FUNCTIONAL SIGNIFICANCE OF SUPEROXIDE DISMUTASE (SOD-1): GENOTYPIC

AND PHENOTYPIC POLYMORPHISM IN CLONAL LINES OF RAINBOW TROUT

by

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of AMBER NICOLE PARRISH find it satisfactory and recommend that it be accepted.

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Abstract

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In the past decade a strong correlation has been drawn between an increase of the endogenous antioxidant enzyme superoxide dismutase (SOD-1) activity, the reduction of reactive oxygen species (ROS), and prevention of oxidative damage accumulation which results in increased disease resistance. The variation seen in antioxidant enzyme activity is a major determinant to how susceptible an organism is to ROS attack and oxidative stress (damage done to DNA, proteins, and lipid membranes), yet little is understood concerning the actual genetic controls underlying the degree of phenotypic variation seen. A recent report found that the *SOD-1* locus among eight clonal lines of rainbow trout (*Oncorhynchus mykiss*) is hypervariable, containing 19 SNPs that result in 4 amino acid substitutions. The purpose of this research was to investigate the functional effects of this variation and the genetic basis of variation in SOD-1 enzyme activity. First we compared the SOD-1 enzyme activity in the liver of five of those clonal lines of rainbow trout and found two lines with significantly divergent activities: Oregon State University (OSU) with high enzymatic activity and Arlee (AR) with low enzymatic activity. Next we tested OSU and AR to see if there was a difference in oxidative damage by comparing liver lipid peroxidation (MDA) accumulation in both young (4 month) and old (52 month) individuals. There was no difference in MDA

concentrations found between young OSU and young AR, but both older OSU and AR were significantly higher than their younger counterparts, and unexpectedly older OSU had a greater amount of MDA than older AR. Finally, doubled haploid progeny produced from an F1 hybrid of these two lines were used to evaluate the possible quantitative trait loci (QTL) for SOD-1 enzyme activity. Composite interval mapping revealed two significant QTL with opposing additive effects, explaining 19% and 34% of the phenotypic variation; neither of these QTL contain the *SOD-1* locus itself. These two QTL, and the surprising inverse relationship between SOD-1 enzyme activity and lipid peroxidation levels, suggest a complex polygenic control for antioxidant activity and oxidative damage accumulation.

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Dedication

This thesis is dedicated to my husband in thanks
for his humor and never-ending patience

CHAPTER 1

INTRODUCTION

Oxidative Damage

Reactive oxygen species (ROS) are formed by an incomplete reduction of oxygen. The most common ROS are superoxide anions (O_2^-), nitric oxide (NO^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-). All of these oxidants are naturally formed within an organism from either environmental or endogenous processes. However when in abundance ROS cause damage to proteins, lipids and nucleotides. Environmental factors that lead to an increase of ROS are exposure to heavy metals, oxygen, UV radiation, insecticides, or smoking (Garcia et al. 2008; Hansen et al. 2006). Diet also can be a factor in ROS production, as high levels of salt and chronic alcohol consumption can cause ROS production (Bailey et al. 2002; Zhu et al. 2007). However, the majority of ROS (about 90%) are produced during normal cellular function (Bailey et al. 2002). Immune response, enzyme function, signal transduction, and electron leak from respiration along the electron transport chain all contribute to an increase of ROS, and likewise, an increase in oxidative damage (Agostini et al. 2002; Ahmed et al. 2008).

When in abundance, ROS can cause oxidative damage in membrane lipids, proteins, and DNA. Polyunsaturated lipid membranes are especially vulnerable to ROS attack because the bonds neighboring a C=C bond will break, disassociate, and produce a lipid radical that can go on to cause further damage and ROS production (Hulbert et al. 2007; Zhu et al. 2007). Oxidative damage accumulates over time causing cellular membranes to lose fluidity, increases in the rate of DNA base mutations, changes in protein structures by oxidizing amino acids, and reduction of telomere length leading to an acceleration of cell death (Droge 2002;

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Hulbert et al. 2006; Valko et al. 2006). Such accumulation of oxidative damage is thought to be a proximal cause of aging as the effects are progressive, harmful, endogenous, and irreversible (Hulbert et al. 2007).

In Atlantic salmon, 6 weeks of moderate hyperoxia resulted in lipid peroxidation, lower enzyme activity in SOD and GPX, and levels of ascorbate were reduced (Lygren et al. 2000). This suggests that increased oxygen within the tank environment not only reduced the salmon's antioxidant defenses, but as a result of that loss suffered more damage and likewise reduced growth (Dabrowski et al. 2004). Another study also measured notable weight and appetite loss in other species of fish exposed to oxidative stress (Wilhelm et al. 2005). More recently individuals exposed to only 3 weeks of hyperoxia showed significant decrease in the rate of food consumption and consequently reduced body mass (Espmark et al. 2009).

ROS attack and oxidative damage cause an increase in apoptosis (Agostini et al. 2002; Ahsan et al. 2003; Baruchel et al. 1992) and decrease effective resistance to bacterial infection in trout (Caldwell et al. 1995). For instance, the effects of autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus are compounded by damage done to DNA from ROS attack. The damage results in macromolecules with structural changes that make them unrecognizable as "self" and subsequently illicit an immune response (Ahsan et al. 2003). Other diseases with a possible causal relationship to oxidative stress have been identified, including amyotrophic lateral sclerosis (ALS), cancer, kidney failure, diabetes, and hypertension (Barber et al. 2006; Cejas et al. 2004; Halliwell 1992; Plavec et al. 2008; Rajneesh et al. 2008; Skrzycki et al. 2007; Ushio-Fukai et al. 2008; Valko et al. 2006; Zhu et al. 2007). There are also direct correlations between tumor growth, DNA mutation and the damage caused by ROS and lipid peroxides (Cejas et al. 2004; Valko et al. 2006). In many

instances the presence of cancer also correlates with elevated quantities of lipid peroxides and

ROS (Plavec et al. 2008; Rajneesh et al. 2008; Ushio-Fukai et al. 2008).

Antioxidants

The body can cope with ROS through a system of scavenging antioxidants. When an

antioxidant comes into contact with a ROS, it will undergo a redox reaction and neutralize the

free radical. This exchange causes the antioxidant to become a radical itself, but due to their

unique nature, they are considered 'stable radicals' and will not damage the body (Hansen et

al. 2006; Kiron et al. 2004). The ratio between antioxidants and ROS would ideally be in

balance, where the rate of ROS production does not exceed the antioxidant's level of defense.

If production of ROS overwhelm antioxidant defenses, they will continue on to cause

oxidative damage until the rate of ROS production either decreases, or the level of

antioxidants increase to meet the demand (Monaghan et al. 2008). Increasing the quantity of

antioxidants can be established by increasing production of antioxidant enzymes, or manually

with dietary antioxidants like vitamins C and E (Hansen et al. 2006).

Both Vitamin C and E are anti-oxidants. Vitamin C is both hydrophilic and an acid.

With dual responsibilities it serves as both an antioxidant and regenerates other antioxidants

(Martinez-Alvarez et al. 2005). Increasing the quantity of dietary vitamin C has shown to

lower blood pressure and increase vascular relaxation (Chen et al. 2001; Zhu et al. 2007).

While it can access a significant variety of systems within the body, it cannot directly protect

the lipid bilayer of cell membranes, and in excess may reduce pH (Kiron et al. 2004). Since

vitamin E is hydrophobic it can better protect these lipid structures. However, this

characteristic also makes it difficult to clear from the body, and excess vitamin E can cause

deleterious effects such as renal failure, hepatotoxicity, and immune suppression (Kiron et al. 2004).

The first antioxidant enzyme, superoxide dismutase (SOD), appeared around 2 BYA as a defense against the increase of atmospheric oxygen, a byproduct of photosynthesis originating roughly 3.5 BYA (Scandalios 2002; Zelko et al. 2002). SOD dismutates superoxide anions (O_2^-) to hydrogen peroxide (H_2O_2). Dismutation is the process by which two like molecules are both reduced and oxidized at the same time. Radicals will dismutate naturally, though it is an incredibly slow process that allows ample time for ROS to cause damage. Enzymatic dismutases scavenge ROS and facilitate a speedier neutralization (Zelko et al. 2002).

After the formation of H_2O_2 there two other antioxidant enzymes, catalase (CAT) and glutathione peroxidase (GPX), that reduce H_2O_2 into O_2 and H_2O. These two enzymes, in combination with SOD, all work together to neutralize ROS and simply having an abundance of one kind cannot prevent oxidative damage without the presence of the others (Trenzado et al. 2005). For instance, if an organism has a lot of SOD but little CAT or GPX, the H_2O_2 produced would react with a metal ion and form a more harmful hydroxyl radical (Scandalios 2002).

Superoxide dismutase and variability

There are three major isozymes of SOD in vertebrates, and each has a primary location and function (Zelko et al. 2002). The most prolific isozyme is Cu/Zn-SOD (SOD-1) which is found in cytosol and has copper and zinc in its catalytic center. Mn-SOD (SOD-2) has manganese located in its catalytic center, and is found with the electron transport chain inside

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mitochondria. EC-SOD (SOD-3) also has Cu and Zn in its catalytic center, but unlike SOD-1 it is only found within the extracellular fluid (Zelko et al. 2002).

Variation in the *SOD-1* gene is frequently associated with variation in SOD-1 enzyme activity, and as a result can cause variation in the accumulation of oxidative damage and the severity of diseases that are at least partly caused by oxidative damage (Pasinelli et al. 2006; Skrzycki et al. 2007). Pasinelli and Brown (2006) found that mutations in the *SOD-1* gene generally, and more specifically mutations in the apoptosis-resistant wild-type *SOD-1* allele, both resulted in an increase of motor neuron death in familial amyotrophic lateral sclerosis. There is also a relationship between cancerous cell growth and reduced antioxidant enzyme function; in combination with other treatments enzymatic antioxidant gene therapy has been found to reduce tumor growth (Weydert et al. 2008).

A surprising amount of SNP variation within the *SOD-1* locus has been found in rainbow trout (Brunelli et al. 2008). Nine homozygous clonal lines are currently being propagated in the Thorgaard lab at Washington State University. Individuals from 8 of these lines were also genotyped for *SOD-1*; all 8 clonal lines tested had unique *SOD-1* SNP genotypes. Specifically within allozyme allele *SOD-1*152*, 4 SNPs were found and within *SOD-1*100*, 15 SNPs were found; these 19 SNPs were caused by variation at 11 different sites. (In contrast, the *LDH-4* locus which is expressed in liver had only 3 SNPs). Base pair substitutions at 4 of these sites result in amino acid substitutions in the SOD-1 enzyme. Research presented in this thesis investigated the functional effects of this *SOD-1* variation in rainbow trout in terms of enzyme activity and oxidative damage.

CHAPTER 2

RESEARCH DESIGN AND METHODOLOGY

INTRODUCTION

Reactive oxygen species (ROS), such as superoxide anions (O_2^-), nitric oxide (NO^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-), are formed by normal cellular function and environmental factors. When in abundance, ROS can cause oxidative damage in membrane lipids, proteins, and DNA. Polyunsaturated lipid membranes are especially vulnerable to ROS attack because the bonds neighboring a C=C bond will break, disassociate, and produce a lipid radical that can go on to cause further damage and ROS production (Hulbert et al. 2007; Zhu et al. 2007). Oxidative damage accumulates over time causing cellular membranes to lose fluidity, increases in the rate of DNA base mutations, changes in protein structures by oxidizing amino acids, and reduction of telomere length leading to an acceleration of cell death (Droge 2002; Hulbert et al. 2006; Valko et al. 2006).

Such accumulation of oxidative damage is thought to be a proximal cause of aging as the effects are progressive, harmful, endogenous, and irreversible (Hulbert et al. 2007). In addition, the accumulation of small but generally irreversible changes and mutations in cell membranes and DNA can lead to an increased risk or severity of tumors, a reduced ability to defend against pathogens, and vascular and neurodegenerative diseases (Barber et al. 2006; Cejas et al. 2004; Halliwell 1992; Skrzycki et al. 2007; Valko et al. 2006). In most instances the presence of cancer also correlates with elevated quantities of lipid peroxides and ROS (Plavec et al. 2008; Rajneesh et al. 2008; Ushio-Fukai et al. 2008).

The enzyme superoxide dismutase (SOD) scavenges superoxide anions (O_2^-) to reduce the occurrence of oxidative damage (Barber et al. 2006). After SOD dismutates O_2^- into

hydrogen peroxide (H_2O_2), two other antioxidant enzymes, catalase (CAT) and glutathione peroxidase (GPX), reduce H_2O_2 into O_2 and H_2O. These three enzymes work together to neutralize ROS; however, simply having an abundance of one enzyme cannot prevent oxidative damage without the others (Scandalios 2002; Trenzado et al. 2005).

SOD first appeared around 2 BYA as a defense against the increase of atmospheric oxygen, a byproduct of photosynthesis originating roughly 3.5 BYA (Scandalios 2002; Zelko et al. 2002). There are three major isozymes of SOD in vertebrates, and each has a primary location and function (Zelko et al. 2002). The most prolific isozyme is Cu/Zn-SOD (SOD-1) which is found in cytosol and has copper and zinc in its catalytic center. Mn-SOD (SOD-2) has manganese located in its catalytic center, and is found with the electron transport chain inside mitochondria. EC-SOD (SOD-3) also has Cu and Zn in its catalytic center, but unlike SOD-1 it is only found within the extracellular fluid (Zelko et al. 2002).

Variation in the *SOD-1* gene is frequently associated with variation in SOD-1 enzyme activity, and as a result can cause variation in the accumulation of oxidative damage and the severity of diseases that are at least partly caused by oxidative damage (Pasinelli et al. 2006; Skrzycki et al. 2007). Pasinelli and Brown (2006) found that mutations in the *SOD-1* gene generally, and more specifically mutations in the apoptosis-resistant wild-type *SOD-1* allele, both resulted in an increase of motor neuron death in familial amyotrophic lateral sclerosis. There is also a relationship between cancerous cell growth and reduced antioxidant enzyme function; in combination with other treatments enzymatic antioxidant gene therapy has been found to reduce tumor growth (Weydert et al. 2008).

A surprising amount of single nucleotide polymorphisms (SNPs) variation within the *SOD-1* locus has been found in rainbow trout (Brunelli et al. 2008). Within allozyme allele

SOD-1*152, 4 SNPs were found and within *SOD-1*100*, 15 SNPs were found; these 19 SNPs were caused by variation at 11 different sites. (In contrast, the *LDH-4* locus which is expressed in liver had only 3 SNPs). Base pair substitutions at 4 of these sites result in amino acid substitutions in the SOD-1 enzyme.

Much of this SNP variation has been captured in experimental clonal lines of rainbow trout (Brunelli et al. 2008). Rainbow trout, along with a number of other fish species, have been utilized in chromosome set manipulation studies. In fishes and amphibians, unlike mammals, individuals with both chromosome sets from a single parent are viable. Chromosome set manipulations which fishes tolerate include induced androgenesis (all-paternal inheritance), gynogenesis (all-maternal inheritance), triploidy, and tetraploidy (Ihssen et al. 1990; Komen et al. 2007; Streisinger et al. 1981). Thermal shocks are typically used to induce retention of the second polar body (to produce triploids) or to block the first cleavage division (to produce tetraploids). When these procedures are combined with irradiation of the eggs or sperm prior to fertilization, androgenesis or gynogenesis are the results.

These chromosome set manipulation methods have been used to generate homozygous clonal lines of rainbow trout. Nine homozygous clonal lines are currently being propagated in the Thorgaard lab at Washington State University. Individuals from 8 of these lines were also genotyped for *SOD-1*; all 8 clonal lines tested had unique *SOD-1* SNP genotypes and 22 of all 28 pairwise comparison of the clonal lines revealed SNP differences that resulted in at least one amino acid substitution in the SOD-1 enzyme, with the Oregon State University (OSU), Hot Creek (HC), Arlee (AR), and Whalerock (WR) lines all having different amino acid substitutions.

The hypervariability of the *SOD-1* locus raises several issues. First, what is the evolutionary significance of the high degree of genetic variation? In order to gain any understanding of this question, the functional effects on SOD-1 activity and oxidative damage must be investigated. Second, are any functional differences among *SOD-1* genotypes influenced by other loci? Given the fact that SOD-1 works in conjunction with other anti-oxidant enzymes like catalase and peroxidase, testing for the effects of other loci is important. Third, given that clonal lines of trout vary in *SOD-1* SNP genotype and *SOD-1* amino acid sequence, can these clonal lines be developed as a model system to investigate the medical significance of genetic and functional variation in SOD-1? Investigations into the first two questions should reveal an answer to this third question.

In order to examine these questions, we tested for differences in SOD-1 activity and oxidative damage among clonal lines of trout that differ in *SOD-1* SNP genotype. We also conducted a QTL study to identify chromosomal regions associated with SOD-1 enzyme activity. We predicted that clonal lines would show differing levels of SOD-1 enzyme activity and that clonal lines with greater expression of SOD-1 enzyme activity would accumulate less oxidative damage than those with low SOD-1 enzyme expression. We also hypothesized that a significant QTL would be found that included the *SOD-1* locus, though we do not rule out the possibility of other QTL as well.

METHODS

Trout Rearing

Formation of Clonal Lines and OSUxArlee:

The Oregon State University (OSU), Arlee (AR), Hot Creek (HC), Clearwater (CW), and Whalerock (WR) clonal populations were originated as described by Parsons and Thorgaard (1985). Double haploids were formed by crossing an OSU female with an AR male. This produced a male OSUxAR F_1 generation, and sperm from the OSUxAR male was used to produce an F_2 generation of double haploids via androgenesis (Parsons et al. 1985). Due to the recombination that occurs, these 93 F_2 individuals are homozygous within themselves, but are genetically different from each other and from the preceding F_1 generation (Young et al. 1998).

Fish Rearing Conditions and Preparation:

All clonal lines were raised in a re-circulating fresh water tank at the Washington State University hatchery. The OSUxAR double haploid individuals were raised for seven months in a re-circulating tank separate from other clonal lines. Trout were fully anesthetized in clove oil and killed by a blow to the head. At time of sampling, individuals were measured for their wet mass (rounded to nearest 0.1 g) and length (rounded to nearest mm).

SOD-1 Activity

Enzyme Assay:

Livers were extracted and immediately placed in dry ice; 5-10 µg of liver tissue was homogenized several weeks later in 1.25 ml of Tris buffer (20 mM, pH 7.4). This dilution was stored at -80 ^{o}C until performing the SOD-1 enzyme and liver protein assays. Liver homogenates were centrifuged at 10,000 x g for 15 minutes at 4 ^{o}C to separate Cu/Zn-SOD from Mn-SOD (Mattiazzi et al. 2002). Since extracellular quantities of Cu/Zn-SOD (SOD-3) is considered negligible when sampling from liver tissue, the remaining Cu/Zn-SOD will be classified as SOD-1.

Supernatant containing SOD-1 was used to measure enzyme activity (SOD Assay Kit-WST Dojindo Molecular Technologies, Inc.) and kit directions were followed. Briefly, WST Working Solution was prepared by diluting 1 ml WST Stock Solution with 19 ml Buffer Solution, and Enzyme Working Solution was made by diluting 15 µl stock Enzyme Solution with 2.5 ml Dilution Buffer. SOD-1 standards were prepared by diluting a known quantity of SOD-1 from a stock of Bovine Serum Albumin (BSA; Sigma-Aldrich, Inc.) with Tris buffer (20 mM, pH 7.4). Standards were made for concentrations of 200 U/ml, 100 U/ml, 50 U/ml, 20 U/ml, 10U/ml, 7.5 U/ml, 5 U/ml, 2.5 U/ml, 1 U/ml, and 0.1 U/ml.

All SOD-1 standards and samples were plated in duplicate at 20 µl to 200 µl WST Working Solution. When all standards, blanks, and samples were plated, 20 µl of Enzyme Working Solution was added to every well except those of Blank 2 and Blank 3. The plate was incubated at 37 $^{\circ}$C for 20 min then read at 450 nm in a Bio-Tek Instruments, Inc. Synergy HT microplate kit. Mean absorbance was calculated for each blank, standard, and sample. The % Inhibition of superoxide reduction was calculated using this formula:

$$\frac{(\text{Blank 1}) - [(\text{Sample} - \text{Blank 2})]}{(\text{Blank 1}) - (\text{Blank 3})}$$

A standard curve was plotted as a function of Inhibition% by SOD-1 concentration. Concentrations of samples could then be determined from their Inhibition% and the known concentrations of the SOD-1 standards. One Unit of SOD-1 is defined as the amount needed to exhibit 50% dismutation, or 50% inhibition of superoxide radicals.

Protein Assay:

Total protein was determined by using the Pierce BCA Protein Assay Kit (Thermal Fisher Scientific, Inc.). Working Reagent (WR) was prepared by mixing 1 ml of BCA Reagent B with 50 ml of Reagent A. Protein standards were prepared from a series of BSA dilutions in Tris buffer (20 mM, pH 7.4). Standards were 2000 µg/ml, 1500 µg/ml, 1000 µg/ml, 750 µg/ml, 500 µg/ml, 125 µg/ml, 25 µg/ml, and one blank consisting only of buffer (0 µg/ml). Samples from the SOD-1 enzyme activity assay were used in the total protein assay to ensure consistency between results.

In a 96-well microplate, 25 µl of each standard, sample, and blank were plated in duplicate with 200 µl of WR, then the plate was incubated at 37 ^{o}C for 30 min. The plate was cooled for approximately 30 minutes then read at an absorbance of 562 nm. Corrected absorbances were calculated by subtracting the mean absorbance of the blank from the mean absorbance of all the standards and samples. A BSA standard curve was plotted as a function of protein concentration. The protein concentration of each sample was calculated by relating its absorbance to the standard curve. SOD-1 activity in U/mg protein was the determined as:

$$U/mg \text{ protein} = [(U/ml) \cdot (\mu g/ml)] \times 1000$$

Oxidative Damage

TBARS Assay:

The thiobarbituric acid reactive substances (TBARS) assay (Cayman Chemical) is a quantitative measure of lipid peroxidation; an endpoint measure of oxidative stress and free radical activity. The assay uses the reaction between MDA (malondialdehyde) and TBA to form a product measurable by spectrophotometry. The greater the membrane damage, the

more MDA reacts, creating a direct relationship identified as MDA µM; the concentration of MDA present in direct relation to the quantity of oxidative byproducts.

Pre-assay preparation involved diluting 40 ml acetic acid in 80 ml of ddH$_2$O to form an acetic acid solution. Ten ml of 10x NaOH was diluted in 90 ml of ddH$_2$O to form a NaOH solution. The color reagent was formed by mixing 25 ml acetic acid solution with 265 mg of TBA. After the TBA fully dissolved, 25 ml of NaOH solution was mixed in. These quantities made enough color reagent for approximately 25 samples or standards.

The MDA standards were formed in a series of dilutions with ddH$_2$O to form concentrations of 50 µM, 25 µM, 10 µM, 5 µM, 2.5 µM, 1.25 µM, 0.625 µM, and a blank of 0 µM. Ten mg of trout liver were homogenized in 150 µl of Tris buffer (20 mM, pH 7.4), then centrifuging at 1600 x g for 10 min at 4 ^{o}C. 100 µl of supernatant was transferred into a clean 1.5 ml tube and kept on ice with the prepared MDA standards. Only the samples and standards were chilled, all other reagents and solutions were stored at room temperature.

Labeled 5 ml vials with caps were filled with 50 µl of their respective sample or standard, and 50 µl of SDS solution. Two ml of color reagent was added forcefully down the sides of each vial and mixed thoroughly. Capped vials were secured in an upright position and placed in a 95 ^{o}C water bath for 60 min, then immediately put on ice for 10 min. Chilled vials were centrifuged at 1600 x g for 10 min at 4 ^{o}C. 150 µl of supernatant was plated in duplicate on a 96-well microplate and read at an absorbance of 540 nm. The mean absorbance of the blank was subtracted from the mean absorbance of all other standards and samples. The corrected absorbance for each standard was plotted as a function of MDA µM, and the following calculation used for determining sample concentrations from their corrected absorbance:

$$\frac{[(Sample) - (y\text{-}intercept)]}{slope}$$

Quantitative Trait Loci (QTL) Studies

Experimental Design:

The five rainbow trout clonal lines of OSU, AR, HC, CW, and WR were raised in a common environment. Average SOD-1 enzyme activity was measured from livers of individuals within each line. In order to choose parental lines for QTL work, comparisons of enzyme activity were then made between the female OSU line and the four male lines; HC, CW, AR, and WR (Figure 1). WR had the greatest difference in SOD-1 enzyme activity from that of OSU, however WR also had too large of a variance to be considered for breeding. AR had the second greatest difference in enzyme activity from OSU with little variation, so AR and OSU were used for QTL studies.

DNA isolation:

Fin clips from samples were rinsed twice with TE (Tris-EDTA) buffer then placed in labeled 1.5 ml centrifuge tube with 300 µl of Cell Lysis Solution and 3 µl of Proteinase K from the Get *pure* DNA Kit-Cell Tissue (Dojindo Molecular Technologies, Inc.). Clips were digested over a period of 1-3 hours at $60\,^{\circ}C$.

An equal amount of 1:1 phenol-chloroform mixture was added to each tube and centrifuged at 13000 x g for 10 min. 300 µl of supernatant was pipetted into a clean 1.5 ml centrifuge tube with an equal volume of 1:1 phenol-chloroform mixture. Centrifugation was repeated for the second phenol-chloroform wash and 300 µl of that supernatant was placed into a third 1.5 ml centrifuge tube. An equal volume of 100% chloroform was added and

centrifuged at 13,000 x g for 10 min. 250 µl of supernatant was pipetted into a clean 1.5 ml centrifuge tube which contained 250 µl of 100% isopropanol.

After inverting the isopropanol mixture each sample was centrifuged at 13,000-16,000 x g for 5 min. The supernatant was carefully poured off and the tubes were blotted dry. 300 µl of 70% ethanol was then added to the precipitated DNA samples which were then vortexed and spun at 16,000 x g for 1 min. The ethanol was poured off the top and each tube of sample DNA was allowed to air dry for 5-10 minutes, and then rehydrated in 100 µl of TE buffer before being stored at 4 ^{o}C.

Polymerase chain reaction (PCR):

Primers for *SOD-1* were based on GenBank accession No. AF469663, and a previous study that noted a polymorphism between the AR and OSU clonal lines (Nichols et al. 2003a). Reverse transcriptase-PCR amplifications were conducted in 20 µl volumes, using 1 unit of Taq polymerase in 10x Taq DNA polymerase PCR buffer, 2 mM MgCl, 0.5 µl of 10mM dNTP solution (Invitrogen), with 1 µl of forward and reverse gene-specific primers (see Appendix). Polymerase chain reaction cycling was done as follows: 94 ^{o}C for 120 sec pre-amplification dwell, 30 cycles of 94 ^{o}C for 50 sec, 54 ^{o}C for 50 sec, 72 ^{o}C for 60 sec, and 72 ^{o}C for 120 sec post-amplification dwell.

Evaluation was done by electrophoresis of 5 µl of RT-PCR amplification product through 2% agarose gels stained with EtBr. Gels were viewed with UV light and photographed by a Gel Logic 200 Imaging System.

Amplified fragment length polymorphism (AFLP):

The restriction enzyme *Eco*RI was used with *Mse*I to digest genomic DNA for selective amplification. Following amplification, reaction products were mixed with 10 µl of

formamide load buffer (98% formamide, 0.5 M EDTA pH 8.0, ddH$_2$O, and bromo phenol blue). The products mixed with load buffer were heated for 6 min at 94 ^{o}C, and then placed on ice.

The polyacrylamide gel was made from 6% acrylamide, 48% urea, 20% 5X TBE (5.4% 50 mM Tris, 2.75% 50 mM Boric acid, 2% 0.5 mM EDTA, 89.8% ddH$_2$O) and 74% ddH$_2$O. To 80 ml of 6% acrylamide gel solution 600 µl of 10% APS and 38 µl of TEMED were added before casting between two glass plates with a 50-well comb on Otter pouring apparatus. Gels were allowed to polymerize for 20 min before combs were removed and gels were pre-run with 1X TBE running buffer at 1200 V until reaching a temperature of 37-40 ^{o}C. Gel wells were flushed of urea before 8 µl of each +3 sample was loaded. Electrophoresis was performed at constant power, 1200 V, for 2.5-3 hours.

After electrophoresis, the siliconized glass plate was removed and gels were scanned and visualized by red fluorescence imaging (Typhoon 9400 Variable Mode Imager serial no. 95965).

Linkage Analysis:

Linkage groups were ordered using Mapmaker/EXP 3.0 (Dr. Eric Lander, Whitehead Institute, Cambridge, MA, U.S.A.). Initial grouping was done using a minimum LOD of 3.0 and a maximum θ of 0.35. Larger groups were further analyzed with an LOD of 4.0 and a maximum θ of 0.30 (Young et al. 1998). Potential scoring errors identified by Mapmaker 3.0 were rechecked and corrected if an error was found, and markers of low quality were removed. A final analysis of each linkage group was done to estimate distances between markers.

QTL:

Composite interval mapping (CIM) via Windows QTL Cartographer Version 2.5 (Zeng 1994). Type I error was set at 0.05 on Model 6 with background control markers, and the genetic map was scanned for a QTL every 8 centiMorgan (cM). Threshold values were determined by the five most significant markers from 500 permutations. Markers with an LOD score greater than the threshold were recorded.

<u>Statistical Analysis</u>

Replicated measures of liver SOD-1 enzyme activity and MDA concentration within each clonal line was averaged then analyzed using analysis of variance (ANOVA) in IBM SPSS Statistics 18.0 for Windows (SPSS Inc., Chicago, IL, USA). Fisher's LSD post-hoc pairwise comparisons were used when a significant ($p < .05$) ANOVA was found.

RESULTS

SOD-1 Activity:

Significant differences in SOD-1 enzyme activity were measured between the female OSU and the male AR ($p = 0.006$), CW ($p = 0.039$), and WR ($p < .0001$) parental lines (Figure 1). WR significantly differed from all other lines. Among the male lines, AR and CW did not significantly differ from each other ($p = 0.640$), but did differ from HC ($p = 0.020$) and WR ($p < .0001$). SOD-1 enzyme activity for the OSUxAR double haploid progeny is shown in Figure 2 with the outlined ranges from the parental lines. Enzyme activity in the double haploid progeny was more variable than in the parental lines, which suggests that mean enzyme activity is affected by more than one genetic region. In addition, SOD-1 activity in OSU was also measured in two different age classes to test for age differences; between 4

month and 52 month old OSU trout there was no significant difference in SOD-1 enzyme activity ($p = 0.167$, Table 1).

Oxidative Damage:

Oxidative damage to membranes is measured as levels of MDA. MDA levels for young and old OSU and AR lines are presented in Figure 3, and were analyzed using factorial ANOVA. A significant main effect difference in MDA levels between OSU and AR clonal lines ($p = 0.005$) was identified, as was a significant difference between the 4 month and 52 month old fish ($p < .0001$); a marginally non-significant interaction between clonal lines and age was also identified ($p = 0.064$, Table 2). Fisher's LSD post-hoc comparisons show no significant difference between the OSU and AR lines at 4 months ($p = 0.205$) but did find a difference between OSU and AR at 52 months ($p < .0001$, Figure 3), with OSU showing higher levels of oxidative damage to cell membranes at 52 months of age than AR.

SOD-1 Activity and the SOD-1 locus:

DNA isolation was successful on 88 of the 93 OSUxAR double haploid samples, and a PCR assay for the *SOD-1* gene was performed to determine the allelic type (OSU vs. AR) for each sample. Samples with an AR *SOD-1* allele (n = 23) had higher SOD-1 enzyme activity than samples with an OSU *SOD-1* allele (n = 36), although this difference was not statistically significant ($p = 0.293$; Table 3).

Linkage and QTL Analysis:

A total of 442 polymorphic markers were scored from 47 AFLP primer combinations. Of the 442 markers, 295 were separated into 42 linkage groups covering a distance of 3648.2 cM with an average interval distance of 12.3 cM. Overall there were 24 large linkage groups of ≥ 5 markers, three groups of 4 markers, two groups of 3 markers, and 13 marker pairs.

Significant deviation from a 50:50 Mendelian inheritance pattern ($x^2_{1,.05}$) was seen in 79 of 295 linked markers; approximately 65% of those deviated towards AR, and 35% deviated towards OSU. Several markers with both normal and severe distribution were matched to previous linkage groups identified for OSUxAR double haploid progeny (Nichols et al. 2003b; Young et al. 1998) and are outlined in bold (see Appendix). QTL for liver SOD-1 enzyme activity were found on the linkage groups OA-10 and OA-12 (Table 4, Figures 4 and 5). 19% of variation is explained at OA-10 and 34% of variation is explained at OA-12. The OSU allele at OA-10 and OA-12 is associated with a decrease in enzyme activity; however the frequency of the OSU allele at OA-10 was significantly less than its frequency at OA-12. Neither of these QTL occur on the OA-I linkage group containing the *SOD-1* gene, indicating that SOD-1 enzyme activity is a polygenic trait.

DISCUSSION

SOD-1 Activity

Three out of the five measured clonal lines had SOD-1 enzyme activities significantly different from each other. This supports the hypothesis that the amino acid substitutions in *SOD-1* cause phenotypic variation in the enzyme activity (Brunelli et al. 2008) . Levels of enzyme activity did not significantly differ across age, and this is consistent with previous findings in trout and liver tissue (Martinez-Alvarez et al. 2005; Passi et al. 1994; Zelko et al. 2002).

The 3 clonal lines measured with significantly different SOD-1 enzyme activities from each other were OSU, AR, and WR. HC was not found to be significantly different from OSU, CW was not found to be significantly different from AR, and WR was significantly

different from every other line. When comparing the enzyme activities to the amino acid substitutions that occur at locations *SOD-1*98*, *SOD-1*122*, *SOD-1*208*, and *SOD-1*399* (Brunelli et al. 2008), we found some interesting trends. No amino acid changes occurred at *SOD-1*122*. OSU and HC, while having statistically similar enzyme averages, had 2 changes at *SOD-1*98* and *SOD-1*208* (threonine:isoleucine and glutamine: lysine) while only 1 change occurred between OSU and AR at *SOD-1*98* (threonine:isoleucine). WR was the only line in this comparison to have glutamic acid instead of aspartic acid at *SOD-1*399*. Because WR has the greatest enzymatic activity, this may be an indication that an amino acid substitution at *SOD-1*399* has a greater impact than changes occurring at *SOD-1*98* or *SOD-1*208*. In addition, perhaps enzyme activity differences due to a substitution at *SOD-1*98* is negated when it is paired with a substitution at *SOD-1*208*. Alternatively, based on the QTL results it is possible the significant variation of SOD-1 enzyme activity is caused by other genes. This could mean that the increased enzyme activity seen in WR is due to it having different alleles at these other loci.

The high levels of SOD-1 activity in the double haploids that exceed the ranges of both parental lines show that the OSUxAR double haploid progeny are more variable. This implies that enzyme activity is regulated by more than just one genetic region (Nichols et al. 2003a; Zimmerman et al. 2005), which also fits with our QTL results (see below). A similar increase of variability was seen for the trait of pyloric caeca number in a double haploid cross of OSUxHC; the parental HC had a greater number of pyloric caeca than the parental OSU, and the double haploid progeny of their cross exceeded both (Zimmerman et al. 2005).

Oxidative Damage

The results of oxidative damage to lipids were unexpected. While young OSU and young AR had similar levels of MDA, older OSU had a significantly higher accumulation than older AR. We had expected that because OSU had a greater enzyme activity, it would be more effective protecting against ROS and thus have a lower accumulation of oxidative damage than that of lines like AR with low levels of SOD-1 enzyme activity. This trend of high enzyme activity and high oxidative damage is seen in individuals with trisomy 21. Due to a replication of chromosome 21, which is the site of the *SOD-1* locus in humans, SOD-1 enzyme activity is increased by about 50%, yet at the same time people with trisomy 21 experience accelerated oxidative stress and neural apoptosis (Brooksbank et al. 1983; De La Torre et al. 1996; Pinto et al. 2002; Turrens 2001). One explanation is that overexpression of other genes along chromosome 21 are responsible for the increase in ROS and oxidative stress, and while SOD enzyme activity is increased as well, there may not enough CAT and GPX to compensate (Pinto et al. 2002).

In addition, although we found similar levels of SOD-1 activity in young and old fish, old fish had significantly higher levels of membrane damage. A similar trend was measured in humans; older men had higher quantities of oxidative damage, yet had the same SOD-1 enzyme activity as that of younger men who had very little oxidative damage (Gianni et al. 2004). In this instance we find our results mirroring this trend; young and old subjects had statistically similar antioxidant enzyme activities, yet the older group showed a higher measure of damage.

A possible explanation of these results can be developed by considering stress from non-oxidative sources. Kestrels fed dietary corticosteroids had the same antioxidant levels as control kestrels, but had a 30% higher rate of oxidative damage (Costantini et al. 2008),

indicating that non-oxidative stressors can induce or exacerbate oxidative stress. In a study measuring whole body cortisol levels in the OSU and AR clonal lines, stress tests found that AR consistently had a lower cortisol stress response than OSU (Drew et al. 2007). Furthermore, in response to thermal stress AR again had consistently lower hsp70 levels than did the OSU line (Heredia-Middleton et al. 2008). While the quantity of heat shock proteins is not analogous to MDA concentrations, they are both measures that are related to reduced longevity and slower growth rate, and their accumulation levels consistently trend with AR having lower quantities of various stress-regulated biomolecules (Heredia-Middleton et al. 2008).

Although SOD-1 enzyme activity levels are important, the levels of the other two antioxidant enzymes, CAT and GPX were not taken into consideration in this study. SOD-1 is a limiting factor as to how much O_2^- can be dismutated to H_2O_2, but even after O_2^- is neutralized, there is a risk that H_2O_2 can revert to OH^-, which on its own can cause significant amounts of lipid peroxidation (Monaghan et al. 2008; Pinto et al. 2002; Scandalios 2002). Hence while it is possible that the results presented are being driven by activities of these other antioxidant enzymes, without measuring the CAT and GPX levels in the same liver in which we measured SOD-1 activity, concluding whether the unexpected accumulation of tissue damage in OSU is correlated to SOD-1 enzyme activity or by one of the other two antioxidant enzymes is not possible.

QTL Studies

Two significant QTL for SOD-1 enzyme activity were found on the OA-10 and OA-12 linkage groups. Even more interesting, neither of these QTL were on the OA-1 linkage group which contains the *SOD-1* gene. Similarly the gene associated with stress response in

trout, carbonyl reductase, also mapped to OA-1, and while multiple QTL for stress response were found, neither of those appeared on OA-1 (Drew et al. 2007). Hence with SOD-1, much like with the cortisol QTL, power to pick up additional QTL for OA-1 may be lacking; alternatively, perhaps none appeared because the trout genome is still incomplete and may lack locations of other significant regulatory genes (Drew et al. 2007; Nichols et al. 2003a).

Segregation distortion was present in about 30% of the mapped makers and are listed in the Appendix along with matched markers from Nichols (2003b), who notably also saw distortion in the same direction and comparable magnitude in the same linkage group which is the largest QTL in this study. Other significant segregation distortions were found in OSUxAR (Nichols et al. 2003b; Young et al. 1998), and OSUxCW crosses (Sundin et al. 2005). It has been reported that both CW and AR have faster growth rates compared to OSU (Robison et al. 1999; Sundin et al. 2005), which may mean trout with CW and AR alleles may have advantage when competing with smaller OSU for food (Sundin et al. 2005), and thus lead to differential mortality among individuals with differing parental alleles. However, in this specific case, it has not yet been confirmed if there is a disadvantage to the OSU genotype or if it truly has a higher mortality rate. While it is promising that other studies with these clonal lines have had severe segregation distortion, it does not completely explain the large degree of distortion we found, and therefore we need to consider the possibility that some of the segregation results we found may be due to a methodological anomaly or error.

Future Directions

The results presented herein suggest a number of directions for future research. First, additional QTL mapping of the OSUxAR cross would be useful. Incomplete alignment of linkage maps between this study and other studies make it difficult to ascertain if QTL

reported here are on the same linkage groups from previous studies. Adding more AFLP and microsatellite markers would refine the results and perhaps reveal with more certainty the genetic variation responsible for the phenotypic differences measured in SOD-1 activity, and additional QTL studies could confirm the direction and magnitude of the segregation distortion identified in this and other studies (Nichols et al. 2003a; Nichols et al. 2003b; Sundin et al. 2005; Young et al. 1998).

Second, it would be worthwhile to explore how variation in SOD-1 activity and oxidative damage influence and are influenced by other types of stressors. Hyperoxia has shown negative effects on trout growth, reduction of their ability to recover from infection, and caused trout to exhibit a greater quantity of cortisol (Caldwell et al. 1994; Caldwell et al. 1995). Previous studies have also shown that stress tests with trout in ozonated water has resulted in an increase of oxidative tissue damage and antioxidant activity (Ritola et al. 2002) but did not consider genetic variation in the trout used.

Third, measuring activity of the CAT and GPX enzymes is important because they work with SOD-1 in reducing ROS. By including these three antioxidant enzymes we would be more able to conclude whether or not AR's low level of SOD-1 enzyme activity is due to higher activities of CAT and/or GPX, and how that compensation might reflect the low levels of accumulated lipid peroxidation.

Fourth, lipid damage between clonal lines may be dependant on their membrane make-up (Hulbert 2005; Markesbery 1997; Mitchell et al. 2007); however it does not address another major marker of oxidative damage: DNA damage. One study showed how differences in DNA breakage levels in vent mussels are an expression of cell viability, enzyme activity, and that younger mussels were more effective at DNA repair than older mussels (Pruski et al.

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2003). Given the unexpected results of lipid peroxidation between older OSU and older AR, including information on whether or not a similar trend in DNA damage would give us a more comprehensive view on how these lines differ in avoiding and repairing oxidative damage. Quantification of DNA and lipid damage along with measuring all 3 major antioxidant enzymes would better answer the question of how much of the variation in oxidative stress and the variation of antioxidant activities, is reflective of the variation seen within an expanded genetic map.

Source of variation	SS	df	MS	F-value	*p*-value
Between groups	3362.00	1	3362.00	2.458	0.167
Within groups	8175.00	6	1362.50		
Total	11537.00	7			

Table 1: ANOVA table comparing the mean SOD-1 enzyme activities between 52 month old OSU (n = 4, m = 162 ± 42 U/mg) and 4 month old OSU (n = 4, m = 121 ± 31 U/mg).

Source of variation	SS	df	MS	F	p – value
Line	2.58	1	2.58	11.47	0.005*
Age	14.33	1	14.33	63.69	< .0001*
Line x Age	.94	1	.94	4.18	0.064
Error	2.7	12	.23		
Total	20.55	15			

Table 2: Two-Way ANOVA for MDA concentrations in liver from young (4 month) and old (52 month) OSU and Arlee clonal lines. * marks significance ($p < .05$).

Source of variation	SS	df	MS	F-value	*p*-value
Between groups	227150.87	2	1357.43	1.246	0.293
Within groups	925992.00	85	10894.02		
Total	953142.86	87			

Table 3: ANOVA table for PCR results on *SOD-1* gene and SOD-1 enzyme activity in OSUxAR double haploid progeny. OSU allele (n = 23, m = 360 ± 96 U/mg), AR allele (n = 36, m = 335 ± 122 U/mg), ambiguous markers (n = 29, m = 314 ± 85 U/mg).

QTL designation	Linkage group	LOD	r^2	Additive effect
SOD-1a	OA-10	3.52	0.19	-97.1
SOD-1b	OA-12	5.38	0.34	147

Table 4: Calculated effects of significant QTL found in OSUxAR double haploid progeny. LODs listed are above the significance threshold of 3.1 for SOD-1 enzyme activity. r^2 is the proportion of variance explained by the QTL, and additive effect is the effect of substituting an AR allele with an OSU allele at the QTL.
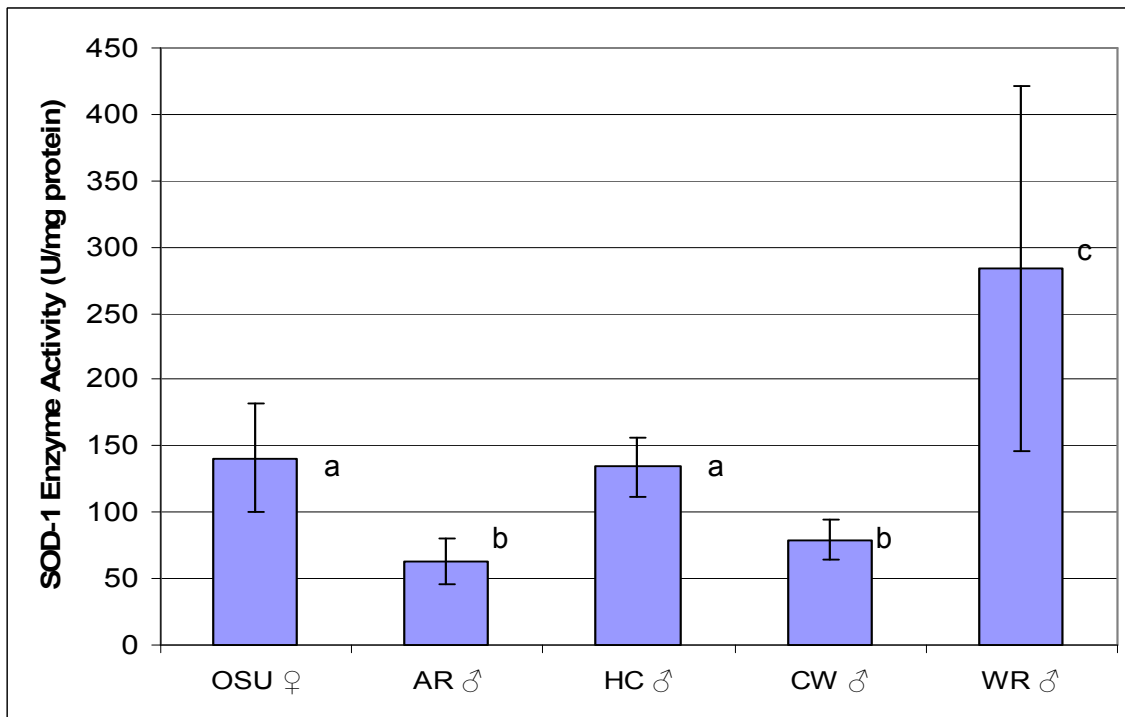
Figure 1: Letters by individual columns identify that clonal line as having a mean enzyme activity level as significantly different from others after conducting a Fisher's LSD ($p < .05$) post hoc comparisons.
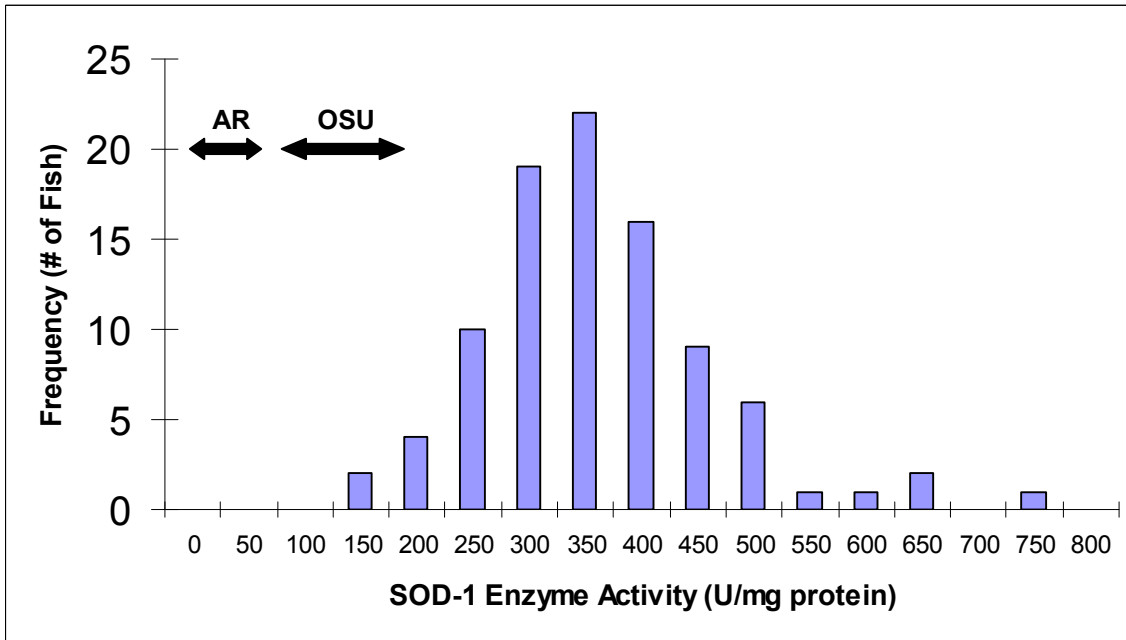
Figure 2: Frequency distribution of SOD-1 enzyme activity from liver tissue of OSUxAR double haploid progeny (n = 93). Ranges of enzyme activity in AR (33 to 92 U/mg) and OSU (110 to 218 U/mg) parental lines are outlined by the arrows.
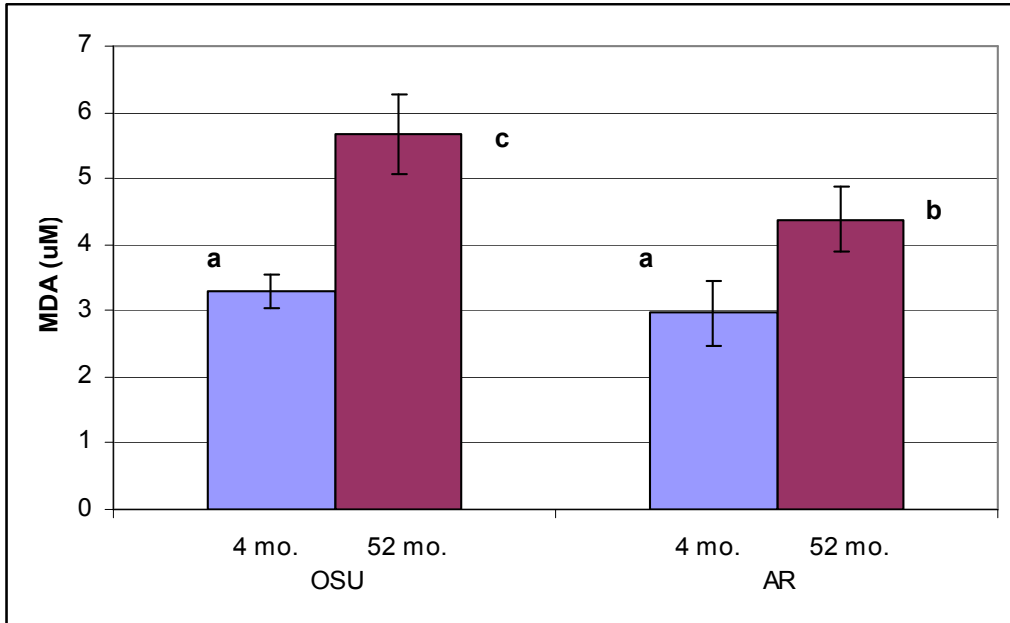
Figure 3: Letters by individual columns identify that clonal line as having a mean MDA concentration as significantly different from others after conducting a Fisher's LSD ($p < .05$) post hoc comparisons.
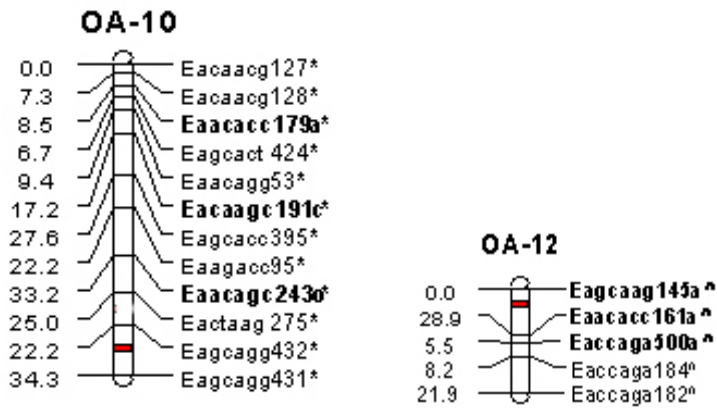
Figure 4: Linkage groups OA-10 and OA-12 identified with significant QTL (band) for the trait of SOD-1 enzyme activity. Markers in bold were matched to previous OA linkage maps (Nichols et al. 2003b; Young et al. 1998). Markers with (*) symbolize severe segregation towards the AR allele, and (^) symbolize distortion towards the OSU allele.
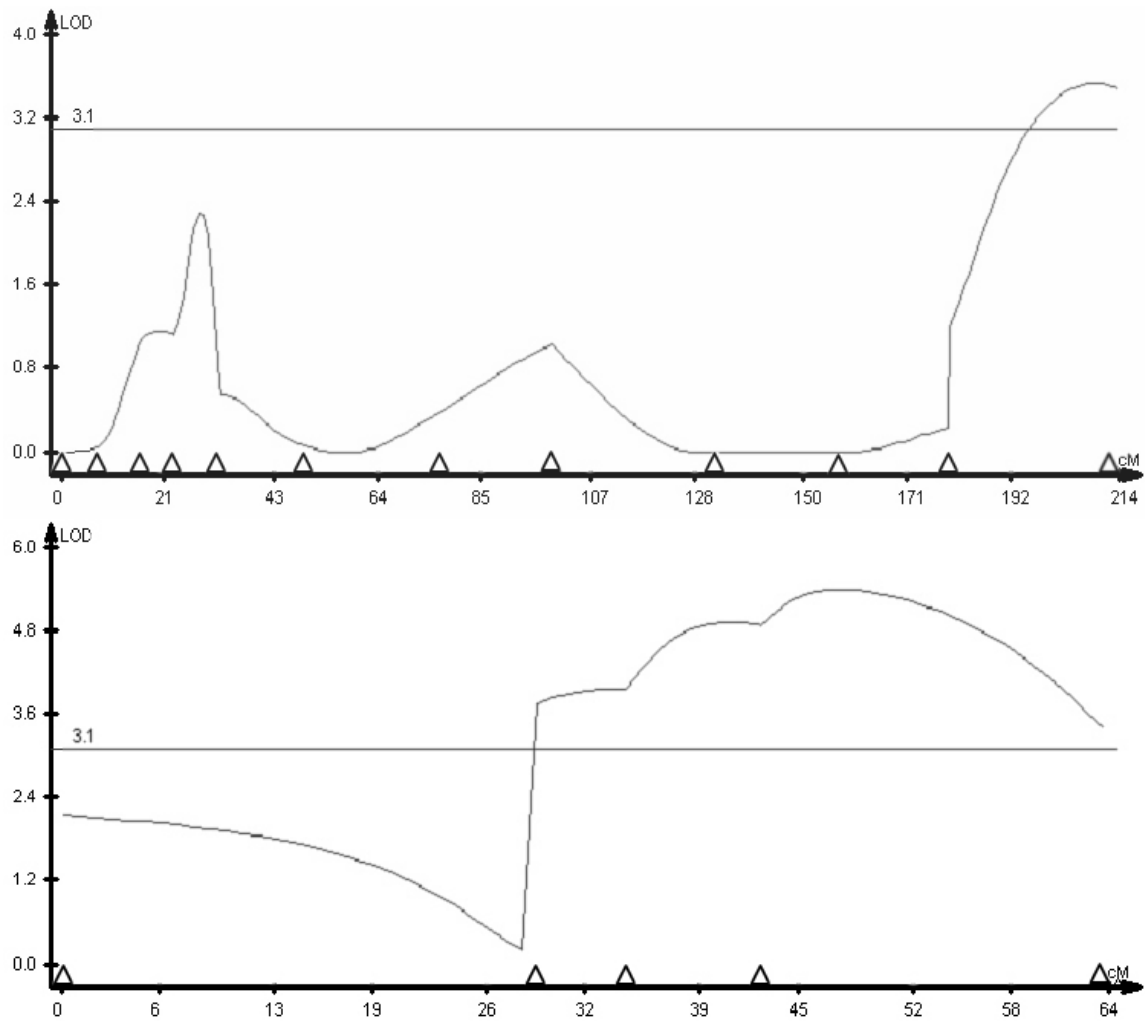
Figure 5: LOD plots for linkage groups OA-10 and OA-12 displaying QTL for the trait of enzyme activity. The threshold for a significant ($p < .05$) LOD score for an SOD-1 QTL is 3.1. Markers are shown on the x-axis as small triangles distributed along each linkage group in centiMorgan (cM).

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SOD-1 SNP DETAILS

SOD-1	98	122	208	240	243	258	330	399	444	447	497
allozyme type	C/T	C/T	A/C	C/G	C/T	C/T	C/T	G/T	C/A	G/T	A/T
allelic variant	T:I	L:P	Q:K					D:E			
152-3 (HC)	T	C	C	G	C	C	C	G	A	G	T
100-10 (AR)	T	C	A	C	C	C	T	G	A	G	A
100-12 (OSU)	C	C	A	C	C	C	T	G	A	G	A
100-13 (WR)	C	C	A	G	C	C	T	T	A	G	T

SOD-1 nucleotide position and SNP alternatives (Brunelli et al. 2008).

SOD1	Cu/Zn-superoxide dismutase 1	Forward	TCCACGTCCATGCTTATGGA
OSUR	CTTCAAAAACTCTCCTCT	ARLR	AACGCTTTCAAAAACTCTG

Forward and reverse primers for the *SOD-1* gene (Brunelli et al. 2008).

PCR PROTOCOL

Reagent	Qty for 100x
PCR buffer (Invitrogen, 10x)	400 µl
MgCl$_2$ (Invitrogen, 50 mM)	160 µl
dNTPs (10 mM)	100 µl
F2	200 µl
ddH$_2$O	2.86 ml
Taq (Gibco BRL)	20 µl
Reverse *SOD* primer (*ARL*R or *OSU*R)	100 µl
Total Master Mix vol.	**3.84 ml**

	temp (^{o}C)	time (min)
pre-dwell	94	2
post-dwell	72	2

No. of cycles	30
4 ^{o}C chill	no

step	temp (^{o}C)	time (sec)
1	94	0
2	94	50
3	54	0
4	54	50
5	72	0
6	72	60

AFLP PROTOCOL

RESTRICTION ENZYME MASTER MIX

Reagent	Qty for 100x
T4 DNA ligase buffer (10x with ATP)	110 μl
0.5 M NaCl	110 μl
1 mg/ml BSA (10x)	55 μl
*Eco*RI	25 μl
*Mse*I	25 μl
1:50 T4 ligase (diluted in ligase buffer)	25 μl
*Eco*RI adapter (10 pmol/μl)	100 μl
*Mse*I adapter (100 pmol/μl)	100 μl
Total Master Mix vol.	**550 μl**

+1 SELECTIVE AMPLIFICATION MASTER MIX

Reagent	Qty for 100x
PCR buffer (Invitrogen, 10x)	200 μl
MgCl$_2$ (Invitrogen, 50 mM)	80 μl
dNTPs (10 mM)	40 μl
*Eco*A (10 pmol/μl)	60 μl
*Mse*A (10 pmol/μl)	60 μl
ddH$_2$O	1.04 ml
Taq (Gibco BRL)	20 μl
Total Master Mix vol.	**1.5 ml**

AFLP +1

	temp (^{o}C)	time (min)
pre-dwell	72	2
post-dwell	72	10

No. of cycles	30
4 ^{o}C chill	yes

step	temp (^{o}C)	time (sec)
1	94	22
2	94	1
3	60	34
4	60	30
5	72	12
6	72	200

+3 SELECTIVE AMPLIFICATION MASTER MIX

Reagent	Qty for 100x
PCR buffer (Invitrogen, 10x)	100 μl
MgCl$_2$ (Invitrogen, 50 mM)	30 μl
dNTPs (10 mM)	20 μl
*Eco*A +3 (50 ng/μl)	25 μl
*Mse*A +3 (50 ng/μl)	150 μl
ddH$_2$O	420 μl
Taq (Gibco BRL)	5 μl
Total Master Mix vol.	**750 μl**

AFLP +3 – 1st

	temp (^{o}C)	time (min)
pre-dwell	94	2
post-dwell	None	

No. of cycles	1
4 ^{o}C chill	No

step	temp (^{o}C)	time (sec)
1	94	0
2	94	1
3	65	29
4	65	30
5	72	7
6	72	200
7	94	22
8	94	1
9	64	30
10	64	30
11	72	8
12	72	200
13	94	33
14	94	1
15	63	31
16	63	30
17	72	9
18	72	200

AFLP +3 – 2nd

pre-dwell	None
post-dwell	None

No. of cycles	1
4 °C chill	No

step	temp (°C)	time (sec)
1	94	22
2	94	1
3	62	32
4	62	30
5	72	10
6	72	200
7	94	22
8	94	1
9	61	33
10	61	30
11	72	11
12	72	200
13	94	22
14	94	1
15	60	34
16	60	30
17	72	12
18	72	200

AFLP +3 – 3rd

	temp (°C)	time (min)
pre-dwell	none	
post-dwell	72	5

No. of cycles	30
4 °C chill	yes

step	temp (°C)	time (sec)
1	94	22
2	94	1
3	59	35
4	59	30
5	72	13
6	72	120

SEGREGATION DISTORTION BY LINKAGE GROUP

OA	Name	O	A	Unk	+%	Nichols, 2003	O	A	Unk	+%
i	Eagaacg7o*	18	**43**	27	20					
	Eaacagc8o*	16	**31**	41	16					
ii	Eaccaca6a*	29	**46**	13	11					
iii	Eacgacg9a^	**50**	30	8	13					
(24)	**Eaccaga6o***	15	**28**	45	15	Eaccaga185o	36	30	12	
	Eagaagg9o*	28	**57**	3	17					
	Eagaagg7o*	28	**47**	13	13					
v	Eaccagc14o*	25	**47**	16	15					
	Eactaag1o*	26	**48**	14	15					
	Eactaag13a*	25	**49**	14	16					
	Eaagact6*	29	**46**	13	11					
vi	Eacgagt6a^	**41**	23	24	14					
	Eaacagc7a*	33	**39**	16	4					
vii	Eaagaca6a^	**49**	30	9	12					
(5)	Eaacagg8o*	56	**27**	5	17					
viii	**Eagcaag2a^**	**55**	18	15	**25**	**Eagcaag145a^**	**46**	24	8	**16**
(12)	**Eaacacc3a^**	**46**	12	30	**29**	**Eaacacc161a^**	**46**	27	5	**13**
QTL	**Eaccaga5a^**	**34**	8	46	**31**	**Eaccaga500a^**	**43**	22	13	**16**
	Eaccaga3a^	**33**	8	47	30					
	Eaccaga1a^	**31**	12	45	22					
ix	Eacaacg3o*	11	**63**	14	35					
(10)	Eacaacg4o*	6	**67**	15	42					
QTL	**Eaacacc6a***	4	**53**	31	43	Eaacacc179a	38	35	5	
	Eagcact13a*	7	**70**	11	41					
	Eaacagg3a*	3	**68**	17	46					
	Eacaagc4a*	10	**54**	24	34	Eacaagc191c	39	34	4	
	Eagcacc2o*	11	**60**	17	35					
	Eaagacc12o*	12	**20**	56	13					
	Eaacagc10o*	10	**37**	41	29	Eaacagc243o	40	33	4	
	Eactaag4a*	19	**55**	14	24					
	Eagcagg5a*	17	**27**	44	11					
	Eagcagg4a*	23	**52**	13	19					
x	Eagaagg5o^	**53**	23	12	20					
(31)										

xi	Eagcaac7a^	**46**	22	20	18					
	Eaccact7a^	**37**	19	32	16					
	Eacgacc5o^	**48**	29	11	12					
	Eaacacg3o^	**46**	23	19	17					
	Eagcacg1o*	29	**46**	13	11					
xiii	Eactaag14a*	23	**39**	26	13					
	Eacgacc1o*	30	**48**	10	12					
	Eagcact8o*	28	**48**	12	13					
	Eagcact14a*	29	**48**	11	12					
	Eagaaag6a*	24	**49**	15	17					
xv (21)	**Eacgact2o***	27	**56**	5	17	Eacgact233o	32	37	9	
xvi	Eagaaag10a*	24	**48**	16	17					
	Eaacaga9a*	10	**29**	49	24					
xvii	Eacgagt2o*	21	**44**	23	18					
xviii (19)	Eaccacg2o*	26	**40**	22	11					
	Eaacagc5a*	17	**32**	39	15					
	Eacgagg2o*	28	**45**	15	12					
xix (6)	**Eaccaga2a***	14	**29**	45	17	Eaccaga286c	37	29	12	
	Eagaagc2a*	14	**41**	33	25					
xxi	Eagcaca4o*	25	**47**	16	15					
xxii (22)	**Eaagaag6a^**	**52**	21	15	21	Eaagaag222a	27	17	34	
	Eaagact4a^	**48**	28	12	13					
	Eaagagg10a^	**51**	27	10	15					
	Eactacg5a^	**48**	30	10	12					
	Eactaga4a^	**48**	28	12	13					
	Eagcact5a^	**49**	25	14	16					
	Eagcacc8a^	**48**	23	17	**18**	**Eagcacc223a^**	**45**	27	6	**13**
xv	Eacgacg2a*	24	**56**	8	20					
	Eaagaca9o*	25	**54**	9	18					
	Eagaaag3o*	27	**46**	15	13					
xxvi (1)	**SOD-1***	23	**36**	29	11	SOD-1	37	39	2	
	Eactaag12o^	**52**	22	14	20					
	Eaagacc3o^	**47**	16	25	25	Eaagacc531o	42	32	4	

	Eaagacc9a^	**45**	17	26	23		*(Continued from previous page)*		
	Eaagacc14a^	**44**	18	26	21				
	Eaagacc2a^	**44**	19	25	20				

xxix	Eaagact3o*	27	**49**	12	14				
	Eagcaca1a^	**56**	27	5	17				
	Eagaaca1a^	**35**	19	34	15				
	Eagaaag12a^	**48**	24	16	17				

xxx	**Eaagagg3o^**	49	31	8	11	Eaagagg209o	31	43	4
(29)	Eacgagt8o*	22	**39**	27	14				

xxxi	Eacaaca3a*	27	**44**	17	12

xxxiii	Eactacc7o*	20	**37**	31	15

xxxv	Eaccacg7o*	23	**43**	22	15
	Eaccacg8o*	17	**55**	16	26

xc	Eacgagt5o*	24	**41**	23	13

List of markers found in this study with severe segregation distortion; linkage groups are ordered by roman numerals unless paired with a linkage group from Nichols (2003b), noted in parentheses. A bolded marker indicates a match to the Nichols (2003b) OSUxAR marker which is listed on the right. A bolded allele is above normal 50:50 Mendelian segregation, and the +% column identifies by how much the bolded allele exceeds 50% segregation.