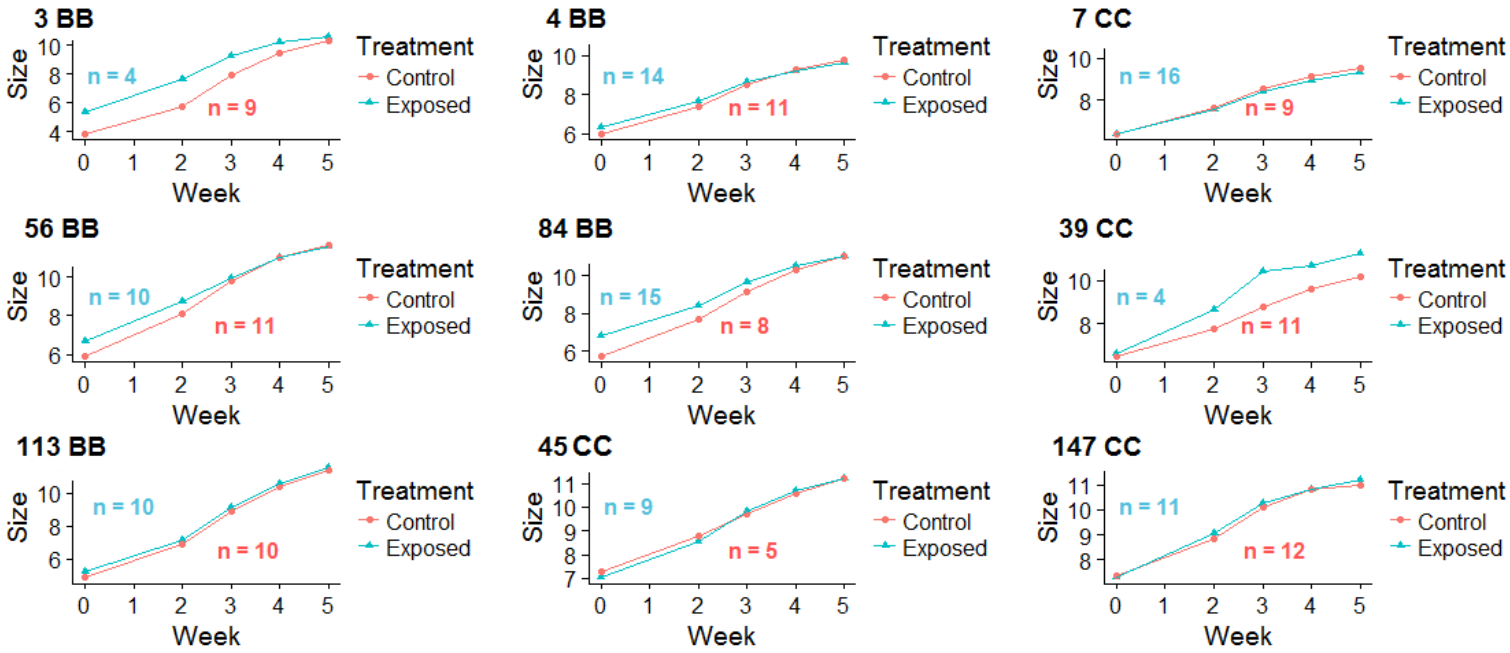


Figure 3: Snail growth through five weeks post exposure. Each frame shows the growth of unexposed (circle points) and exposed (triangle points) for an inbred line. Frame labels are inbred line number and *SOD1* genotype. Sample size is indicated on each panel in the color of the corresponding treatment.

Figure 4:

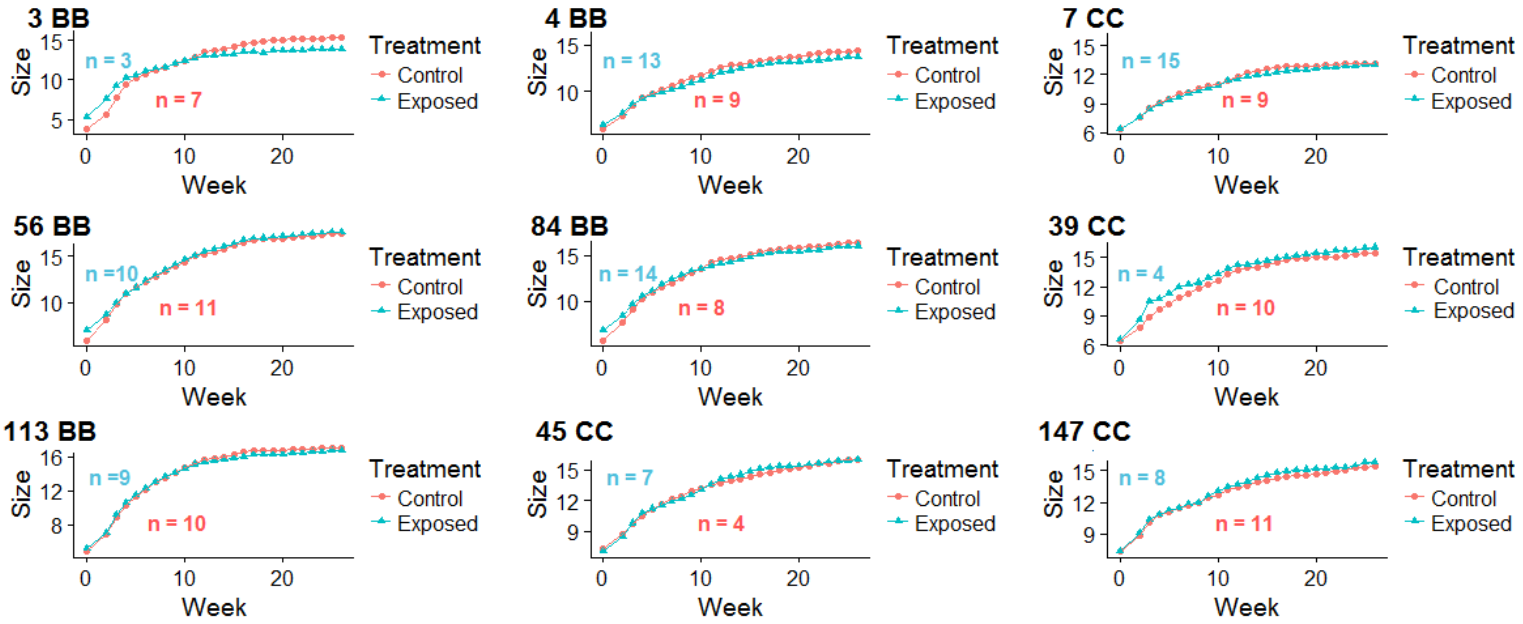


Figure 4: Snail growth through 26 weeks post exposure. Each frame shows the growth of unexposed (circle points) and exposed (triangle points) for an inbred line. Frame labels are inbred line number and *SOD1* genotype. Sample size is indicated on each panel in the color of the corresponding treatment.

Figure 5:

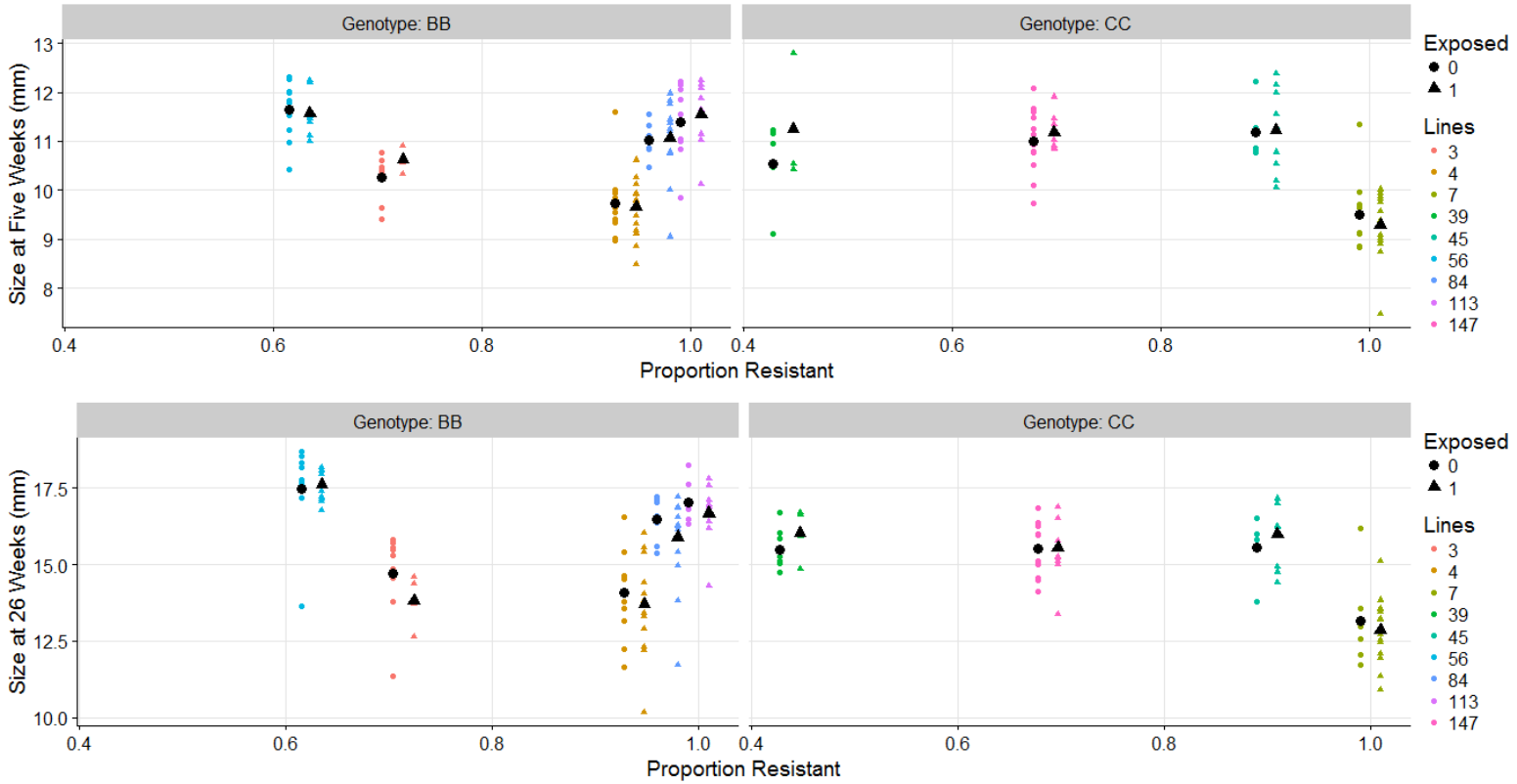


Figure 5: Snail size at five weeks (top panel) and 26 weeks (bottom panel) post exposure. Left panels show size of unexposed snails at five and 26 weeks. Snails are grouped by inbred line, and plotted against proportion resistant on the horizontal axis. Only the nine lines used for statistical analysis are included in Figure 5.

Figure 6:

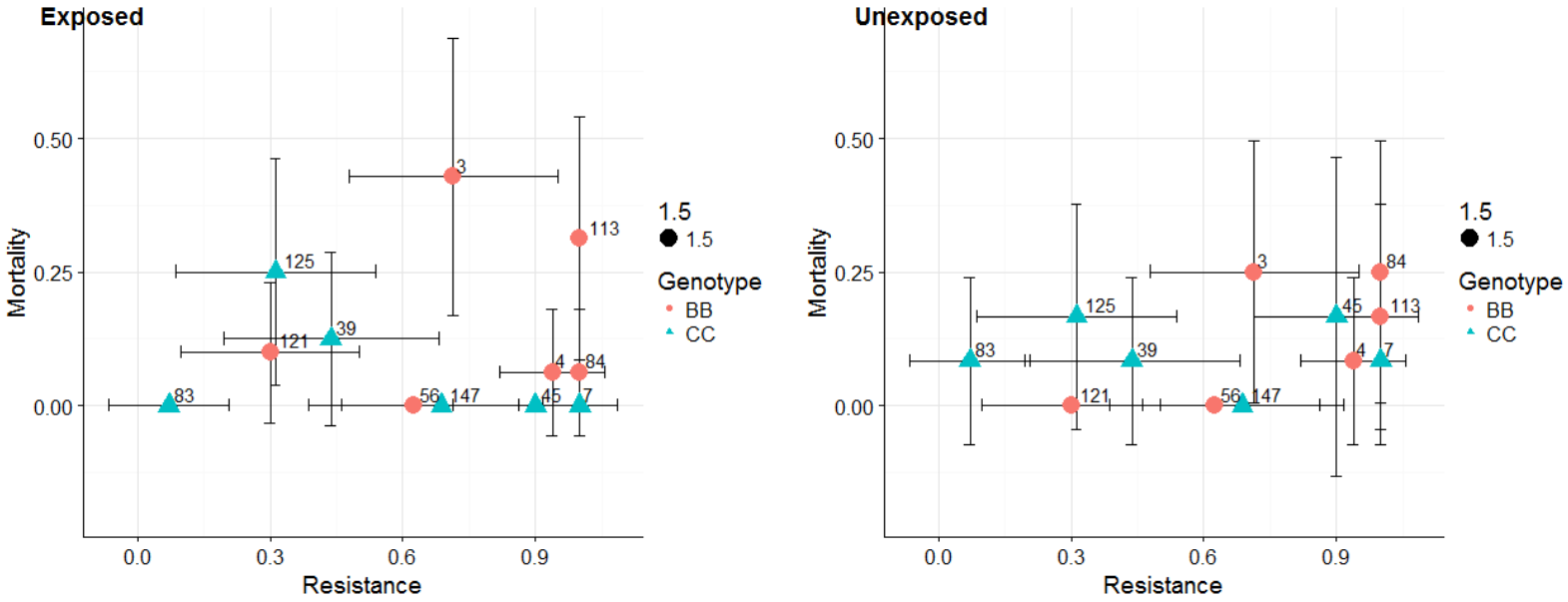


Figure 6: Snail resistance to infection by *S. mansoni* and mortality. 95% mortality confidence intervals shown as vertical bars, 95% resistance confidence intervals shown as horizontal bars. Points are labelled with snail line number.

**Tables:**

Table 1:

Table 1: A general summary of snail lines used in this study and the outcomes for each line of snails. Only snail death that occurred prior to week 11 is included in the died column.

| Line | Genotype | Exposed | Died | Infected | Survived Uninfected | Percent Resistant |
|---|---|---|---|---|---|---|
| 3 | BB | Yes | 6 | 4 | 4 | 71.4 |
| | | No | 3 | 0 | 9 | |
| 4 | BB | Yes | 1 | 1 | 14 | 93.75 |
| | | No | 1 | 0 | 11 | |
| 7 | CC | Yes | 0 | 0 | 16 | 100 |
| | | No | 1 | 0 | 9 | |
| 39 | CC | Yes | 3 | 9 | 4 | 43.75 |
| | | No | 1 | 0 | 11 | |
| 45 | CC | Yes | 0 | 1 | 9 | 90 |
| | | No | 1 | 0 | 5 | |
| 56 | BB | Yes | 0 | 6 | 10 | 62.5 |
| | | No | 0 | 0 | 11 | |
| 83 | CC | Yes | 0 | 13 | 1 | 7.1 |
| | | No | 1 | 0 | 11 | |
| 84 | BB | Yes | 1 | 0 | 15 | 100 |
| | | No | 3 | 0 | 8 | |
| 113 | BB | Yes | 5 | 0 | 10 | 121 |
| | | No | 2 | 0 | 9 | |
| 121 | BB | Yes | 2 | 14 | 2 | 30 |
| | | No | 0 | 0 | 12 | |
| 125 | CC | Yes | 4 | 11 | 1 | 31.3 |
| | | No | 2 | 0 | 10 | |
| 147 | CC | Yes | 0 | 5 | 10 | 68.8 |
| | | No | 0 | 0 | 12 | |

# Chapter 3

## General Conclusions

In Chapter 2, we tested for costs to *B. glabrata* of resisting infection by *S. mansoni*, using growth and early mortality as measures of fitness. We found no apparent costs to *B. glabrata* of resisting infection. We did, however, find a significant effect of proportion resistant on snail growth, regardless of whether a snail had been exposed to *S. mansoni* miracidia or not. We also found that snails with a *BB* genotype were more likely to die during the experiment, regardless of whether a snail had been exposed or not. Although resistant lines were smaller at both the five and 26 week time points and more likely to die in the first ten weeks post exposure, these results provide no evidence *B. glabrata* bear costs of resisting infection by *S. mansoni*.

These results do indicate resistant snails may grow slower, or attain smaller overall size, than more susceptible snails. Although we found no significant difference in size between *BB* and *CC* snails at five and 26 weeks post exposure, we also did not find perfect correlation in the inbred lines used for the study in Chapter 2 and proportion resistant. Previous studies have shown *SOD1* genotype is associated with resistance to infection, with snails possessing a B allele being more resistant to infection than snails with A or C alleles (Blouin et al. 2013; Goodall et al. 2006;

Tennessen, Bonner, et al. 2015). Given our results and those of other recent studies, it is possible that *B. glabrata* do incur fitness costs when resisting infection by *S. mansoni*, but that costs may be associated with a more complicated immune response than just activation of SOD1 (Hanington et al. 2012; Tennessen, Th??ron, et al. 2015).

Although costs of resistance have been thought by ecologists to be common among organisms across various taxa, recent evidence suggests costs may be context dependent or mediated by resource availability or acquisition (Sheldon & Verhulst 1996; Lochmiller & Deerenberg 2000; Anon n.d.; Schmid-Hempel & Ebert 2003; Brace et al. 2015; Schulenburg et al. 2016; Labbé et al. 2010; Bashir-tanoli & Tinsley 2014). Studies in Daphnia have shown that costs of resistance are the exception rather than the rule (Labbé et al. 2010). Other studies have shown that costs of resistance may only be apparent under starvation conditions, or may be due to reduced resource acquisition rather than resource allocation (Anon n.d.; Bashir-tanoli & Tinsley 2014). The snails we used in this experiment were kept in favorable conditions and fed *ad libitum*. It is possible that wild resistant snails bear costs of resisting infection by *S. mansoni* due to limited food resources or poorer environmental conditions that are not apparent under laboratory conditions.

We interpret the results of Chapter 2 as showing no apparent cost to *B. glabrata* of resisting infection by *S. mansoni*, as measured by differences in growth and mortality between *BB* and *CC* genotype snail lines. Although we have not provided conclusive evidence that no costs of resisting *S. masoni* infection exist in *B. glabrata*, our results are supported by other studies in *B. glabrata* and other organisms (Bonner et al. 2012; Labbé et al. 2010). These results, taken with results from

molecular and genetic studies on *B. glabrata*, suggest that *S. mansoni* resistant *B. glabrata* could be an effective control strategy to use in an integrated approach to controlling Schistosomiasis transmission (Rollinson et al. 2013; Ross et al. 2014; Evan Secor 2014; Tennessen, Th??ron, et al. 2015; Tennessen, Bonner, et al. 2015; Hanington et al. 2012). Research groups have recently had success modifying mosquitoes using CRISPR in efforts to control mosquito-transmitted diseases (Gantz et al. 2015; Alphey 2016; Champer et al. 2016). Although the mechanisms used to control schistosome transmission thorugh *B. glabrata* would likely be different than those used in mosquitoes, the CRISPR technology used to modify mosquitoes could be adapted to modify snails.

Our results are interesting in both ecological and human disease contexts. Ecologically, our results provide further evidence that costs of resistance may be less ubiquitous than previously thought (Sheldon & Verhulst 1996; Lochmiller & Deerenberg 2000; Anon n.d.; Labbé et al. 2010; Bonner et al. 2012; Bashir-tanoli & Tinsley 2014; Brace et al. 2015; Schulenburg et al. 2016). These results also provide additional evidence *B. glabrata* may be an effective biological control method for Schistosomiasis (Bonner et al. 2012; Tennessen, Th??ron, et al. 2015; Tennessen, Bonner, et al. 2015).

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