Differential Allele Expression of \textit{SOD1} in \textit{Biomphalaria glabrata}

by

Samantha M. Carolla

A PROJECT

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Biology (Honors Associate)

Presented May 18, 2010
Commencement June 12, 2010
AN ABSTRACT OF THE THESIS OF:

Samantha M. Carolla for the degree of Honors Baccalaureate of Science in Biology presented on May 18, 2010. Title: Differential Allele Expression of SOD1 in Biomphalaria glabrata.

Abstract Approved: __________________________________________________________
Christopher J. Bayne, OSU Zoology Department

The freshwater snail Biomphalaria glabrata serves as the intermediate host of the blood fluke Schistosoma mansoni. Previous studies using the 13-16-R1 strain of B. glabrata demonstrated an association between the gene encoding Cu/Zn superoxide dismutase (SOD1) and susceptibility/resistance to the parasite. This association is assumed to involve a polymorphism that regulates transcription since individuals possessing the B allele have higher transcript levels of SOD1 than individuals without the B allele. The objective of this project was to determine if SOD1 alleles are differentially expressed in B. glabrata heterozygous hemocytes. This study detected no evidence of allelic imbalance, implying that transcription of SOD1 alleles is likely controlled by a trans-acting regulator (secondary gene) rather than a cis-acting regulator.

Keywords: Biomphalaria glabrata, Schistosoma mansoni, SOD1, regulatory polymorphism, differential allele expression

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APPROVED:

Mentor, representing Biology

Committee Member, representing Biology

Committee Member, representing Biology

Chair, Department of Zoology

Dean, University Honors College

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

Samantha M. Carolla, Author
ACKNOWLEDGMENTS

Christopher Bayne, Ph.D. and Randy Bender for patiently serving as mentors and committee members throughout this project.

Virginia Weis, Ph.D. for her support as a committee member.

Maureen Larson and Kaitlin Bonner for assisting in practice defenses and for providing encouragement and feedback.

Tyler Abel for pushing me to stay focused and lovingly scolding me when I wasn’t.

Josh Klein and Jill Brown for having been there before.

My family and friends for all their love and support.
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INTRODUCTION

Schistosomiasis is an infectious human disease caused by the trematode blood flukes: *Schistosoma mansoni*, *Schistosoma haematobium*, and *Schistosoma japonicum*. These parasites are predominantly found in tropical regions of Africa, South America, and Southeast Asia and the disease afflicts 207 million people worldwide (King, 2009). Symptoms of schistosomiasis vary, and many afflicted individuals are initially asymptomatic. Typically, an itchy rash develops at the site of skin penetration by the schistosome, and within months a person can experience fever, chills, cough, and muscle aches. However, the most detrimental effects occur within the liver, spleen, and intestines (Van der Werf et al, 2002). In school-aged children, schistosome infection is also associated with anemia, a factor which can profoundly affect physical and mental development (Mahgoub et al., 2009). It is estimated that schistosomiasis causes 200,000 deaths annually (Van der Werf et al, 2002).

Although schistosome-related morbidity and mortality have decreased in recent years due to use of the drug Praziquantel, reinfection and drug resistance have become increasingly widespread (Bayne, 2009; King, 2009). Because of this, additional strategies are being developed to prevent the disease. One potentially meritorious approach is to interrupt the parasite life cycle at the level of its intermediate host. Schistosome eggs are normally introduced into tropical freshwater streams or lakes by the feces or urine of infected humans and other mammals. The eggs soon hatch and release larvae (miracidia) that infect specific species of snails. In susceptible individuals of the infected snail, miracidia shed their ciliated covering and develop into mother sporocysts. Each mother sporocyst asexually reproduces, yielding daughter sporocysts which subsequently
produce cercariae. Approximately one month after being infected, the snail begins to shed thousands of cercariae into the water. Humans contract the parasite by coming into contact with the infected water, typically by wading or bathing. The cercariae penetrate the skin and require less than half an hour to fully enter the epidermis. Within 24 hours, the schistosomes (as schistosomules) enter the peripheral circulation and pass to the heart. From here, the schistosomules enter the pulmonary capillaries and access the systemic circulation. The parasites spend about 3 weeks developing in the liver before travelling to the blood vessels of the intestines or bladder, where they mature and begin to reproduce. The parasite’s life cycle is perpetuated when infected humans pass the schistosome eggs in feces or urine. Since both resistant and susceptible snails exist in natural populations (Stein, 1979; Morand et al, 1996), humans can become infected only when susceptible snails shed cercariae. Thus, a better understanding of the biochemical and molecular components that dictate snail resistance/susceptibility (R/S) may provide insights into methods that could be developed to disrupt the life cycle. To this end, laboratory strains of *Schistosoma mansoni* and its intermediate host, *Biomphalaria glabrata*, have been used in attempts to discover which factors determine the outcome of a snail-schistosome encounter (Richards & Merritt, 1972; Bayne, 2009).

The *B. glabrata* strain (13-16-R1) used in the work presented here is predominantly resistant to *S. mansoni* (PR-1 strain). Both in vivo and in vitro studies have demonstrated that phagocytic hemocytes in resistant snails’ hemolymph encapsulate and kill parasites within 48 hrs (Jourdane, 1982; Loker et al, 1982; Hahn et al, 2001). In vitro studies involving hemocyte-sporocyst interactions have illustrated that, like mammalian leukocytes, hemocytes undergo a respiratory burst and generate reactive
oxygen species (ROS) (Adema et al, 1994; Fig 1). Furthermore, hydrogen peroxide ($H_2O_2$) was shown to be a decisive constituent in parasite killing (Hahn et al, 2001). These results suggest that enzymes and pathways involved in the production or consumption of $H_2O_2$ likely play vital roles in determining R/S.

Cytosolic copper/zinc superoxide dismutase (SOD1) catalyzes the production of hydrogen peroxide ($H_2O_2$) from superoxide ($O_2^-$). Within the 13-16-R1 population, there are three major alleles (A, B, C) of the SOD1 gene, which encode for the superoxide dismutase enzyme (Goodall et al, 2006). Coordinated phenotyping (R/S) and genotyping experiments have revealed a significant association between the B allele and resistance, as well as an association between the C allele and susceptibility to S. mansoni infection (Goodall et al, 2006). However, these alleles do not account fully for R/S, indicating that other loci are involved. In a subsequent study, hemocyte SOD1 expression was measured in 13-16-R1 individuals, and the B allele was found to be associated with higher SOD1 expression (Bender et al, 2007). Together, these results imply that the B allele is not merely a marker locus for resistance, but that it has a functional association. The association of the B allele with resistance, and higher SOD1 transcript levels in R snails, imply its apparent linkage to a polymorphism that influences transcription.

The work presented here focuses on the specific type of putative regulatory polymorphism that may influence SOD1 expression. Such regulatory variants can be classified as either a cis-acting or trans-acting (Wittkopp, 2005). Cis- regulatory regions can be located on a gene’s promoter region, within an intron, or further upstream on the mRNA (Hubner et al., 2005). In contrast, trans- regulatory regions are genes that have secondary effects on other genes (Yvert et al., 2003). As a first step toward determining the
putative regulatory region and in order to classify the SOD1 regulatory polymorphism, we set out to establish whether or not, in heterozygous individuals, SOD1 alleles are expressed differentially (allelic imbalance). The presence of allelic imbalance would be evidence for a polymorphism that affects a single allele (a cis-acting variant). The absence of allelic imbalance would imply that any polymorphism of a regulatory element affects all alleles (a trans-acting variant), indicating that another gene influences SOD1 transcription. The goals of this project were to implement a protocol for detecting allelic imbalance, and to examine heterozygous B. glabrata individuals for evidence of differential allelic expression in hemocytes from BC heterozygotes.
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MATERIALS & METHODS

Hemocyte collection, RNA extraction and cDNA synthesis

Hemolymph (approx. 75 µL) was collected from individual 13-16-R1 snails via cardiac puncture, placed on Parafilm® to allow shell debris to settle (approx. 1 min), transferred to an untreated 96-well cell culture plate, and spun for 10 min at 400 rpm. Hemocytes were allowed to adhere for 15 min at 26°C and then washed three times with 150 µL Chernin’s balanced salt solution (CBSS; Chernin, 1963). Each well was examined microscopically and those wells with low numbers of hemocytes were excluded. Following CBSS removal, hemocytes were lysed in 150 µL Trizol® (Invitrogen, Carlsbad, CA) and transferred to a 0.65 mL tube. RNA was purified as detailed below and at each step the organic and aqueous phases were thoroughly mixed and then separated by centrifugation. First, 30 µL of chloroform was added to the lysate. After being mixed, sample were centrifuged for 15 min at 12,000 x g. Next, 100 µL of the pink organic layer was removed and discarded. Due to the difficulty of this extraction, the two phases were again centrifuged to more easily collect 65 µL from the aqueous layer. This extraction was added to a new tube containing 65 µL of acid phenol/chloroform (USB, Cleveland, Ohio). After another round of mixing and centrifugation (5 min at 12,000 x g), 30 µL was collected from the aqueous layer and added to 30 µL of chloroform-isoamyl alcohol (1:1), 3 µL 3M sodium acetate, and 2 µL Pellet Paint® (Novagen, Darmstadt, Germany). The Pellet Paint® facilitates the extraction of small amounts of RNA by making the pellet more visible. The RNA was allowed to precipitate at room temperature for 10 min, pelleted (15 min at 16,000 x g) and the pellet
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rinsed with 100 µL of 70% ethanol. All liquid was removed and the pellet was allowed to 
air dry for 5 min, and dissolved in 10 µL of RNase-free water. Each RNA sample was 
treated with DNAase (Ambion., Foster City, CA) following manufacturer’s instructions 
to remove any contaminating DNA. Purified RNA was converted to cDNA using both 
oligo-dT and random hexamer primers with Sprint Powersonscript Reverse Transcriptase® 
(Clontech, Mountain View, CA) following the manufacturer’s protocol.

**Headfoot collection and genomic DNA isolation**

Immediately after hemolymph collection, the headfoot from each individual was 
removed for subsequent DNA extraction as previously described (Goodall et al, 2006). 
Briefly, each headfoot (approximately 35 mg each) was excised with a new razor blade 
and placed into a tube containing 200 µL digestion buffer made up of 2% w/v 
hexadecyltrimethyl-ammonium bromide, 1.4M NaCl, 0.2% v/v-mercaptoethanol, 20mM 
EDTA, 100mM Tris–HCl pH 8, and 200µg/mL proteinase K (Winnepenninckz et al, 
1993). The tubes were incubated on a shaking platform for 16 h at 37ºC, followed by 
centrifugation at 15,000 x g for 5 minutes. Next, 125 µL was extracted from each sample 
and placed into a new tube. The remaining 75 µL of crude sample was stored and 
preserved at -80 ºC as a reserve sample. The DNA samples were not pooled and each 
individual was placed in a labeled tube at each step. The resulting digests were each 
moved to a new tube and mixed with an equal volume (125 µL) of 
phenol/chloroform/isoamyl alcohol (25:24:1), then centrifuged at 15,000 x g for 5 
minutes (Goodall et al, 2006). From this, 100 µL of the aqueous layer was extracted and 
added to a new tube with 100 µL of chloroform-isoamyl alcohol (24:1), and once more
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Centrifuged at 15,000 x g for 5 minutes (Goodall et al, 2006). For the final extraction 60 µL of the aqueous layer was removed and placed in a new tube. Finally, the DNA was precipitated by adding 2/3 volume (40 µL) isopropanol, pelleted by centrifugation, air dried and dissolved in 200 µL distilled water. Individual snails were genotyped as previously described (Goodall et al, 2006).

\textit{Quality control check of cDNA synthesis}

In order to determine if the RNA extraction and cDNA synthesis protocols (above) yielded sufficient quantities and quality of cDNA, Polymerase Chain Reaction (PCR) was performed to amplify \textit{SOD1}. The process was done as previously described (Goodall et al, 2006). All primers were designed using Primer 3 (Rozen & Skaletsky, 2000). The PCR master mix components (amount per reaction) were 10.5 µL of RNA-free water, 8 µL of 2.5x Eppendorf master mix, 0.5 µL of the previously designed forward primer S1 (TTCTATCATTGGTCGCAGCTT), and 0.5 µL of the previously designed reverse primer S2 (CACCACAAGCTAAGCGAGGT). Next, 19.5 µL of the PCR master mix was mixed with 0.5 µL of cDNA template for a final volume of 20 µL for each sample. The PCR profile was as follows: 95 ºC for 2 minutes, 95 ºC for 20 seconds, 56 ºC for 20 seconds, and 72 ºC for 20 seconds. This process was repeated for 40 cycles. Samples were then run on a 1 % agarose gel with a sodium borate buffer (Goodall et al, 2006). The presence of an amplicon with the expected size of the S1- S2 amplicon (112 nt) was the basis used for determining whether or not cDNA was present. Pictures of the cDNA quality control checks and other descriptions of this project can be
found in the associated lab notebook in Cordley 3009.

*Genomic DNA contamination check of cDNA*

In order to determine if any genomic DNA contamination was present in cDNA samples, PCR was performed. The process was done as previously described (Goodall, 2006). The PCR master mix components (amount per reaction) were 10.5 µL of deionized water, 8 µL of 2.5x Eppendorf master mix, 0.5 µL of the *SOD1* forward primer S11 (GACCTTTGACTTACAGCCATGAA, intron 4), and 0.5 µL of the *SOD1* reverse primer S12 (AACAGTCCTGTCTAGCC, intron 4). Next, 19.5 µL of the PCR master mix was mixed with 0.5 µL of cDNA template for a final volume of 20 µL for each sample. The PCR profile was as follows: 95°C for 2 minutes, 95°C for 20 seconds, 55°C for 20 seconds, and 72°C for 20 seconds. This process was repeated (without the initial 2 min at 95 °C) for 40 cycles. Samples were then run on a 1% agarose gel with an SB buffer (Goodall et al., 2006). Both a no-template-control (water substituted in a well) and a positive genomic DNA control (known genomic DNA sample) were used as the determinants for the presence of genomic DNA contamination. Pictures of the genomic DNA contamination checks and other descriptions of this project can be found in the associated lab notebook in Cordley 3009.

*Amplification of SOD1 cDNA and genomic DNA by polymerase chain reactions (PCR)*

In this study, cDNA and genomic DNA were amplified using PCR and were processed as previously described (Goodall et al, 2006). The PCR master mix components (amount per reaction) were 10.5 µL of deionized water, 8 µL of 2.5x Eppendorf master mix, 0.5 µL of the *SOD1* forward primer AE4 (AACATTGTGGCTG
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GTGATGA), 0.5 µL of the SOD1 reverse primer AE 5 (AAGCTGCGAC CAATGATAGAA). Next, 19.5 µL of the PCR master mix was mixed with 0.5 µL of either cDNA or genomic DNA as a template for a final volume of 20 µL for each sample. The PCR profile was as follows: 95 ºC for 2 minutes, 95 ºC for 20 seconds, 55 ºC for 20 seconds, and 72 ºC for 20 seconds. Thermal cycling was repeated for 44 cycles. Samples were then run on a 1 % agarose gel with SB buffer (Goodall et al., 2006).

After the PCR, the samples were each treated with shrimp alkaline phosphatase (SAP) and an exonuclease (EXO1), as described in the protocol for the SNaPshot® kit (Applied Biosystems). The SAP treatment removes the primers from the sample, whereas EXO1 removes the unincorporated dNTPs. The EXO1 was diluted (for each sample) by adding 0.2 µL stock EXO1 to 0.8 µL EXO1 buffer (80 mM Tris, pH 9; 2mM MgCl2). Next, 1.0 µL of diluted EXO1 was added to 5 µL of SAP and 2.3 µL of 10x SAP buffer, and was used for the 15.5 (0.5 µL each) PCR products. Finally, the samples were incubated at 37 ºC for 1 hour, followed by 75 ºC for 15 minutes.

Single base pair extension of cDNA and genomic DNA using the SNaPShot® protocol

Allele expression was measured using the SNaPshot® reaction (Applied Biosystems). This procedure is a primer extension method involving the addition of a fluorescently labeled terminator nucleotide (ddNTP) at the site of a single nucleotide polymorphism (SNP) that discriminates between the SOD1 B and C alleles. Thus, when analyzing samples from a heterozygote, the end product of this reaction essentially contains a primer labeled with either one of two fluorescent tags. The SNaPshot® kit (Applied Biosystems) contains four different ddNTPs each labeled with a different
colored fluorophore. After capillary electrophoresis and scanning, the peak area of each fluorophore is measured, and this is proportional to the amount of each amplified allele. Since each fluorophore has unique properties in terms of its fluorescent intensity and its influence on incorporation of the ddNTP, the peak areas are not always identical between two alleles of equal abundance (Anderle et al, 2004). Therefore, the allelic ratios of genomic DNA are used to normalize the ratios of cDNA, since heterozygote genomic DNA is assumed to contain equal amounts of each allele.

The base pair extension mix was made of the following components: 2.0 µL of SNaPshot® reaction mix, 1.0 µL of the forward extension primer (TGATGATGGTGTTGCTGA), 6.0 µL of deionized water, and 1.0 µL of EXO1 and SAP treated PCR product. The thermocycler was programmed for the single base pair extension reaction as follows: 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 30 seconds. Thermal cycling was repeated for 25 cycles and put on hold at 4°C. The SNaPshot® kit was handled and implemented following the manufacturer’s instructions (Applied Biosystems). In order to remove the primers used in the single base pair extension, another SAP treatment was performed. For each sample, 1.0 µL of SAP and 1.0 µL of 10x SAP reaction buffer were used. The samples were then incubated at 37 °C for 1 hour, followed by 75 °C for 15 minutes. Finally, the products of the treated single base pair extension reactions were sent to the OSU CRBG Core Lab for processing by capillary electrophoresis. This lab used AB 3730 Gene Scanner and Genemapper 3.7 (Applied Biosystems) to obtain measured peak areas from the cDNA and genomic DNA samples. Peak areas were identified based on the corresponding fluorophore of the incorporated ddNTP.

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RESULTS

Correlation between fluorescence peak area and allelic ratios of genomic DNA

Control experiments were done to assess the accuracy and reliability of the assay; these examined if the peak area ratios were proportional to the known amounts of each allele added (Figure 1). Genomic DNA from BB and CC homozygous individuals were used to create specific dilutions of the SOD1 B allele to the C allele (1:1, 1:2, 1:3, & 1:4). After amplification, each allele was fluorescently labeled with a specific ddNTP fluorophore during single base pair extension using the SNaPshot kit. Capillary electrophoresis was used to separate components of the reaction mix, and electrophoregrams were scanned to detect the fluorescent markers in the form of allele-specific peak areas which were measured. Peak area ratios were determined as the ratio of the B allele to the C allele for each individual’s genomic DNA. The dilutions were used as standards to assess the power of the assay to accurately quantitate the allele expression (peak area) from each sample. Each dilution was run in triplicate; only mild deviations were observed between samples and this deviation is represented by the error bars (Figure 1). The assay was deemed to accurately reflect the ratios of alleles in genomic DNA ($r^2 = 0.977$). Therefore these data validated the use of the same methodology to determine the extent of allelic imbalance in SOD1 in the BC individuals.

Normalized peak area ratios in BC heterozygous individuals

We identified 4 BC snail individuals that were appropriate for analysis of allelic expression. After amplification and treatment, the DNA each individual was subjected to the single base pair extension and capillary electrophoresis. Genomic DNA and cDNA from each snail were run in triplicate in order to reveal any variability within
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the assay. The quantity of mRNA representing each allele was determined by comparing the quantities of each fluorophore. In each case, fluorophore-labeled amplicons of the B and C alleles flurophores were the highest peak areas. Weak peaks of the incorrect corresponding fluorophore were determined to be background and deemed not relevant to this study. The area of the peak representing the B allele and that representing the C allele were used to calculate the peak area ratios (B/C) (Figure 2a). Normalized peak area ratios (cDNA/gDNA) were obtained from genomic DNA and cDNA from the same individual and were run within the same assay (Figure 2b). If allelic imbalance is present in a sample, then the normalized peak area should deviate clearly from unity, which is represented as a 1:1 ratio of cDNA/gDNA (Zhang, 2005). As a group, the normalized peak area ratios for our samples approached unity, mean normalized peak ratios = 0.94 ± 0.17 (Figure 2a). For individual samples, it is more difficult to determine deviation from unity. Since each bar represents the triplicates of the normalized peak areas per individual, variation (both biological and technical) among samples of the triplicates becomes more apparent. It is for this reason that we decided to evaluate the assay for the introduction of technical variance (differences in fluorescence) due to both the single base pair extension step and the capillary electrophoresis step. When allelic expression of *SOD1* was previously investigated in homozygous 13-16-R1 snails, the ratio of allele expression (B allele to C allele) was found to equal 1.66 times (Bender et al, 2007). Since allelic ratios in our data do not approach this value, it appears that the B allele is not imbalanced with the C allele. The only individual in which the allelic ratio diverged somewhat from unity was AE 60 (Figure 2b), and this may imply that the C allele is in very slight imbalance relative to the B allele. This marginal imbalance is considered
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unlikely to be of biological significance in determining R/S and SOD1 activity and therefore was not further analyzed.

*Technical variation due to single base pair extension and capillary electrophoresis*

While testing for allelic imbalance (Figure 2), variation was apparent within triplicate samples of the individual snails. In order to determine if the extent to which the assay protocol might be responsible for the differences among samples, we evaluated the two critical steps: single base pair extension and capillary electrophoresis. Samples AE 56 and AE 60 were used to test these procedures since sufficient quantities were available of both the cDNA and genomic DNA. The single base pair extension was tested by separating amplified cDNA and gDNA into triplicates prior to performing the single base pair extensions. After undergoing the same capillary electrophoresis, the triplicates for AE 56 and AE 60 appeared to deviate little within each trio (Figure 3). The capillary electrophoresis was tested by dividing the AE 56 and AE 60 samples only after they underwent the same PCR and single base pair extension. These results also varied little among the aliquots comprising the triplicates of each individual sample (Figure 4).
Figure 1: Correlation between fluorescence peak area and allelic ratios of genomic DNA. Genomic DNA was isolated from snails that were homozygous at the SOD1 locus (i.e. BB or CC individuals). Different amounts of gDNA were used to obtain B:C ratios of 1:1, 1:2, 1:3, 1:4. The gDNA mixtures were amplified and the alleles were labeled using the SNaPshot kit. The peak area for each allele was determined by capillary electrophoresis. The peak area ratios were plotted against the known gDNA ratios, demonstrating that the assay is quantitative ($r = 0.977$).
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### a) Observed and Normalized Peak Area Ratios

<table>
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<tr>
<th>Samples</th>
<th>Normalized cDNA/gDNA</th>
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<tbody>
<tr>
<td>AE 31</td>
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</tr>
<tr>
<td>AE 50</td>
<td>0.888</td>
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<tr>
<td>AE 56</td>
<td>1.086</td>
</tr>
<tr>
<td>AE 60</td>
<td>0.902</td>
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<table>
<thead>
<tr>
<th>Mean ± S.D.</th>
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<td>3.71 ± 0.33</td>
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### b) Normalized Peak Area Ratios in BC Heterozygous Individuals

Figure 2:

- **a)** *Observed and normalized peak area ratios for cDNA and genomic DNA in BC heterozygous samples.* The SNAPshot reaction and capillary electrophoresis were employed to measure peak areas of the B allele over the C allele in both genomic DNA and cDNA. Data were normalized by dividing cDNA peak area ratios by genomic DNA peak area ratios.

- **b)** *Normalized peak area ratios in BC heterozygous individuals.* The bars represent the mean (± standard deviation) of the normalized ratios of three separate assays per individual. Samples are compared to unity which is defined as the lack of allelic imbalance (i.e. cDNA: gDNA = 1:1).
Figure 3: Variation among replicates of the SNaPshot reaction. A single PCR reaction and subsequently three SNaPshot reactions were prepared from cDNA and genomic DNA from two individuals (AE 56 and AE 60). Peak areas were measured by capillary electrophoresis and peak area ratios (B allele: C allele) were calculated. Each bar represents the mean peak area ratio (± standard deviation).
Figure 4: **Variation among replicates of capillary array electrophoresis.** A single PCR reaction and subsequently a single SNaPshot reaction were prepared from cDNA and genomic DNA from two individuals (AE 56 and AE 60). Peak areas were measured in triplicate by capillary electrophoresis and peak area ratios (B:C) were calculated. Each bar represents the mean peak area ratio (± standard deviation).
DISCUSSION

**Significance of regulatory variants**

Regulatory variants of a gene are sequence polymorphisms that modify the expression level of a transcript, and can be classified as either *cis*-acting or *trans*-acting elements. By definition, a *cis*-regulatory polymorphism would be found within the SOD1 locus and a *trans*-regulatory polymorphism would be another gene (Wittkopp, 2005). Consequently, the presence of allelic imbalance would constitute evidence for a *cis*-acting polymorphism, whereas the absence of allelic imbalance would suggest a *trans*-acting polymorphism (Pastinin & Hudson, 2004). Based on our research, the B and C alleles of *B. glabrata* SOD1 do not appear to be so imbalanced as to imply a *cis*-acting variant. This implies that a *trans*-acting variant is involved. It is important note that, due to the small number of samples (n=4) and because of individual differences, our work may have failed to detect allelic imbalance that is, in reality, present; however the available evidence suggests that this is not the case. Further research will be needed in order to definitively determine this as well to determine what other gene might be involved if a *trans*-acting polymorphism regulates transcription of SOD1 in this mollusc.

**Statistical analysis**

In this project no formal statistical analysis was performed. Firstly, the sample size (n=4) was too small to use traditional parametric tests (i.e. *t*-test), and, due to time constraint, it was impractical to isolate more genomic DNA and cDNA of the appropriate genotype (BC). Secondly, a similar analysis is needed for AB and AC heterozygous snails. This would allow a more robust inference as to the presence or
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absence of *cis* or *trans*-regulation of transcription. The AB samples that were originally procured, were contaminated during PCR steps and could not be used in the project. Lastly, although the Oregon State University Statistics Department was consulted in order to implement a non-parametric test, and the mentor and author determined that it was beyond the scope of this thesis and would likely invoke a type-I error or reject the null hypothesis (no allelic imbalance) when there is actually no differential expression. This thesis is therefore strictly empirical and observational, and any inferences are based on logical reasoning rather than a statistical analysis.

*Next steps to explore and consider*

It is evident that although the thesis is complete, the project is not. In tandem with the presentation of these observational findings, AB genotyped individuals are currently being processed. It is also likely that AC genotyped individuals will be available for study. Within the next couple of months more genomic DNA and cDNA will be isolated and analyzed in order to increase the sample number and diversify the genetics. In order to measure allelic expression with greater accuracy and precision, a new quantitative Polymerase Chain Reaction (qPCR) instrument will used. This will enable us to better detect and measure the relative allelic expression when normalized to genomic DNA. With these objectives pursued, the project will provide a more definitive answer as to whether a *cis-* or *trans-* polymorphism is regulating SOD1 expression in *B. glabrata.*
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