Identification of phenotypic variation across levels of cSOD activity in *Drosophila melanogaster* reveals that phenotypes are most influenced by second chromosome genetic background and sex at high cSOD activity

by

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Defining the effects of sex and genetic background on variation in Superoxide dismutase (cSOD) related phenotypes in Drosophila melanogaster
Abstract

This thesis examined how variable the effects of changes in cSOD activity were on phenotypes across genetic backgrounds and between sexes. Analysis of variance (ANOVA), and the effect size partial eta squared ($\eta^2_p$) were used to partition the amount of variation attributable to sex, cSOD activity, and genetic background across the distal and proximal phenotypes assayed. The absence of cSOD activity results in pervasive changes in phenotypic expression, and these changes are only slightly modified by sex or genetic background. Higher levels of cSOD activity generally result in phenotypic expression closer to wild-type levels, though phenotypes were more susceptible to modification depending on sex and genetic background when some cSOD activity was present. Results here indicate that the cSOD-null syndrome is pervasive, and the significant influence of sex and genetic background across phenotypes supports the utilization of both sexes and multiple genetic backgrounds in genetic analyses.

Keywords

Cu,Zn Superoxide Dismutase, Genetic Background, Sex, Oxidative Stress, Reactive Oxygen Species, cSOD-null Syndrome, *Drosophila melanogaster*
Co-Authorship Statement

Chapter 1 is an introduction to the broader concepts of free radicals, reactive oxygen species (ROS), antioxidants, sex, and genetic background, as well as a more specific introduction to superoxide dismutase (SOD), with an emphasis on the cSOD-null syndrome. Thomas J.S. Merritt (TJSM) and Tony L. Parkes (TLP) provided editorial and organizational feedback.

Chapter 2 covers the methodology used in the performance of this study. TJSM and TLP provided editorial feedback, as well as contributing to the conception and experimental design of the study. Christine Bernard (CB) provided troubleshooting advice for the SOD assay.

Chapter 3 outlines the results obtained in this study. TJSM and TLP provided editorial and organisational feedback.

Chapter 4 is an in depth discussion of the results obtained in this study. TJSM and TLP provided editorial and organisational feedback.

Chapter 5 is a summary of the general conclusions obtained, including several novel biochemical phenotypes for the cSOD-null syndrome. Suggestions for three further projects are outlined for further investigation of cSOD phenotypes, as well as an expansion of potential genetic background studies that can be performed.
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List of Abbreviations

6PGD - 6-phosphogluconate dehydrogenase
CF - Partition coefficient
CRISPR - Clustered regularly interspaced short palindromic repeat
crRNA - CRISPR RNA
cSOD - Cytosolic (copper/zinc) superoxide dismutase
DGRP - *Drosophila melanogaster* genetic reference panel
dsx - Doublesex
EMS - Ethyl methanesulfonate mutagenesis
ETC - Electron transport chain
FMN - Flavin mono-nucleotide
fru - Fruitless
G6PD - Glucose-6-phosphate dehydrogenase
GWAS - Genome wide association studies
H$_2$O$_2$ - Hydrogen peroxide
IDH - Isocitrate dehydrogenase
MEN - Malic enzyme
MnSOD - Mitochondrial (manganese) superoxide dismutase
NADH - Nicotinamide adenine dinucleotide
NADPH - Nicotinamide adenine dinucleotide phosphate
NO - Nitric oxide
NHEJ - Nonhomologous end joining
NOX - NADPH oxidases
$\eta^2_p$ - Partial eta squared

$^1\text{O}_2$ - Singlet oxygen

$\text{O}_2^-$ - Superoxide

ONOO$^-$ - Peroxynitrite

PI - Performance index (negative geotaxis)

QTL - Quantitative trait loci

RNAi - RNA interference

RNS - Reactive nitrogen species

ROS - Reactive oxygen species

Sxl - Sex lethal

sgRNA - Synthetic guide RNA

SNP - Single nucleotide polymorphism

TOR - Target of rapamycin

tracrRNA - Trans-acting crRNA

WT - Wild-type
Chapter 1 Introduction

1.1 Overview

Phenotypes are not the function of alleles at one locus, but rather a combination of alleles, genotype across the rest of the genome (genetic background), and the environment (Chandler, et al., 2013, and Chari and Dworkin, 2013). More complex biological networks are likely more susceptible to genetic background effects than simple networks (Chandler, et al., 2013, and Chari and Dworkin, 2013). The dynamic and complex nature of reactive oxygen species (ROS) metabolism presents a model system to use in the study of genetic background effects. There are different types of ROS, including free radicals, molecules that contain an unpaired electron that makes them highly reactive to biological macromolecules with the degree of reactivity depending on the radical species (Dröge, 2001). Superoxide is a free radical ROS generated as a by-product of energy metabolism (Cadenas and Davies, 2000) and other cytosolic enzymatic reactions, including NADP(H) oxidases (NOX; Ozcan and Ogun, 2015). It is well established that ROS can cause damage to cellular macromolecules, however they are also used in the maintenance of normal cell homeostasis (Ozcan and Ogun, 2015). Oxidative stress can result if there is an imbalance towards the production of ROS (Staveley et al., 1991).

Cytosolic superoxide dismutase (cSOD) is an antioxidant enzyme responsible for the detoxification of the superoxide radical, which functions as the initial step of the antioxidant enzyme defence network (Phillips et al., 1989). In Drosophila melanogaster, flies lacking cSOD activity, cSOD-null homozygotes, exist in a state of chronic oxidative stress resulting from an inability to metabolize cytosolic superoxide (O$_2^-$; Phillips et al.,
1989). Oxidative stress associated with the cSOD-null allele results in a series of pathological conditions, collectively known as the cSOD-null syndrome (Parkes et al., 1998, and Phillips et al., 1989). We hypothesized that differences in the ROS state between cSOD-null and wild-type (WT) flies may manifest in a difference in response to genetic background at the different levels of cSOD activity, with different genetic backgrounds potentially ameliorating or further impairing the phenotypes of the cSOD-null syndrome.

1.2 Free Radicals and the Generation of Free Radicals

Free radicals are molecules that contain one unpaired electron, and are present in biological systems (Halliwell, 1994, and McCord, 2000). The unpaired electron in radicals makes these molecules reactive to biological macromolecules, with the level of reactivity dependent upon the radical species (Halliwell, 1994). If two radicals meet, the unpaired electron on each radical can react to form a covalent bond, generating a non-radical molecule (Halliwell, 1994). Conversely, if a radical meets a non-radical, the radical's free electron can react with the non-radical and generate a new radical, potentially causing a chain of redox reactions, in which each successive reaction produces a free radical that triggers the next step in the chain (Buonocore, et al., 2010, and Halliwell, 1994). The high reactivity of radicals can present a threat in biological systems, in which most molecules are non-radicals, by triggering reaction cascades within biological macromolecules, such as DNA or enzymes, which can prematurely trigger signal transduction pathways, such as those involved in apoptosis (Buonocore, et al., 2010, and Halliwell, 1994). Free radicals can be classified into different groups depending on their atomic composition, including reactive nitrogen species (RNS) and
reactive oxygen species (ROS; Dröge, 2001), though classifications are not necessarily mutually exclusive. ROS are a biologically important class of molecules in living systems, as they are produced as natural metabolic products, and make up components of both cell signalling and homeostasis (Ozcan and Ogun, 2015).

The mitochondrial electron transport chain (ETC) is the largest producer of free radicals, primarily ROS, including superoxide, though ROS are also produced in the outer mitochondrial membrane independent of respiration (Cadenas and Davies, 2000). Electrons are transferred from electron carriers (i.e. NAD(H)) to the ETC, and passed step-wise down the series of enzyme complexes, alternating in oxidation and reduction reactions, ultimately converting the energy in the electrons into ATP (Cadenas and Davies, 2000 and Murphy, 2009). The ETC protein complexes are bound with electron carriers, such as the flavin mono-nucleotide (FMN) cofactor, and the reaction of oxygen with these electron carriers, outside of normal ETC function, produces the partially reduced superoxide anion as a reaction by-product (Cadenas and Davies, 2000 and Murphy, 2009). The first stage of the ETC transfers electrons from NADP(H) to the FMN cofactor in complex I, and this complex produces the majority of the superoxide anion generated by the ETC (Cadenas and Davies, 2000, Finkel and Holbrook, 2000, and Murphy, 2009). ETC complex III, which channels electrons to cytochrome c, also produces superoxide anions via side reactions, however, it produces much less than complex I (Murphy, 2009).

Although the ETC is the primary producer of free radicals, cytosolic enzymes, such as NADP(H) oxidase (NOX), dihydrorotate dehydrogenase, aldehyde oxidase, and xanthine oxidase, are also large producers of the superoxide anion (Gandhi and Abramov,
NOX enzymes function as transmembrane electron transporters, transferring electrons from NADP(H) to molecular oxygen and thereby generating superoxide (Krause, 2006). Other cellular pathways, such as those used by phagocytes as part of the immune response, can also produce targeted bursts of ROS (Ozcan and Ogun, 2015). The overall cellular concentration of free radicals is determined by the balance of their rate of production (both by-product and not) to their rate of clearance by different antioxidant molecules (both enzymatic and not; Dröge, 2001, and Finkel and Holbrook, 2000). Balance is crucial; high concentrations of free radicals can cause cellular damage, while low or moderate levels of free radicals can have beneficial effects on cellular functions (Buonocore, et al., 2010).

1.3 Reactive Oxygen Species (ROS)

There are two main potential effects of increasing the cellular concentration of oxidants: cellular component damage resulting from oxidative stress, or activation of specific cell signalling pathways (Finkel and Holbrook, 2000). When ROS react with biological macromolecules such as lipids, proteins, and nucleic acids, the reaction can damage these molecules, potentially initiating degradative cellular processes, e.g. apoptosis (Cadenas and Davies, 2000). Superoxide, one of the most commonly produced free radicals, is not a highly reactive free radical, however it is one of the more toxic ones because of its propensity to generate other radicals (McCord, 2000, and Ozcan and Ogun, 2015). Increased steady state concentrations of superoxide can reduce transition metals, which in turn can react with hydrogen peroxide (H$_2$O$_2$) to produce the hydroxyl radical (OH; Turrens, 2003). Additionally, superoxide can react with other free radicals, such as nitric oxide (NO$^-$), and generate much more cytotoxic free radicals such as peroxynitrite
Superoxide can also react with, and ultimately damage, major classes of biological molecules, including DNA (McCord, 2000, and Ozcan and Ogun, 2015). However, because superoxide does not readily pass through cell membranes it primarily produces local effects and is relatively short-lived in cells (Ozcan and Ogun, 2015). The relative toxicity of superoxide is increased by its capacity to be converted into other, more reactive, non-radical and radical species, such as singlet oxygen (${\text{^1O}}_2$), hydroxyl radical, and peroxynitrite (Dröge, 2001).

Though the accumulation of ROS can cause oxidative stress, ROS are also necessary for the maintenance of normal cellular homeostasis. ROS have been implicated in host defence mechanisms, regulation of the proliferative response, and they function as part of signalling pathways (Finkel and Holbrook, 2000). In higher organisms, superoxide and nitric oxide are produced as ROS bursts by macrophages as part of the inflammatory response (Finkel and Holbrook, 2000 and Valko et al., 2007).

While the above processes and functions are crucial and part of a healthy biological system, oxidative stress results when there is an imbalance between the production and elimination of ROS; such an imbalance has been implicated in a series of pathological conditions, as well as in the overall processes of ageing and senescence (Staveley et al., 1991).

### 1.4 Free Radical Defences

Aerobic organisms employ both enzymatic and non-enzymatic defences to scavenge free radicals in cellular systems (Parkes et al., 1998b). Superoxide is detoxified primarily by the antioxidant enzyme superoxide dismutase (SOD; McCord and Fridovich, 1969). SOD functions as part of an antioxidant enzyme defence network, the first step of
which is the dismutation of superoxide into hydrogen peroxide by SOD; the hydrogen peroxide is then further reduced into water by catalase and peroxidases (Phillips et al., 1989). The cytotoxicity of the superoxide radical is largely credited to its ability to promote Fenton chemistry, which results in generation of the highly reactive hydroxyl radical, and other secondary ROS (Gutteridge, 1985). The efficiency of superoxide scavenging by the antioxidant defence network reduces the concentration of superoxide available to generate more reactive radicals (e.g. hydroxyl radical; Gutteridge, 1985).

SOD is ubiquitous in organisms exposed to oxygen. All SOD enzymes include a metal ion core that functions in electron transfer (Bafana et al., 2011) and four types of metal ion cores have been identified in isozymes of this enzyme: copper/zinc- (Cu,Zn-), manganese- (Mn-), iron- (Fe), and nickel- (Ni-) SODs (Bafana et al., 2011, and Miller, 2012). The Mn- and Fe- core SODs are often grouped in a single family due to their high amino acid sequence similarity, with Mn-SODs likely being derived from the more primitive Fe-SODS, or Fe/Mn-SODs that can substitute either Fe- or Mn- ions as their cores (Bafana et al., 2011, and Miller, 2012). Ni-SOD has been identified in several prokaryotes, including cyanobacteria, however, overall this group of SODs is still poorly characterized (Bafana et al., 2011). Cu,Zn-SOD and Fe/Mn-SODs evolved independently in prokaryotes several billion years ago (Bafana et al., 2011, Miller, 2012). Mn-SOD is both the prokaryotic SOD isozyme and the SOD isozyme located in the mitochondria of eukaryotes (mtSOD or SOD2; Bafana et al., 2011, and Miller, 2012). Two distinct forms of Cu,Zn-SOD exist in many eukaryotes, which are coded for by separate genes (Bafana et al., 2011). One form of Cu,Zn-SOD is located in the cytosol, mitochondrial intermembrane space, lysosomes, and the nucleus (cSOD or SOD1; Bafana
cSOD dismutates superoxide via a stepwise reaction in which the copper ion gains and loses electrons from superoxide and hydrogen to ultimately convert superoxide into hydrogen peroxide (Franco et al., 2013, and Smirnov and Roth, 2006).

1. \( \text{Cu}^{2+} + \text{O}_2^- \rightarrow \text{Cu}^+ + \text{O}_2 \)
2. \( \text{Cu}^+ + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{Cu}^{2+} + \text{H}_2\text{O}_2 \)

The dismutation rate of SOD isoforms relies almost solely on the rate of diffusion of superoxide, as the electron transfer between the substrate and the active site is highly efficient (Bafana et al., 2011, and Smirnov and Roth, 2006). Similar to other essential antioxidant enzymes, such as catalase, glutathione reductase, and glutathione peroxidase, the production of SOD isoforms is regulated by the transcription factor nuclear erythroid-related factor 2 (Nrf2), which responds to variation in oxidative stress levels (Buonocore, et al., 2010). This pattern of regulation is broadly distributed, in vertebrates and invertebrates, including *D. melanogaster* (Buonocore, et al., 2010, Lakhan, et al., 2009, and Sykiotis and Bohmann, 2008).

### 1.5 *Drosophila melanogaster* cSOD

In *Drosophila melanogaster*, the locus for the cSOD enzyme is located on the left arm of the 3rd chromosome. This gene codes for a 151 amino acid monomer (Sáez et al., 2002), and the active cSOD enzyme is a homodimer comprised of two identical monomers bound together (Campbell et al., 1986). In *D. melanogaster*, there are two known naturally occurring cSOD allozymes, *cSod-fast* and *cSod-slow*, and the allozymes differ biochemically, although they only differ compositionally by a single amino acid,
with the \textit{cSod-fast} allele occurring at the much higher frequency in natural populations (Campbell \textit{et al.}, 1986 and Sáez \textit{et al.}, 2002). The \textit{cSod} null allele used in this thesis was generated from a \textit{cSod-fast} allele (Campbell \textit{et al.}, 1986).

The \textit{cSod}^{n108} null allele was generated via the process of ethyl methanesulfonate (EMS) mutagenesis (Campbell \textit{et al.}, 1986), and subsequently recombined onto a 3\textsuperscript{rd} chromosome bearing the larval marker \textit{red}, with \textit{Oregon R} as the background strain, and the chromosome was re-classified as \textit{cSod}^{n108}, \textit{red} (Campbell \textit{et al.}, 1986, Parkes \textit{et al.}, 1998b, and Phillips \textit{et al.}, 1989). The EMS-generated \textit{cSod}^{n108} null allele results from a missense mutation that substitutes a serine for a glycine at the 49\textsuperscript{th} amino acid position (Phillips \textit{et al.}, 1995). There are two pairs of symmetric main-chain hydrogen bonds that form across the dimer interface, and the Gly -> Ser substitution breaks one of the symmetric pairs of main-chain hydrogen bonds, destabilizing the dimer assembly (Phillips \textit{et al.}, 1995). Dimer destabilization is presumably the mechanism that leads to the loss of cSOD activity.

(Parkes et al., 1998b), ix) increased spontaneous mutation rate in somatic and germ line cells (Woodruff et al., 2004), as well as x) an 'enfeebled' phenotype that is variably expressed and includes bloated abdomens, and crinkled or incompletely expanded wings (Phillips et al., 1989). Recent biochemical work on cSod-null Drosophila has added four more phenotypes to this list: xi) metabolomic differences between cSOD-null and cSOD+ flies (Knee et al., 2013), xii) differences in NADP(H) enzyme activity between cSOD-null and cSOD+ males (Bernard et al., 2011), xiii) an increase in lipid concentration in cSOD-null compared to cSOD+ males (Bernard et al., 2011), and xiv) starvation resistance in cSOD-null compared to cSOD+ males (Bernard et al., 2011).

1.6 cSOD-Null Mechanism

Overexpression of human cSOD in D. melanogaster motorneurons extends normal lifespan, as well as restoring the lifespan of the short lived cSOD-null mutant D. melanogaster to nearly WT (Parkes et al., 1998a). The connection between cSOD activity and lifespan, and the influence of cSOD overexpression within a unique cell type, suggests that the overall lifespan of the entire organism is a function of the lifespans of some 'critical' cell types, i.e. motorneurons (Parkes et al., 1998a). The free radical theory of aging hypothesizes that an accumulation of oxidative damage to cellular macromolecules results in the progressive decline, and eventual death, of cells (Harman, 1956). In this model, increased oxidative stress from the higher concentration of superoxide anion in motorneurons, of cSOD-null D. melanogaster, causes premature failure of the motorneurons and, ultimately, premature organism death (Parkes, et al., 1999, and Phillips, et al., 2000).
While ROS can cause intracellular damage, the ubiquitous presence of oxygen in aerobic organisms, and the resulting generation of ROS, has provided conditions where ROS can be exploited by organisms, as seen by their roles in signal transduction pathways, gene regulation during development, and differentiation, as well as overall cell homeostasis (Lin, *et al.*, 1998, and Parkes, *et al.*, 1999). ROS are key factors mediating growth factor signalling using G-protein coupled receptors, as well as Notch and Wnt, which control downstream cascades for MAPK, JAK-STAT, FoxO, NF-kB, ERK, and PI3K/AKT (Buetler, *et al.*, 2004, Owusu-Ansah and Banerjee, 2009, and Zhou *et al.*, 2012). Specifically, ROS (including the superoxide anion and hydrogen peroxide) are involved in vascular formation (Buetler, *et al.*, 2004, and Zhou *et al.*, 2012), haematopoietic differentiation (Owusu-Ansah and Banerjee, 2009), cell growth and proliferation (Buetler, *et al.*, 2004), insulin synthesis (Weidinger and Kozlov, 2015) and TOR signalling (Blagosklonny, 2008, and Patel and Tamanoi, 2006), as well as cell migration (Bloomfield and Pears, 2003). Therefore, an alternate theory for the mechanism of the cSOD-null syndrome would be that the altered cellular ROS dynamic, in cSOD-null flies, influences cell and tissue physiology by altering signal transduction pathways, and ultimately manifesting in the suite of cSOD-null phenotypes (Parkes, *et al.*, 1999, and Phillips, *et al.*, 2000). For example, cSOD-null flies have an increased concentration of the superoxide anion, which can react with nitric oxide to generate the free radical peroxynitrite (Weidinger and Kozlov, 2015). Peroxynitrite is known to negatively influence a number pathways involving MAPK, STAT3, ERK, and PKC pathways, which control processes such as apoptosis and differentiation (Weidinger and Kozlov, 2015). Similarly, cSOD activity changes over the course of organismal
development (Radyuk, et al., 2004), so changes in cell and tissue physiology as a result of the cSOD-null condition, may result in phenotypic changes over the course of fly development (i.e. the cSOD-null syndrome).

1.7 Phenotypes

The cSOD-null syndrome is characterized by a suite of phenotypes that range from behaviour (i.e. changes in locomotor ability) and organism fitness, to metabolic and enzymatic differences. The phenotypes encompassed by the cSOD-null syndrome can be classified into two broad categories: distal phenotypes and proximal phenotypes. Broadly speaking, proximal phenotypes are changes “close” to the mutation, changes that are direct, or nearly direct, results of the mutation, and distal phenotypes are changes more “distant” from the mutation, phenotypes that result from changes across a network or pathway. Seven phenotypes were examined in this thesis that spanned both of these classifications.

1.7.1 Distal Phenotypes

Distal phenotypes are the result of the downstream interactions of multiple genes (such as behaviour and IQ; Scriver, 1994 and Scriver, 1995), and can be modified by mutations across the underlying interacting genes. For example, the distal phenotype triglyceride content, is correlated with changes in malic enzyme (Men) activity (Merritt, et al., 2005). I examined four distal phenotypes that cover different aspects of Drosophila life history (genotypic viability, and longevity) and locomotion (negative geotaxis, and climbing ability), which are influenced by the lack of cSOD activity
1.7.1.a Life History

Life history traits are a function of a series of genetic and environmental factors which influence an organism’s overall ability to survive and reproduce. Two life history related phenotypes are longevity and viability. Longevity, which examines how long-lived organisms are, is influenced by multiple genetic loci, which are components of many genetic pathways (i.e. oxidative stress resistance pathways), and as such single gene mutations can influence longevity (Vijg and Suh, 2005). Longevity in D. melanogaster is depressed in flies homozygous for the cSod<sup>108</sup>,<i>red</i> allele, reflecting their state of chronic oxidative stress (Parkes <i>et al</i>. 1998b, and Phillips <i>et al</i>. 1989), and flies overexpressing cSOD in their motorneurons have increased longevity (Parkes, <i>et al</i>. 1999). These differences in longevity in cSOD-null, and cSOD overexpressed, flies suggest that longevity will also be influenced by intermediate levels of cSOD activity.

Viability, in essence, is the ability of an organism to survive, though overall, a number of different, but interrelated types of viability can be measured, and all contribute to life history. Viability is expressed as an index (Keightley, 1994), with the components of the index varying depending on the type of viability measured. Types of viability include egg-to-adult viability, which is the proportion of eggs that result in eclosed adults (Kern <i>et al</i>. 2001); larva-to-adult viability, which is the proportion of larva that result in eclosed adults (Kern <i>et al</i>. 2001); offspring viability, which encompasses egg hatching success and larva-to-adult survival (Kern <i>et al</i>. 2001); and relative genotypic viability, which is the relative ability of different genotypes to successfully eclose (Keightley,
1994, and Merritt et al., 2005). cSOD-null *Drosophila* have reduced egg-to-adult viability compared to WT *Drosophila* (Sun and Tower, 1999). Relative genotypic viability measures the differences in survivability of distinct genotypes (Keightley, 1994, and Merritt et al., 2005), and the reduced egg-to-adult viability in cSOD-null homozygotes, compared to WT viability, may suggest that the genotypic viability of cSOD-null homozygotes is also reduced compared to that of WT flies.

1.7.1.b Locomotion

There are multiple locomotor phenotypes, and locomotion, in general, is influenced by multiple interacting loci, most with small individual effects, which are in turn influenced by environmental conditions (Jordan et al., 2007). Negative geotaxis and climbing ability are two locomotor phenotypes that measure different, but related, aspects of locomotion. Negative geotaxis is a startle response against gravity, and it measures the immediate, reflexive, response of flies to a startle stimulation (Jordan et al., 2012). Climbing ability was measured by partitioning flies based on differing endurance responses to a startle stimulation (Benzer, 1967, and Petersen et al., 2013). Accelerated age-related locomotor impairment in negative geotaxis has been observed in flies with low cSOD activity driven by a ubiquitously expressed RNAi construct (Martin, et al., 2009). cSOD-null flies have not been previously tested on the countercurrent apparatus, so their locomotor ability on this assay is unknown, though other types of locomotion, including negative geotaxis, experience similar declines in locomotor performance with age (Jones and Grotewiel, 2011). Although similar locomotor types (negative geotaxis and startle response) have similar overall responses, differences in the underlying genetic
mechanisms of the traits can result in variable locomotor sensitivities to factors such as genetic background and oxidative stress (Jordan et al., 2012).

### 1.7.2 Proximal Phenotypes

Proximal phenotypes are the direct result of a change in a single gene (such as enzyme activity and metabolic phenotypes; Scriver, 1994, and Scriver, 1995), and can be modified by mutations in the single gene, or in closely related genes. For example, the proximal phenotypes of enzyme activity (V\text{max}) and substrate binding affinity (K\text{m}) are influenced by single nucleotide polymorphisms in the Men gene, with the phenotypes varying depending on the nucleotide present (Merritt et al., 2005). I examined three proximal phenotypes that cover the activities of three NADP(H) enzymes (Malic enzyme - Men, Isocitrate dehydrogenase - Idh, and Glucoe-6-phosphate dehydrogenase - G6PD), which have reduced activity in the absence of cSOD activity in male flies (Bernard, et al., 2011).

#### 1.7.2.a NADP(H) Enzymes

The NADP(H)/NADP\textsuperscript{+} pools in cells are primarily maintained by four key cytosolic enzymes; MEN, IDH, G6PD, and 6-phosphogluconate dehydrogenase (6PGD), and significant interactions are present across the four (Merritt et al., 2005, 2009, Rzezniczak and Merritt, 2012). However, NADP(H) enzyme activities are also influenced by the absence of cSOD activity (Bernard et al., 2011), likely because NADP(H) is required by catalase and glutathione-dependent antioxidants to scavenge hydrogen peroxide, produced as the product of SOD (Kanzok et al., 2001). NADP(H) binds and stabilizes catalase to promote proper function (Kirkman et al., 1999), while
NADP(H) is used for redox cycling by glutathione reductase (Halliwell, 1994), and thioredoxin reductase (Kanzok et al., 2001), as well as by non-enzymatic antioxidants, such as vitamin C and E (Sies and Stahl, 1995), all of which function in antioxidant defences. In cSOD-null male flies, MEN, IDH, and G6PD activities were significantly lower than in WT flies, with the magnitude of effect varying across the three enzymes (Bernard, et al., 2011). Given this interaction, we hypothesized that intermediate levels of cSOD activity will similarly result in changes in NADP(H) enzyme activity.

1.8 Sex

Organisms are categorized as male or female according to the occurrence of primary and secondary sexual characteristics; sex specific phenotypic variation is known as sexual dimorphism (Assis, et al., 2012, and Fairbairn and Roff, 2006). Originally, sexual dimorphism only referred to differences in morphology; however, this term has been expanded to encompass sex specific differences in morphology, as well as in physiology and biochemistry, behaviour, and life history strategy (Assis, et al., 2012, and Fairbairn and Roff, 2006). The occurrence of sexual dimorphism is ubiquitous among higher eukaryotes (Assis, et al., 2012, and Ranz et al., 2003), including Drosophila. Previous research in Drosophila has demonstrated sexual dimorphism in phenotypes such as longevity (Pasyukova et al., 2000, and Spencer et al., 2003), locomotion (Jordan et al., 2007, and Yamamoto et al., 2009), and NADP(H) enzyme activity (Merritt et al., 2009) and expression (Gnad and Parsch, 2006). Overall, a large portion of the Drosophila transcriptome displays sex-specific regulation, though sex-biased transcription may not translate into functional genetic differentiation between sexes (Connallon and Clark, 2011, and Ranz et al., 2003). In D. melanogaster, genes that exhibit male- or female-
biased expression seem to differ in their tissue specificity; male-biased genes are expressed in sex-specific tissues, while female-biased genes are more broadly expressed, and more pleiotropic in nature (Assis, et al., 2012).

Sex determination, or the hierarchy of events that results in the development of sexual characteristics in organisms, is broadly controlled by a few key genes (Clough and Oliver, 2012), in many organisms. The underlying genomes in males and females are predominantly the same, so sex determination relies on the differential utilization of shared genes, through processes such as differential gene expression, and alternate gene splicing (Connallon and Clark, 2011, and Rhen, 2000). For organisms possessing sex chromosomes, sex determination is initiated by genes on the sex chromosomes; however, responding genes are also distributed on the autosomes, such that phenotypic effects of sex-linked genes are often controlled via the regulation of autosomal gene expression (Fairbairn and Roff, 2006). Sex differential gene expression in adults is controlled via two (known) mechanisms where gene regulation in adults is either actively regulated by the sex hierarchy, or gene expression in adult tissues is not actively regulated by the sex hierarchy, but follows regulatory patterns set down during development (Arbeitman et al., 2004).

Sex determination, controlled by the sex hierarchy, in *D. melanogaster*, is initiated by *sex lethal (Sxl)*, which is differentially produced in males and females (Kopp et al., 2002). The presence (females) or absence (males) of Sxl triggers production of the genes *doublesex (dsx)* and *fruitless (fru)*, which undergo alternative splicing in males and females, with sex specific isoforms of each protein being generated (Clough et al., 2014, and Kopp et al., 2002); *fru* and *dsx* control different branches of sex determination.
In *D. melanogaster* the *dsx* gene is required for sexual dimorphism in morphology, physiology and biochemistry, and behaviour, though the full mechanism by which this occurs is not fully understood (Clough *et al.*, 2014). *dsx* regulation of loci with minor, but polygenic, effects on development and biochemistry, may contribute to genetic background induced variation in sex-related phenotypes (Clough *et al.*, 2014).

**1.9 Genetic Background**

Phenotypes are context dependent; the effects of a mutation on a gene of interest depend on the specific allele, the genetic background, as well as the external environment (Chandler, *et al.*, 2013, and Chari and Dworkin, 2013). Genetic background refers to the complete genetic context of an organism, or an organism's complete genotype (Chandler, *et al.*, 2013). Organisms that have similar overall phenotypes may vary dramatically at the genetic level, but differences are buffered under 'normal' conditions, such that the overall phenotypes are approximately the same (Chandler, *et al.*, 2013, and Chari and Dworkin, 2013). When environments, or genes are perturbed, then the ability of different genetic backgrounds to buffer against these perturbations may vary, resulting in phenotypic variation which doesn't exist under 'normal' conditions (Chandler, *et al.*, 2013, Dworkin *et al.*, 2008). Genetic background specifically can influence expression of neomorphic (novel gene function or expression *i.e.* DER-Ellipse; Polaczyk, *et al.*, 1998), hypomorphic (reduced gene function or expression *i.e.* HSP90; Sangster *et al.*, 2007), and amorphic (complete loss of gene function or expression *i.e.* EGFR; Threadgill *et al.*, 1995) mutant alleles, although the magnitude of the influence varies according to the genetic background present, the environmental factors, and likely the type of allele
(Chari and Dworkin, 2013). Similarly, complex traits, which are the result of multiple interacting genes, are also influenced by genetic background (e.g. longevity; Spencer et al., 2003; transvection Bing et al., 2014).

The cSOD-null phenotype is sensitive to genetic background, but the extent of the variation in phenotypes across backgrounds has not been quantified. The cSOD-null allele was originally generated on a 3rd chromosome with the genotype \( cSod^{m108}, sr, e^f, ca \), and the cSOD-null related phenotypes identified with this chromosomal genotype included high death rate during metamorphosis, and subsequently very low adult viability, adult sterility, lifespan of 2-3 days, and no detectable cSOD activity (Campbell et al., 1986). When the \( cSod^{m108} \) allele was subsequently recombined onto a 3rd chromosome carrying the larval marker \( red \), cSOD activity was still undetectable, and larval development was slightly delayed, however, survivability to adulthood was improved as was adult lifespan, though not to WT levels (Phillips et al., 1989). Similarly, when recombinant lines were subsequently generated to remove the \( red \) marker, though the lines remained cSOD-null, several of the recombinant lines were more viable and fertile than the parent line (Hilliker, A.J., Parkes, T.L., Duyf, B., and Phillips, J.P., unpublished data).

Though much has been done to define the \( cSod \)-null syndrome of \( D. melanogaster \), one limitation of this work is that much of it has been done using only the \( Oregon \ R \) genetic background in which the \( cSod^{m108} \) allele was generated (see Campbell et al., 1986, Parkes et al., 1998b, Phillips et al., 1989, Woodruff et al., 2004, Knee et al., 2013, and Bernard et al., 2011), or cSOD-null organisms within isogenic genetic backgrounds (Parkes et al., 1998b, and Radyuk, et al., 2004), such that potential
background effects could not be detected. However, it is known that cSOD activity
varies across lines, and although the cSOD gene is located on the 3\textsuperscript{rd} chromosome, cSOD
concentration and activity can be modified by elements found on the 2\textsuperscript{nd} chromosome
(Arking, 2001, Graf and Ayala, 1986, and Hernández et al., 1988). Furthermore, when
the effect of overexpression of cSOD was examined across a series of wild-caught long-
lived genetic backgrounds, although lifespan increased on average, the magnitude of the
effect was dependent on the genetic background as well as the sex of the fly (Spencer et al., 2003).
In addition, the Oregon-R line has been maintained in the laboratory since
before 1925 (Bridges and Brehme, 1944) and has evolved to lab conditions resulting in
altered phenotypes such as early fecundity and shortened longevity (Spencer et al., 2003),
which may exacerbate the phenotypes of the cSOD-null syndrome.

\textbf{1.10 Project Outline}

Understanding the translation of genotype to phenotype relies on understanding
the complex interactions that occur between loci. Some of the greatest challenges to
defining the interactions that occur between loci are the limited conclusions that can be
made using single isogenic backgrounds. Further, it can be difficult to accurately define
and quantify appropriate phenotypes by which to assess background effects. Our current
understanding of genetic interactions between loci is largely based on the examination of
the effects of mutant alleles on phenotypes in controlled genetic backgrounds and
environments, which simplifies the way mutational effects are analyzed, but potentially
biases how allelic effects are interpreted (reviewed in Chandler, et al., 2013). Variation
in genetic background contributes to phenotypic variation, even when examining the
influence of a single mutant allele (e.g. Rzezniczak and Merritt, 2012 and Bing et al.,
The acknowledgement of the limitations of studies examining interactions within relatively simple genetic systems has led to the realization of the need to improve our understanding of how genes function in complex, and biologically relevant, genetic systems. Within this thesis, I have examined phenotypic variation, across a broad range of phenotypes, associated with changes in the level of a cytosolic antioxidant enzyme, superoxide dismutase (cSOD). This thesis focused broadly on examining the influence of genetic background, sex, and cSOD activity on phenotypic variation, allowing the quantification of the influence of each factor on phenotype. This research complements previous studies performed in the Merritt laboratory that demonstrated phenotypic variation associated with the cSOD locus (Bernard, et al., 2011), and biological complexity in the NADP(H)-enzyme network related to variation in 3rd chromosome genetic background (Rzezniczak and Merritt, 2013).

In the work presented here, I document the responses of seven phenotypes to two ranges of cSOD activity, sex, and eight genetic backgrounds, to define the relative influence of each factor on phenotypic variance. I found that the absence of cSOD activity results in pervasive changes in phenotypic expression, relative to WT phenotypes, and these changes are only slightly enhanced or suppressed by sex or genetic background, although the degree does vary between phenotypes. However, when cSOD activity varies across low and high ranges, higher levels of cSOD activity generally result in phenotypes closer to WT levels, though phenotypes were more susceptible to enhancement or suppression depending on sex and genetic background when some level of cSOD activity was present.
Chapter 2 Materials and Methods

2.1 Fly Stocks and Lines

Chromosome substitution lines involve the replacement of one or more chromosomes, with chromosomes derived from a separate source via a crossing scheme (Mackay, 2009). Second chromosome substitution lines were generated using marker-assisted introgression, which uses marker loci to accelerate the introduction of a target gene (following Merritt et al., 2006) and a strategically selected subset of eight isofemale lines (derived from a single gravid female) from the Drosophila melanogaster Genetic Reference Panel (DGRP) established by the Mackay Lab (Suite of fully sequenced D. melanogaster inbred lines; Mackay et al., 2012): 304, 307, 313, 324, 335, 517, 705, and 820. The cSOD-null syndrome is characterized by changes in many phenotypes, including longevity, fitness, paraquat sensitivity, and startle response, and the subset of DGRP lines were chosen to include a high expression and low expression line for each of the listed phenotypes, as indicated by available DGRP data (Mackay et al., 2012). The eight 2nd chromosome extraction lines were genetic background replaced to make them isogenic except for the DGRP 2nd chromosome present: w1118; DGRPi/CyO; VT83 with “i” being the 2nd chromosome from one of the DGRP lines and VT83 being a 3rd chromosome isolated from the wild (Merritt et al., 2006).

The cSOD- and cSOD+ lines used in this study were the T0 (w+; T0/T0; csodn108,red/TM3, Sb) and T5 (w+; T5/T5; csodn108,red/csodn108,red) lines, respectively. The cSOD-null allele, csodn108, was generated by Campbell et al., (1986), via ethyl methanesulphonate (EMS) mutagenesis, and was subsequently introduced into the Oregon R strain and recombined with the 3rd chromosome larval marker red. A cSOD-
null line was generated by introgressing a 2nd chromosome, lacking transgenes, into the original csod\textsuperscript{n108,red} stock, creating the T0 stock (Parkes et al., 1998). A parallel cSOD\textsuperscript{+} control line, the T5 stock, was generated by inserting a cSOD transgene under the control of the native cSOD promoter, into a 2nd chromosome matched to that in the T0 stock, which was then introgressed into the original csod\textsuperscript{n108,red} stock, creating a whole organism transgenic rescue line (Parkes et al., 1998). When homozygous, the transgene restores approximately 60-70\% of wild type cSOD activity (Parkes et al., 1998) and T5/T5; cSOD\textsuperscript{n108,red}/SOD\textsuperscript{n108,red} flies have been phenotypically indistinguishable from WT cSOD\textsuperscript{+} flies in all studies subsequent to their generation.

The T0 and T5 lines were crossed to the iso 2nd chromosome lines to quantify the average effects of cSOD activity, sex, and genetic background (line) on a series of phenotypes across two ranges of cSOD activity: 0\% and 50\%; 30\% and 80\%. Distinct schemes were necessary for the T0 (Figure 1.1) and T5 (Figure 1.2) derived lines because the T0 and T5 cSOD constructs involve different chromosomes (2nd for the transgene and 3rd for the cSOD gene). The T0 crosses created progeny with either 0\% or 50\% of WT cSOD activity, while the T5 crosses created progeny with either 30\% or 80\% of WT cSOD activity. The 0\% (w\textsuperscript{+}; T0/DGRP\textit{i}; csod\textsuperscript{n108,red}/csod\textsuperscript{n108,red}) and 50\% (w\textsuperscript{+}; T0/DGRP\textit{i}; csod\textsuperscript{n108,red}/VT83) (Figure 1.1ci and 1.1cii) of WT cSOD activity flies that possess the same DGRP\textit{i} chromosome only differ in genotype by one csod\textsuperscript{n108,red} chromosome, as do the 30\% (w\textsuperscript{+}; T5/DGRP\textit{i}; csod\textsuperscript{n108}/csod\textsuperscript{n108}) and 80\% (w\textsuperscript{+}; T5/DGRP\textit{i}; csod\textsuperscript{n108}/VT83) (Figure 1.2ci and 1.2cii) of WT cSOD activity flies. For each assay, five replicate crosses of each DGRP genetic background with both the T0 and T5 lines, were used.
Figure 1: Crossing schemes used to generate T0 (1) and T5 (2) derived flies. In all crosses adults were placed on fresh cornmeal-yeast-agar media and allowed to mate and lay eggs for one week, then moved to fresh media for one week, and discarded. Crosses a) and b) were set up using two male and two virgin female flies. Single males were crossed to two virgin females in cross c) as male progeny from cross b) expressing the curly wing phenotype could have possessed one of two genotypes, only one of which was...
desired for cross c). Use of single males prevented mixing males with different genotypes, and allowed crosses that had been set up with the incorrect genotype to be discarded. Cross 1c) generated i) \( w^+; T0/DGRP_i; csod^{n108}/csod^{n108} \) (0% WT CSOD activity) and ii) \( w^+; T0/DGRP_i; csod^{n108}/VT83 \) (50% WT cSOD activity). Cross 1c) also produces \( w^+; T0/CyO; csod^{n108}/csod^{n108} \), \( w^+; T0/CyO; csod^{n108}/VT83 \), \( w^+; T0/CyO; csod^{n108}/TM3, ser \), \( w^+; T0/CyO; VT83/TM3, ser \), \( w^+; T0/DGRP_i; csod^{n108}/TM3, ser \), and \( w^+; T0/DGRP_i; VT83/TM3, ser \) genotypes. Cross 2c) generated i) \( w^+; T5/DGRP_i; csod^{n108}/csod^{n108} \) (30% WT cSOD activity) and ii) \( w^+; T5/DGRP_i; csod^{n108}/VT83 \) (80% WT cSOD activity). Cross 2c) also produces \( w^+; T5/CyO; csod^{n108}/csod^{n108} \), and \( w^+; T5/CyO; csod^{n108}/VT83 \) progeny. The four genotypes carrying the TM3, Sb third chromosome balancer, produced by 1c), are distinguished by a stubble bristles phenotype and have been removed from analysis as the four genotypes encompass two phenotypes which are visually indistinguishable and possess different levels of cSOD activity. The genotypes carrying the CyO chromosome, produced in 1c) and 2c) have been excluded from the analyses as preliminary results suggested the balancer chromosome was falsely driving background differences.

2.2 Genotypic Viability Assay

Genotypic viability, which measures the frequencies of survival of distinct genotypes, was assessed as described by Merritt et al., (2006) using progeny generated from crosses 1.1c and 1.2c (Figure 1 - Above). Cross 1.1c quantified viability in flies possessing 0% or 50% WT CSOD activity. Virgin female \( w^+; T0/T0; csod^{n108}, red/ TM3, Ser \) were crossed with males derived from the eight DGRP \( i \) lines (\( w^{1118}; DGRP_i/CyO; csod^{n108}, red/VT83 \)). Cross 1.2c quantified viability in flies possessing 30% or 80% WT cSOD activity. Virgin female \( w^+; T5/T5; csod^{n108}, red/ csod^{n108}, red \) were crossed with males derived from the eight DGRP \( i \) lines (\( w^{1118}; DGRP_i/CyO; csod^{n108}, red/VT83 \)). Adult progeny from each cross were counted from day 11 through day 16, and the frequencies of occurrence of each phenotype were calculated. Statistical analyses were performed on the calculated frequencies.

2.3 Longevity Assay

Longevity (lifespan) was measured as described by Parkes et al., (1998). Adult male and female flies of each genotype were collected 48 hours post eclosion and transferred to standard shell vials containing cornmeal-yeast-agar media, with a maximum of 20 flies per vial. Longevity was measured (based on genotype mortality) in
single sex and single genotype vials, and both sexes were assumed non-virgin. Approximately 100 flies per genotype were assayed overall, but the number varied slightly across the genotypes. Vials were maintained at 25°C, 12hr light:dark, and mortality was recorded every two days. Flies were transferred to fresh media every four days until no living flies remained. Statistical analyses were performed on the recorded longevities.

2.4 Negative Geotaxis Assay

Directional startle response of adult flies was measured using a negative geotaxis assay modified from Sofola et al., (2010), and Patel and Tamanoi, (2006). Briefly, groups of 15 adult male and 15 adult female flies of each specific genotype were collected 48 hours post eclosion, and aged for three days on cornmeal-yeast-agar medium, in single sex, single genotype vials. Following aging, the groups of flies were transferred into empty 25x95mm shell vials marked at a height of five centimetres, and allowed 30 seconds to recover. Following recovery, flies were tapped down to the bottom of the vial and allowed 10 seconds to climb. After 10 seconds, the number of flies above, and below, the five centimetre mark were quantified. For each vial, three tap down trials were performed at one minute intervals and the mean number of flies above, and below, the line were calculated. A performance index (PI), an estimate of the probability that a fly will respond to being startled, was calculated for each sex and genotype following Sofola et al., (2010). PI ranges from zero to one; if the PI is close to one it indicates that flies have a high response to a startle stimulus, while if the PI is close to zero it indicates that flies have a poor response to a startle stimulus (Sofola et al., 2010). PI was calculated as $PI = \frac{1}{2}(n_{TOTAL} + n_{TOP} - n_{BOTTOM} / n_{TOTAL})$ where $n_{TOTAL}$ is the
total number of flies, $n_{\text{TOP}}$ is the number of flies above the five centimetre line, and $n_{\text{BOTTOM}}$ is the number of flies below the five centimetre line (Sofola et al., 2010). Statistical analyses were performed on the calculated PIs.

### 2.5 Countercurrent Climbing Assay

![Figure 2](image)

**Figure 2:** Photo A) and schematic diagram B) showing the countercurrent apparatus as modified by Petersen et al., (2013). Flies enter the apparatus at vial 1, in the bottom vial set, as indicated by the grey circles, and vial 8, in the top vial set, is inverted over vial 1 (Petersen et al., 2013). The vials are tapped down and the flies are given one minute to climb (Petersen et al., 2013). Following one minute the top set of vials was shifted over, and the flies were tapped down, and allowed to climb for one minute (Petersen et al., 2013). This procedure was repeated a total of seven times, and vials that were not opposed by other vials were plugged to prevent flies from escaping (Petersen et al., 2013). Flying climbing ability was designated poor, moderate, or good dependent on the vial the flies were in at the end of the assay (vial designations marked in B); Petersen et al., 2013).

Climbing ability of adult flies was measured using a countercurrent climbing assay as modified by Petersen et al., (2013). While the negative geotaxis assay measures the immediate fly response to a startle stimulus, the countercurrent assay measures fly climbing endurance in response to startle stimuli. The countercurrent apparatus consists of two sets of four shell vials that are taped together, with the bottom set labelled 1 to 4,
and the top set labelled 5 to 8 (Petersen et al., 2013) (Figure 2). Adult male and adult female flies of each genotype were collected at 48 hours post eclosion, and aged for three days on cornmeal-yeast-agar medium in single sex, mixed genotype vials. Aged flies were loaded into vial 1, in the bottom vial set, and vial 8, in the top vial set, was inverted over vial 1 (Figure 2B), the vials are then tapped down and the flies given one minute to climb. Following one minute, the top set of vials was shifted over, and the flies were tapped down and allowed to climb for one minute (Figure 2B). This process was repeated a total of seven times, and vials that were not opposed by other vials were plugged to prevent flies from escaping (Figure 2B). Flies were classified by climbing ability based on the vials that they were located in at the end of the trial: poor = vial 1, moderate = vials 2-4, or good = vials 5-8 (Figure 2B). A partition coefficient (CF), an estimate of the probability that a fly will climb out of their starting vial at each trial, was calculated for each sex and genotype, where 0 < CF < 1 (Kamikouchi et al., 2009). A CF close to one indicates that flies tend to climb up, while a CF close to zero indicates that flies tend to not climb (Kamikouchi et al., 2009). The partition coefficient was calculated as $CF = \sum N_k(k - 1)/(n - 1)\sum N_k$, where $n$ = the number of climbing classes, $N_k$ is the number of flies in the $k^{th}$ climbing class, and the climbing classes are assigned the $k$ values poor ($k = 1$), moderate ($k = 2$), and good ($k = 3$; Kamikouchi et al., 2009).

Statistical analyses were performed on the calculated CFs.

### 2.6 Enzyme Activity Assays

For all enzyme assays, groups of adult male and adult female flies were collected 48 hours post eclosion, aged for three days, and frozen at -80°C in single sex and single genotype groups of four flies prior to assaying.
2.6.1 MEN, IDH, and G6PD Enzyme Activity Assays

Assays were performed as described by Bernard *et al.*, (2011), and Merritt *et al.*, (2006). Fly samples were weighed to the nearest 0.01mg with a microbalance (MX5 Balance, Mettler Toledo AG, Greifensee, Switzerland) and then homogenized in 100μL of homogenizing buffer per fly (0.1M TRIS-HCL, pH 7.4, 0.01M NADP+), centrifuged at 13 000 rpm for 12 minutes at 4°C, and the supernatant was collected. Enzyme reactions were performed in a standard 96-well microtiter plate. Within each well, the reaction used 10μL of the sample supernatant and 100μL of the assay solution (MEN - 0.1M TRIS-HCL, pH 7.4, 10mM malate, 5mM MnCl$_2$, 0.34mM NADP+; IDH - 0.1M TRIS-HCL, pH 8.6, 1.37mM isocitrate, 0.84mM MgSO$_4$, 0.1mM NADP+; G6PD - 20mM TRIS-HCL, pH 7.4, 3.5mM G6P, 0.2mM NADP+). NADP(H) produced was quantified as an increase in absorbance and was measured at 340nm with a spectrophotometer (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA, USA). For the MEN and IDH reactions, absorbance was measured at 25°C every 9 seconds for three minutes, while for the G6PD reaction absorbance was measured at 25°C every 9 seconds for five minutes. Three technical replicates were used to determine the enzyme activity of each biological sample, and statistical analyses were performed on the calculated activities for each biological sample.

2.6.2 cSOD Enzyme Activity Assay

cSOD activity was quantified using a commercial assay kit (Cayman Chemical Superoxide Dismutase Assay Kit, Ann Arbor, MI, Product Number: 706002). Prior to homogenization, fly samples were weighed to the nearest 0.01mg with a microbalance (MX5 Balance, Mettler Toledo AG, Greifensee, Switzerland). Samples were
homogenized in chilled 20mM Hepes buffer (pH 7.4, 1mM EDTA, 210mM mannitol, 70 mM sucrose) at a ratio of 100μL/fly, then diluted to a total ratio of 400μL/fly, homogenized flies were then centrifuged at 4°C for 10 minutes at 3 000 rpm, and the supernatant collected. Prior to assaying, the supernatant was diluted to a ratio of 1:4 supernatant with the assay kit sample buffer, as the SOD activity in the supernatant is too high to assay without being diluted. Assay kit reaction solutions were prepared following the manufacturer's protocol, and the enzyme reactions were performed in a standard 96-well microtiter plate. For each trial, two distinct reactions were performed to determine cSOD activity. The first reaction measures total SOD (Mitochondrial (manganese) SOD - MnSOD, and cSOD) activity, and within each well the reaction used 10μL of the diluted sample supernatant, 200μL of the radical detector, and 20μL of xanthine oxidase. The second reaction measures MnSOD activity, and within each well the reaction used 10μL of the diluted sample supernatant, 190μL of the radical detector, 20μL of xanthine oxidase, and 10μL of 5mM of sodium cyanide. All reactions were incubated at room temperature on the lab bench for 30 minutes prior to reading the absorbance. Endpoint absorbance was measured at 25°C and 450nm with a spectrophotometer (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA, USA). Each sample was assayed twice for each reaction (total SOD activity, and MnSOD activity) and SOD activities were calculated based on comparisons with the SOD standards. The cSOD activity was calculated as the difference between the mean total SOD activity and the mean MnSOD activity. Statistical analyses were performed on the calculated cSOD activities.
2.7 Total Protein Concentration

For each enzyme activity assay, total protein concentration was quantified with the bicinchoninic acid (BCA) assay using a commercial assay kit (Pierce, Thermo Scientific, Rockford, IL, Product Number 23225) with modifications as described by Rzezniczak and Merritt, (2012). In brief, the reactions were performed in a standard 96-well microtiter plate, and within each well the reaction used 10μL of sample supernatant and 100μL of reagent. The 96-well plates were then incubated at 37°C for 20 minutes, and allowed to cool prior to absorbance readings. Endpoint absorbance was measured at 562nm and at 25°C, and total protein concentrations were calculated based on comparisons with the standard curve generated with bovine serum albumen standards. The protein standard concentrations used were changed from those in the manufacturer's protocol to 1200μg/mL, 800μg/mL, 400μg/mL, and 100μg/mL (Rzezniczak and Merritt, 2012). Each sample was assayed three times and the mean protein concentration was determined. Preliminary analyses standardizing enzyme activity by fly weight and mean protein concentration were similar, so protein concentration was used to standardize enzyme activities as it accounts for size differences between individuals, and as well as possible homogenization differences between samples.

2.8 Statistical Analysis

The results of each assay were analyzed using analysis of variance (ANOVA), which allowed us to partition variation into phenotypic variation between sexes, 2nd chromosome genetic background, cSOD activity, as well as the sex-by-background interaction, the sex-by-cSOD activity interaction, the background-by-cSOD activity interaction, the sex-by-background-by-cSOD activity interaction, and the error variance
Post hoc comparisons were carried out using Tukey's HSD test. Separate analyses were performed on data from the T0 (0% and 50% cSOD activity) and T5 (30% and 80% cSOD activity) crosses as flies from these crosses can differ by more than one chromosome, due to the locations of the cSOD transgene and the cSOD gene on the 2nd and 3rd chromosomes, respectively.

To calculate the amount of variation attributable to each factor and interaction, across levels of cSOD activity, the effect size measure partial eta squared ($\eta^2_p$) was used. $\eta^2_p$ measures the proportion of variation that is attributable to a particular factor, while removing variance that is explained by other predictor variables, and it is calculated using the equation $\eta^2_p = SS_{\text{Factor}} / SS_{\text{Factor}} + SS_{\text{Error}}$ (Pierce, Block, and Aguinis, 2004). $\eta^2_p$ was calculated for each factor, and interaction, for each analysis using the Sum of Squares (SS) values produced in the ANOVA output.
Chapter 3 Results

The primary purpose of this study was to examine how variable the effects of changes in cSOD activity were on phenotypes across genetic backgrounds and between sexes. The use of multiple DGRPi genetic backgrounds, as well as male and female flies, allowed me to partition the amount of variation attributable to genetic background, sex, cSOD activity, and their interactions, for each phenotype. The effect size partial eta squared ($\eta^2_p$) was used to determine the proportion of variation attributable to each factor, and $\eta^2_p$ values and statistics are summarized in Figure 8. All $\eta^2_p$ values and their associated statistics can be found in the Appendices, Tables A1 to A7. Specific line effects, maximum and minimum pooled cSOD activity measures, and maximum and minimum line measures, for each phenotype are illustrated in Table A8, Table A9, and Table A10 respectively.

3.1 cSOD activity is primarily influenced by the transgenic and third chromosome genotypes, but it is not affected by DGRPi genetic background

Using two separate three-step crossing schemes (Figure 1) flies across a series of cSOD activity levels were generated; one scheme created 0% and 50% WT cSOD activity flies and the second created 30% and 80% WT cSOD activity flies. The four levels of cSOD activity were generated using genetic constructs that differed at the 2nd and 3rd chromosomes, with large scale differences in cSOD activity resulting from the presence or absence of a knockout mutation on the 3rd chromosome, and the presence or absence of a cSOD transgene on the 2nd chromosome. Comparisons were made within each range of cSOD activity (0% and 50% WT cSOD activity, or 30% and 80% WT
cSOD activity), not across the four levels of activity, as those genotypes differed by more than one chromosome.

The cSOD activity for each genotype was estimated according to the transgenic and 3rd chromosome genotypes: T0/ DGRPi; cSod\textsuperscript{n108},red/ cSod\textsuperscript{n108},red - 0% cSOD activity; T0/ DGRPi; cSod\textsuperscript{n108},red/ VT83 - 50% cSOD activity; T5/ DGRPi; cSod\textsuperscript{n108},red/ cSod\textsuperscript{n108},red - 30% cSOD activity; and T5/ DGRPi; cSod\textsuperscript{n108},red/ VT83 - 80% cSOD activity. cSOD activity can, however, be influenced by 2nd chromosome genetic background (Graf and Ayala, 1986), so the actual cSOD activity may vary across the DGRPi genetic backgrounds. To quantify this variation, I assayed the amount of cSOD activity across eight DGRPi genetic backgrounds, within each transgenic and 3rd chromosome genotype. There was no significant variation across the backgrounds; large-scale differences in cSOD activity are a function of the transgenic and 3rd chromosome genotype with only small, statistically insignificant variation across the DGRPi genetic backgrounds (Figure A1). For the 0% cSOD activity flies and the 50% cSOD activity flies, variation in cSOD activity was due only to the differences in 3rd chromosome genotype (Figure 3A, Table A9), and cSOD activity was much lower, essentially absent to the level of detection of the assay, in the 0% cSOD activity flies, consistent with the genotype estimate. Interestingly, while cSOD activity in the 30% cSOD activity flies and 80% cSOD activity flies was primarily a function of the transgenic and 3rd chromosome genotype, there was also a significant sex effect (Figure 3B, Table A9); cSOD activity is lower in males than females in the 30% and 80% cSOD activity flies. Activities are corrected for protein content, so this difference does not simply reflect the larger size of females; males have proportionately, per unit protein, lower cSOD activity than females.
Given the results above showing no significant differences in cSOD activity across the *DGRPi* 2nd chromosome genetic backgrounds, the *T0/DGRPi; cSod<sup>n108</sup>,red/ cSod<sup>n108</sup>,red*, *T0/DGRPi; cSod<sup>n108</sup>,red/VT83*, *T5/DGRPi; cSod<sup>n108</sup>,red/ cSod<sup>n108</sup>,red*, and *T5/DGRPi; cSod<sup>n108</sup>,red/VT83* flies will be defined according to their genotype derived activities - 0%, 50%, 30%, and 80% cSOD activity respectively - as the measured activities of these genotypes are approximately equivalent to their estimated activities.

**Figure 3:** Mean ± SEM of the measured cSOD activities, standardized by protein concentration, (units/μg protein) for adult male and adult female flies pooled across the eight *DGRPi* genetic backgrounds within each genotype. A) cSOD Activity - F<sub>1,127</sub> = 84.1, p < 0.0001, η<sup>p</sup><sup>2</sup> = 0.40 B) cSOD Activity - F<sub>1,124</sub> = 158, p
3.2 Mean phenotypes were obtained by pooling responses across DGRPi genetic backgrounds within each third chromosome genotype

To test for phenotypic variation driven by large-scale differences in cSOD activity, phenotype data was pooled across DGRPi genetic backgrounds within each cSOD activity genotype. The pooled data was then analyzed for differences across cSOD activities and between the sexes.

3.2.1 Expression of distal phenotypes is proportional to the amount of cSOD activity

Across the four distal phenotypes examined (longevity, viability, negative geotaxis, and climbing ability) scores were lowest in 0% cSOD activity flies, and were higher in high cSOD activity flies, with scores being approximately equivalent between the 50% and 80% cSOD activity groups. The effect of sex on phenotypic expression depended on the phenotype examined and the amount of cSOD activity present.

Viability was only affected by cSOD activity, across both cSOD activity comparisons (Figure 4, Table A9). Viability was lower in low cSOD activity flies, but did not vary significantly between males and females. Similar to viability, there was a significant cSOD effect on climbing ability across both comparisons (Figure 5, Table A9), however, in the high cSOD activity comparison, there was also a significant sex effect on countercurrent ability (Figure 5B). Across both cSOD comparisons there was a trend with males having better countercurrent ability than females. Conversely, there was a significant sex-by-cSOD activity interaction effect on longevity in the low cSOD activity comparison (Figure 6A, Table A9), where the level of sexual dimorphism varied
at the different cSOD activities. In the high cSOD activity comparison there were significant sex and cSOD activity effects on longevity, but the interaction effect was not significant (Figure 6B, Table A9). Across both cSOD activity comparisons females lived longer than males. Similar to longevity, there were significant sex and cSOD activity effects on negative geotaxis, but the interaction effect was not significant in either comparison (Figure 7, Table A9). Across both cSOD comparisons, males had better negative geotaxic ability than did females.

cSOD activity was a determining factor for all of the distal phenotypes observed, however, the relative importance of genetic background and sex varied. In viability, longevity, negative geotaxis, and climbing ability, the effect size of cSOD activity was smaller in the high cSOD comparison (Figure 8); i.e. small differences at low levels of cSOD had the greatest effect on phenotype. In the high cSOD comparison, cSOD was still the most important factor affecting viability and climbing ability. In contrast, sex had the largest affect on longevity in the high cSOD comparison, while the sex-by-genetic background interaction had the largest affect on negative geotaxis (Figure 8). The effect size of sex either remained approximately constant, or was higher in the high cSOD comparison, although its effect was not always significant (Figure 8). Interestingly, one sex did not perform consistently “better” than the other in the distal phenotypes; males showed “better” performance in some distal phenotypes, and females in others.
Figure 4: Mean ± SEM of viability (frequency) for adult flies pooled across the eight DGRPi genetic backgrounds within each third chromosome genotype. A) cSOD Activity - $F_{1,143} = 107$, $p < 0.0001$, $\eta^2_p = 0.43$ B) cSOD Activity - $F_{1,148} = 11.2$, $p = 0.001$, $\eta^2_p = 0.070$. Brackets enclose significantly different groups.
Figure 5: Mean ± SEM of the climbing partition coefficient (CF) for adult male and female flies pooled across the eight DGRPi genetic backgrounds within each third chromosome genotype. A) cSOD Activity - $F_{1,269} = 501$, $p < 0.0001$, $\eta^2_p = 0.65$ B) cSOD Activity - $F_{1,175} = 61.6$, $p < 0.0001$, $\eta^2_p = 0.26$, Sex - $F_{1,175} = 20.8$, $p < 0.0001$, $\eta^2_p = 0.11$. Brackets enclose significantly different groups.
Figure 6: Mean ± SEM longevity (days) for adult male and female flies pooled across the eight DGRP\textit{i} genetic backgrounds within each third chromosome genotype. A) Sex-by-cSOD Activity - $F_{1,2227} = 35.2$, $p < 0.0001$, $\eta^2_p = 0.016$ B) cSOD Activity - $F_{1,1673} = 64.9$, $p < 0.0001$, $\eta^2_p = 0.038$, Sex - $F_{1,1673} = 66.1$, $p < 0.0001$, $\eta^2_p = 0.038$. Brackets enclose significantly different groups.
Figure 7: Mean ± SEM of the negative geotaxis performance index (PI) for adult male and female flies pooled across the eight DGRPi genetic backgrounds within each genotype. A) cSOD Activity - $F_{1,156} = 170, p < 0.0001, \eta_p^2 = 0.52$. Sex - $F_{1,156} = 4.83, p = 0.0294, \eta_p^2 = 0.030$ B) cSOD Activity - $F_{1,100} = 7.33, p = 0.008, \eta_p^2 = 0.068$. Sex - $F_{1,100} = 8.80, p = 0.0038, \eta_p^2 = 0.081$. Brackets enclose significantly different groups.
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| 30% and 80%  | Sex          | 0.038     | NS        | 0.081             | 0.11     | 0.12    | 0.016   | 0.36     |              |              |
|              | Bkgd         | 0.023     | NS        | 0.088             | NS       | 0.088   | 0.095   |          |              |              |
|              | cSOD         | 0.037     | 0.070     | 0.068             | 0.26     | 0.014   | NS      |          |              |              |
|              | Sex X Bkgd   | 0.012     | NS        | 0.17              | NS       | 0.074   | 0.055   | NS       |              |              |
|              | Bkgd X cSOD  | 0.025     | NS        | 0.17              | NS       | 0.073   | NS      | 0.062    |              |              |
|              | Sex X cSOD   | NS        | NS        | 0.036             | NS       | NS      |          |          |              |              |
|              | Sex X Bkgd X cSOD | NS | NS        | NS              | NS       | NS      |          |          |              |              |

**Figure 8:** Partial eta squared ($\eta_p^2$) for longevity, viability, negative geotaxis, countercurrent (climbing), malic enzyme (MEN), isocitrate dehydrogenase (IDH), and glucose-6-phosphate dehydrogenase (G6PD) calculated for each factor across cSOD activity conditions. Larger values represent larger effects (Orange), and smaller values represent smaller effects (Purple). NS, non-significant interactions.

### 3.2.2 The magnitude of NADP(H) enzyme activity is modified by sex and cSOD activity

Enzyme activities are themselves proximal phenotypes and in the three enzymes surveyed (MEN, IDH, and G6PD) activity was strongly influenced by sex and cSOD activity (Figure 8). In all three of these NADP⁺ reducing enzymes, there are significant sex-by-cSOD activity interaction effects in the 0% and 50% cSOD comparison (MEN - Figure 9A, IDH - Figure 10A, G6PD - Figure 11A; Table A9); the magnitude of difference in enzyme activity between males and females varied across cSOD activities. In the 30% and 80% cSOD comparison, the effect of cSOD activity on NADP(H) enzyme activities tended to be small, while the effect size of sex tended to be large (MEN - Figure 9B, IDH - Figure 10B, G6PD - Figure 11B; Table A9). In IDH and G6PD, the effect size of cSOD activity was smaller in the high cSOD comparison, while in MEN, the effect size of cSOD activity was larger in the high cSOD comparison (Figure 8). NADP(H)
enzyme activities in males were lowest in the 0% cSOD activity flies and higher in the high cSOD activity flies, with NADP(H) enzyme activities being approximately equivalent between the 50% and 80% cSOD activity groups. Conversely, female NADP(H) enzyme activities tended to be higher in the 0% cSOD activity flies, although there was less variation in NADP(H) enzyme activities in females, across cSOD activities, compared to males. As long as some cSOD activity was present, male NADP(H) enzyme activity tended to be greater than that in females, and there was also a greater change in NADP(H) enzyme activity across cSOD activities in males compared to females. Overall, the changes in effect size for sex and cSOD activity were much larger in IDH and G6PD activity, than in MEN activity.
Figure 9: Mean ± SEM of MEN activity standardized by protein concentration (units/μg protein) for adult male and female flies pooled across the eight DGRPi genetic backgrounds within each third chromosome genotype. A) Sex-by-cSOD Activity - $F_{1,265} = 31.8, p < 0.0001, \eta^2 = 0.11$ B) Sex-by-cSOD Activity - $F_{1,280} = 10.5, p = 0.0013, \eta^2 = 0.036$. Brackets enclose significantly different groups.
**Figure 10:** Mean ± SEM of the IDH activity standardized by protein concentration (units/μg protein) for adult male and female flies pooled across the eight *DGRP* genetic backgrounds within each genotype. 

A) Sex-by-cSOD Activity - $F_{1,265} = 25.5$, $p < 0.0001$, $\eta^2_p = 0.088$  

B) Sex - $F_{1,280} = 4.58$, $p = 0.0333$, $\eta^2_p = 0.016$. 

Brackets enclose significantly different groups.
Figure 11: Mean ± SEM of G6PD activity standardized by protein concentration (units/μg protein) for adult male and female flies pooled across the eight DGRPi genetic backgrounds within each third chromosome genotype. A) Sex-by-cSOD Activity - $F_{1,265} = 19.6$, $p < 0.0001$, $\eta^2_p = 0.069$ B) Sex - $F_{1,280} = 155$, $p < 0.0001$, $\eta^2_p = 0.36$. Brackets enclose significantly different groups.
3.3 Phenotypic response to genetic background is modified by phenotype and cSOD activity

In addition to quantifying the average phenotypic effects across the eight *DGRPi* genetic backgrounds (above), I also quantified genetic background effects using analysis of variance (ANOVA) to partition variation between cSOD activity, sex, genetic background, and the interactions between each factor. The effect size $\eta^2_p$ was used to determine the changes in the proportion of variation attributable to each factor within each phenotype as cSOD activity changes.

3.3.1 Longevity is sensitive to cSOD activity, sex, and genetic background, but the magnitude of the effect varies most significantly with cSOD activity

Longevity varied in the magnitude of sexual dimorphism across genetic backgrounds in the low cSOD comparison. There was a significant sex-by-genetic background-by-cSOD activity interaction effect on longevity in the 0% and 50% cSOD comparison (Figure 12A). Longevity in males and females was higher at 50% cSOD activity than at 0% cSOD activity across genetic backgrounds, and there was more variation in longevity, in males and females, at 50% cSOD activity than at 0% cSOD activity (Table A10). The sex-by-genetic background-by-cSOD activity interaction effect was not significant in the high cSOD comparison, so longevity was primarily affected by this interaction in the low cSOD comparison (Table A8).

The effect of genetic background on longevity varied across cSOD comparisons, with larger effects in the high cSOD comparison (Figure 8). There were significant sex-by-genetic background interaction and genetic background-by-cSOD activity interaction effects on longevity across the cSOD comparisons, though the effect size of the former
was larger in the low cSOD comparison, while the effect size of the latter was larger in the high cSOD comparison (Figure 12B). The differences in effect size for the interactions indicates that the magnitude of sexual dimorphism in longevity varies across cSOD activity comparisons and genetic background (Table A8). Longevity in females was higher than that in males across the spectrum of cSOD activities and, at 50% and 80% cSOD activity the variation in longevity in males and females was approximately equivalent (Table A10).

**Figure 12:** Ratio of the mean ± SEM of low cSOD: high cSOD activity longevities for adult male and female flies across the eight *DGRPi* genetic backgrounds. Values closer to 1 indicate more similar longevities at the levels of cSOD activity being compared, and confidence intervals are at 95% and represent the ratio of the low cSOD activity and high cSOD activity SEMs. A) Sex-by-Genetic
Background-by-cSOD Activity - $F_{7,2227} = 4.38, p < 0.0001, \eta_p^2 = 0.014$  
B) Sex-by-Genetic Background - $F_{7,1673} = 2.91, p = 0.0049, \eta_p^2 = 0.012$. Genetic Background-by-cSOD Activity - $F_{7,1673} = 6.03, p < 0.0001, \eta_p^2 = 0.025$.

3.3.2 Viability is sensitive to changes in cSOD activity, but is not affected by sex or genetic background

Viability was not significantly affected by genetic background in either sex or at any cSOD activity (Figure 13). Viability was only significantly influenced by cSOD activity (Figure 8); sex and genetic background had no significant effect (background specific differences can be seen in Tables A8, A10).
Figure 13: Ratio of the mean ± SEM of the ratio of low cSOD: high cSOD activity viability (frequency) for adult male and female flies across the eight DGRPi genetic backgrounds. Values closer to 1 indicate more similar PI values at the levels of cSOD activity being compared, and confidence intervals (CI) are at 95% and represent the ratio of the low cSOD activity and high cSOD activity SEMs. A) cSOD Activity - F_{1,143} = 107, p < 0.0001, \eta^2_p = 0.43 B) cSOD Activity - F_{1,148} = 11.2, p = 0.001, \eta^2_p = 0.070.

3.3.3 Sensitivity of negative geotaxis to cSOD activity and genetic background was highest in the low cSOD activity comparison

Across cSOD comparisons, negative geotaxis differed between males and females, and the magnitude of this sexual dimorphism varied across DGRPi genetic backgrounds. There was a significant sex-by-genetic background-by-cSOD activity interaction effect on negative geotaxis in the 0% and 50% cSOD comparison (Figure 14A). Negative geotaxis was higher at 50% cSOD activity than at 0% cSOD activity across DGRPi genetic backgrounds in both sexes, but there was more variation in negative geotaxis at 0% cSOD activity than at 50% cSOD activity (Table A10). The sex-by-genetic background-by-cSOD activity interaction was not significant in the high cSOD comparison, so negative geotaxis was primarily influenced by this interaction in the low cSOD comparison (Table A8).

Negative geotaxis varied in the magnitude of sexual dimorphism across genetic backgrounds in the high cSOD comparison. There was a significant sex-by-genetic background interaction effect on negative geotaxis across the spectrum of cSOD activities (Figure 14B), and the effect size was larger in the high cSOD comparison (Figure 8, Table A8). Sex had a larger effect on negative geotaxis in the high cSOD activity comparison, though the magnitude of sexual dimorphism varied across genetic backgrounds (Figure 8). Negative geotaxis was higher in male flies across cSOD activities, however, there was more variation in negative geotaxic ability in female flies in the 30% and 80% cSOD activity comparison (Table A10).
Figure 14: Ratio of the mean ± SEM of the ratio of low cSOD: high cSOD activity negative geotaxis performance index (PI) values for adult male and female flies across the eight DGRP<sub>i</sub> genetic backgrounds. Values closer to 1 indicate more similar PI values at the levels of cSOD activity being compared, and confidence intervals (CI) are at 95% and represent the ratio of the low cSOD activity and high cSOD activity SEMs. A) Sex-by-Genetic Background-by-cSOD Activity - F<sub>1,156</sub> = 4.95, p < 0.0001, η<sup>p2</sup> = 0.18 B) cSOD Activity - F<sub>1,100</sub> = 7.33, p = 0.008, η<sup>p2</sup> = 0.068, Sex-by-Genetic Background - F<sub>1,100</sub> = 3.02, p = 0.0064, η<sup>p2</sup> = 0.17.

3.3.4 Countercurrent locomotion is sensitive to cSOD activity across comparisons, and to sex in the high cSOD activity comparison

Low cSOD activity flies (i.e. 0% cSOD activity) are poor climbers and to improve detection of possible background effects in these flies I reduced the distance that the flies had to climb in the negative geotaxis assay. This change may, however, have reduced the
sensitivity of the assay to detect differences in negative geotaxic ability in the high performance flies (i.e. possessing high cSOD activity). The reduced scale of the assay may have resulted in apparently equivalent negative geotaxis in high cSOD activity flies across genetic backgrounds, potentially negatively driving down the discernible background effects. Therefore, a second locomotor assay was performed to attempt to further resolve background effects at high cSOD activities.

Climbing ability responds differently in males and females in the high cSOD comparison, but there are no significant genetic background effects across cSOD comparisons (Figure 15). Climbing ability was significantly influenced by cSOD activity, though the effect of cSOD activity was smaller in the high cSOD comparison (Figure 8). Conversely, the effect of sex on climbing ability was larger than that of cSOD activity in the high cSOD comparison (Figure 8). Genetic background did not have a significant effect on climbing ability in either cSOD comparison (background specific differences can be seen in Table A8 and A10).
Figure 15: Ratio of the mean ± SEM of the ratio of low cSOD: high cSOD activity climbing ability partition coefficient (CF) for adult male and female flies across the eight DGRPi genetic backgrounds. Values closer to 1 indicate more similar PI values at the levels of cSOD activity being compared, and confidence intervals (CI) are at 95% and represent the ratio of the low cSOD activity and high cSOD activity SEMs. A) cSOD Activity - $F_{1,269} = 501$, $p < 0.0001$, $\eta_p^2 = 0.65$ B) Sex - $F_{1,175} = 20.8$, $p < 0.0001$, $\eta_p^2 = 0.11$, cSOD Activity - $F_{1,175} = 61.6$, $p < 0.0001$, $\eta_p^2 = 0.26$.

3.3.5 Sensitivity of Malic enzyme activity to genetic background is higher in the high cSOD activity comparison

The response of MEN activity to cSOD activity differs between males and females across cSOD comparisons. There was a significant sex-by-cSOD activity
interaction effect on MEN activity in the 0% and 50% cSOD comparison, and in 30% and 80% cSOD comparison (Figure 16). MEN activity in females is higher in 0% cSOD activity flies than 50% cSOD activity flies, while in males the opposite trend is observed (Table A10). Furthermore, there is a trend for higher MEN activity in males compared to females in the 30%, 50%, and 80% cSOD activity flies (Table A10). There were significant sex-by-cSOD activity interaction effects on MEN activity across cSOD comparisons, though the interaction effect was smaller in the high cSOD comparison (Figure 8).

MEN activity responds differently to genetic background in males and females in the high cSOD comparison. There were significant sex-by-genetic background interaction and genetic background-by-cSOD activity interaction effects on MEN activity in the 30% and 80% cSOD comparison (Figure 16B). MEN activity was higher in male 30% and 80% cSOD activity flies across genetic backgrounds, though MEN activity in males is higher at 30% cSOD activity than at 80% cSOD activity, while the opposite trend is observed in females (Tables A8, A10). Further, there was a greater amount of variation in the magnitude of MEN activity observed in males between 30% and 80% cSOD activity flies, than was observed in females. The effect sizes for the genetic background interactions were not significant in the low cSOD comparison, indicating that more variation in MEN activity is attributable to genetic background in the high cSOD comparison (Figure 8).
**3.3.6 Sensitivity of Isocitrate dehydrogenase activity to genetic background is higher in the high cSOD activity comparison**

IDH activity is not affected by genetic background in the low cSOD comparison, though male and female response does differ. There was a significant sex-by-cSOD activity interaction effect on IDH activity in the 0% and 50% cSOD comparison (Figure 16).
IDH activity in males and females was higher in 0% cSOD activity flies than in 50% cSOD activity flies, though IDH activity was lower in flies with 0% cSOD activity, compared to those with 50% cSOD activity (Figure A10). However, there was a greater amount of variation in the magnitude of IDH activity observed in males in the 0% and 50% cSOD activity comparison, than in females (Table A9). The sex-by-cSOD activity interaction was not significant in the high cSOD comparison, indicating that variation in IDH activity is more attributable to this interaction in the low cSOD comparison (Figure 8).

Sexual dimorphism in IDH activity varied across genetic backgrounds. There was a significant sex-by-genetic background interaction effect on IDH activity in the 30% and 80% cSOD comparison (Figure 17B). IDH activity was higher in females than in males across genetic backgrounds, though there was more variation in the magnitude of IDH activity in males than in females across genetic backgrounds (Table S8, S9). There was no significant effect of cSOD activity on IDH activity in the 30% and 80% cSOD comparison in either males or females (Table A8, A10). The sex-by-genetic background interaction was not significant in the low cSOD comparison, indicating that more variation in IDH activity is attributable to genetic background in the high cSOD comparison (Figure 8).
Figure 17: Ratio of the mean ± SEM of the ratio of low cSOD: high cSOD activity IDH activity standardized by protein concentration (units/μg protein) for adult male and female flies across the eight DGRPi genetic backgrounds. Values closer to 1 indicate more similar PI values at the levels of cSOD activity being compared, and confidence intervals (CI) are at 95% and represent the ratio of the low cSOD activity and high cSOD activity SEMs. A) Sex-by-cSOD - $F_{1,265} = 25.5$, $p < 0.0001$, $\eta^2_p = 0.088$ B) Sex-by-Genetic Background - $F_{7,280} = 2.31$, $p = 0.0263$, $\eta^2_p = 0.055$.

3.3.7 Sensitivity of Glucose-6-phosphate dehydrogenase activity to genetic background is smaller in the high cSOD activity comparison, while sensitivity to sex is higher

The magnitude of sexual dimorphism in G6PD activity varies across genetic background in the low cSOD comparison. There was a significant sex-by-genetic
background-by-cSOD activity interaction effect on G6PD activity in the 0% and 50% cSOD comparison (Figure 18A). G6PD activity in male and female flies was higher at 50% cSOD activity than at 0% cSOD activity across genetic backgrounds; furthermore, male and female G6PD activity was approximately equivalent in 0% cSOD activity flies, while at 50% cSOD activity G6PD activity was greater in male flies (Table A8, A10). A greater amount of variation in the magnitude of G6PD activity was observed between 0% and 50% cSOD activity males, compared to females (Table A10). The sex-by-genetic background-by-cSOD activity interaction effect was not significant in the high cSOD comparison, indicating that more variation in G6PD activity is attributable to this interaction in the low cSOD comparison (Figure 8).

The magnitude of variation in G6PD activity in the high cSOD comparison varied across DGRP$i$ genetic background, in males and females. Sex, and the genetic background-by-cSOD activity interaction significantly affected G6PD activity in the 30% and 80% cSOD comparison (Figure 18B). G6PD activity was higher in male 30% and 80% cSOD activity flies than in female flies, and there was more variation in G6PD activity across genetic backgrounds in male than in female flies (Tables A8, A10). The genetic background-by-cSOD activity interaction effect was only significant in the high cSOD comparison, however, sex had the largest effect of any factor, in the high cSOD comparison, indicating that most variation in G6PD activity in the high cSOD comparison is attributable to sex (Figure 8).
Figure 18: Ratio of the mean ± SEM of the ratio of low cSOD: high cSOD activity G6PD activity standardized by protein concentration (units/μg protein) values for adult male and female flies across the eight DGRP$i$ genetic backgrounds. Values closer to 1 indicate more similar PI values at the levels of cSOD activity being compared, and confidence intervals (CI) are at 95% and represent the ratio of the low cSOD activity and high cSOD activity SEMs. A) Sex-by-Genetic Background-by-cSOD Activity - $F_{7,265} = 2.57$, $p = 0.0142$, $\eta^2_p = 0.063$ B) Sex - $F_{1,280} = 155$, $p < 0.0001$, $\eta^2_p = 0.37$, Genetic Background-by-cSOD Activity - $F_{7,280} = 2.65$, $p = 0.0114$, $\eta^2_p = 0.062$. 
Chapter 4 Discussion

4.1 Magnitude of phenotypic expression varies with the influence of cSOD activity, genetic background, and sex

In *D. melanogaster*, the absence of cSOD activity leads to a characteristic suite of phenotypes, known as the cSOD-null syndrome (Parkes *et al.*, 1998b). Previous analyses demonstrate that the cSOD-null syndrome encompasses a broad array of characteristics, which include changes in a range of both proximal (phenotypes that respond directly to changes in the gene of interest *i.e.* enzyme activity) and distal (phenotypes that are a function of downstream genetic interactions *i.e.* longevity) phenotypes. While complete loss of cSOD results in this broad suite of effects, 50% of WT levels of cSOD activity has proven sufficient to fully ameliorate these phenotypes. (Bernard *et al.*, 2011, Jones and Grotewiel, 2011, Knee *et al.*, 2013, Parkes *et al.*, 1998b, Phillips *et al.*, 1989, Sun and Tower, 1999, and Woodruff *et al.*, 2004). Evidence suggests that the expression of proximal and distal phenotypes is influenced by their genetic context (Jordan *et al.*, 2007, Laurie-Ahlberg *et al.*, 1982, Pasyukova *et al.*, 2000, and Yamamoto *et al.*, 2009), but many of the defined phenotypic changes associated with cSOD activity have only been documented within single isogenic backgrounds (Bernard *et al.*, 2011, Jordan *et al.*, 2007, Knee *et al.*, 2013, Laurie-Ahlberg *et al.*, 1982, Parkes *et al.*, 1998b, and Pasyukova *et al.*, 2000). In the work presented here, I document the responses of seven phenotypes to two ranges of cSOD activity, sex, and eight genetic backgrounds, to define the relative influence of each factor on phenotypic variance.

The phenotypes observed were most significantly influenced by the amount of cSOD activity the flies possessed. Phenotypic expression tended to be most greatly modified by a complete lack of cSOD activity and was less sensitive to moderate changes
in cSOD, as some cSOD activity was still present, although these changes did have significant effects on specific phenotypes. In flies with low cSOD (i.e. 30% cSOD) activity, performance in assays of distal phenotypes (longevity, viability, negative geotaxis, climbing ability) was impaired relative to that in flies with 50% or 80% cSOD activity. However, quantitative assessments of the proximal phenotypes (MEN, IDH, and G6PD activity) in flies with low cSOD (i.e. 30% cSOD) activity may be approximately equivalent to, or lower than, that in flies with 50% or 80% cSOD activity, although this effect was dependent on the sex of the fly. This modification in phenotypic response to different cSOD activities indicates that the threshold of cSOD activity to generate WT phenotypes varies between proximal and distal phenotypes.

The absence of cSOD activity results in pervasive changes in phenotypic expression, relative to WT phenotypes, and these changes are only enhanced or suppressed by sex or genetic background, although the degree does vary between phenotypes. When cSOD activity varies between the 0% and 50% cSOD, and 30% and 80% cSOD, activity comparisons phenotypes also varied, with higher levels of cSOD activity resulting in phenotypes closer to WT levels in each case. Phenotypes were more susceptible to enhancement or suppression by sex and genetic background as long as some cSOD activity was present, compared to phenotypes in the absence of cSOD activity.

4.2 Phenotypes

Complex phenotypes are influenced by multiple genes. Study of these phenotypes using isogenic backgrounds may eliminate potential confounding epistatic effects, but likely oversimplifies the actual genetic interactions underlying phenotypes (Spencer et
The cSOD-null condition influences a series of distal, as well as proximal, phenotypes in *D. melanogaster*, and many of these phenotypes are known to be influenced by genes on the 2nd and 3rd chromosomes (Jordan *et al.*, 2007, Laurie-Ahlberg *et al.*, 1982, Leips and Mackay, 2000, and Spencer *et al.*, 2003). Considering that distal phenotypes result from the downstream interactions of multiple genes, and that the combined physiological effects of even subtle changes in multiple proximal phenotypes might be manifested as dramatic changes in any particular distal phenotype, I expected that differences in cSOD activity, sex, and genetic background would have larger effects on distal phenotypes than on proximal phenotypes. Though, the existence of redundancies in phenotypic mechanisms may conversely serve to buffer any dramatic responses to changes in genetic background. Interestingly, cSOD activity, sex, and genetic background greatly influenced the expression of both the distal and proximal phenotypes, with the effect sizes for sex, cSOD activity, and genetic background generally following similar patterns of change across cSOD activity ranges. Overall, the distal phenotypes were more sensitive to smaller differences in cSOD activity than the proximal phenotypes; the threshold of cSOD activity at which distal phenotypes were restored to near WT levels was higher than that for proximal phenotypes.

### 4.2.1 Longevity and Genotypic Viability

Longevity and viability are two components of life history and both are influenced by cSOD activity. Consistent with earlier work focused on the complete lack of cSOD activity (Parkes *et al.*, 1998b, and Sun and Tower, 1999), longevity and genotypic viability were both significantly reduced in flies with lower amounts of cSOD activity, in a dose-dependent manner (Figure 19A and B). Previous research has
demonstrated that longevity is significantly influenced by sex (Pasyukova et al., 2000, and Spencer et al., 2003) and second chromosome quantitative trait loci (QTL; Pasyukova et al., 2000) and results here indicate that sex and 2nd chromosome modifiers interact with cSOD levels to influence these characteristics (Figure 19A, 19C). Strikingly, there were no significant differences in longevity across genetic background or sex in the 0% cSOD activity flies, though there was an overall trend where females lived longer than males across cSOD activities (Figure 19C), similar to other studies in Drosophila (Bonduriansky et al., 2008, and Spencer et al., 2003), possibly reflective of differing reproductive strategies between the sexes (i.e. increased hormone production in males stimulates the expression of secondary sex traits improving reproductive fitness, but reducing longevity; Bonduriansky et al., 2008). The reduction in longevity observed in the absence of cSOD activity was not significantly suppressed or enhanced by sex or genetic background. In contrast, when some cSOD activity is present (30% or higher in this study), sex and genetic background both significantly affect the magnitude of longevity. Like longevity, viability was sensitive to levels of cSOD activity and higher cSOD activity resulted in higher viability. In contrast to longevity, across the range of cSOD activities viability was not sensitive to sex or genetic background; only cSOD activity had a significant effect on this phenotype (Figure 19B and D). Viability, but not longevity, is robust to sex and to changes in second chromosome dominant modifiers.

Some life history traits have been observed to be robust to differences in genetic background (Fry, 2008), although genetic variation in basic life history traits is present (Fry et al., 1998). Longevity and viability are both life history traits, but in this study, they differed in sensitivity to genetic background perturbation. Longevity was heavily
influenced by sex and genetic background (Figure 8), whereas viability was more robust
to these variables, suggesting that longevity is more sensitive to small changes in genetic
variation. If the underlying mechanism of viability contains independent, or redundant,
components, then viability would be more robust to genetic background perturbations
(Stearns, et al., 1995), conceivably contributing to the low variation observed in viability,
compared to longevity, which is known to be sensitive to perturbation (Spencer et al.,
2003). Surprisingly, longevity and viability in the 80% cSOD activity male and female
flies, is lower than that in the 50% cSOD activity flies (Figure 19A,B), possibly driven by
the presence of the transgene in the 80% cSOD activity flies, as genetic background can
influence the effect of different cSOD activities on some phenotypes (Seto et al., 1990).
Phenotypes are recovered to WT levels at 50% cSOD activity (Phillips et al., 1989,
Parkes et al., 1998b, and Bernard et al., 2011), so the presence of the transgene in a fly
(in combination with the WT cSOD⁺ allele producing 50% cSOD activity) could effect
phenotype expression if there is an interaction between the WT cSOD allele and the
transgene. Although the T5 transgene is under the control of the native cSOD promoter
(Parkes et al., 1998b, and Seto et al., 1990), if transgene expression is driven
indiscriminately in a particular tissue, the redox state of that tissue may be perturbed,
compared to flies with just the WT cSOD⁺ allele, ultimately inhibiting tissue function.
Flies with the combination of the cSOD transgene and the native cSOD⁺ allele (80%
cSOD activity), may then have reduced longevity and viability, compared to flies
possessing just the native cSOD⁺ allele (50% cSOD activity), based on the presence of
the transgene. Response to sex, genetic background, and cSOD activity followed similar
pattern across both traits, though sensitivity to each factor varied (the change in sex effect
was smallest in both viability and longevity and was ~1.5x different; the difference in
background effect in the two traits was ~2-3x different; while the change in the cSOD
effect size was largest in the two traits at ~6-21x different from 0%-50% cSOD to 30%-
80% cSOD activity).

Viability and longevity differ in sensitivity to sex and genetic background,
suggesting that related traits may be differentially maintained, i.e. more variation in one
trait, may more negatively impact overall fitness. High sensitivity of viability to sex
would cause sex imbalances in populations, which could decrease overall population
reproductive fitness. Sexual dimorphism in longevity likely reflects differences in the
reproductive strategies between the two sexes (Bonduriansky et al., 2008). Variation in
the level of redundancy, or the degree of allelic polymorphism, underlying the
mechanisms of the two traits, likely underlies the differences in sensitivity of viability
and longevity to genetic background.
Figure 19: Summary of the mean ± SEM data pooled across backgrounds for longevity A) and viability B)
across the four levels of cSOD activity surveyed showing the trend of phenotypic response to changes in cSOD activity. Summary of the mean ± SEM background data for longevity C) and viability D) across the four levels of cSOD activity surveyed showing the trend of phenotypic response to changes in cSOD activity.

4.2.2 Locomotion

Locomotion, measured using two complementary assays, was sensitive to variation in cSOD activity, and sex, but only one type of locomotion was sensitive to genetic background. Flies lacking cSOD activity had reduced performance in both negative geotaxis (Figure 20A and B), which evaluates the immediate startle response against gravity, and countercurrent locomotion, which evaluates the endurance of the adult climbing response following startle stimuli. Locomotor performance is known to be sensitive to naturally segregating genetic variation, including sexual dimorphism and second chromosome genetic background (Jordan et al., 2007 and Yamamoto et al., 2009). The general poor performance in locomotion at 0% cSOD activity is consistent with previous reports that cSOD-nulls exhibit an accelerated rate of age-related locomotor impairment (Jones and Grotewiel, 2011). Performance in the negative geotaxis assay was affected by genetic background, sex, and cSOD activity (Figure 20C). Unlike longevity, which showed no variation attributable to sex and genetic background at 0% cSOD activity, negative geotaxis was influenced by both factors in the cSOD-null flies. Negative geotaxis was significantly influenced by sex across the spectrum of cSOD activities, but there was less variation in negative geotaxis across genetic background at high levels of cSOD activity compared to at low levels. The reduced variation observed may, however, reflect the nature of the negative geotaxis assay (see below). Consistent with negative geotaxis, flies with higher levels of cSOD activity had greater climbing ability, and males had greater ability than did females across both assessments of
locomotion. In contrast to negative geotaxis, however, although climbing ability did change with cSOD activity levels it was robust to variation in genetic background across the range of cSOD activities (Figure 20D). Also in contrast to negative geotaxis, climbing ability was only different in male and female flies in the high cSOD comparison (Figure 20D).

I found that the two locomotor assays responded in similar, but distinct, ways to variation in sex, genetic background, and cSOD activity. These results are consistent with previous research on two similar but distinct reactive locomotor phenotypes, startle response and negative geotaxis, in which similarities and differences between the phenotypes, and different genetic mechanisms for each phenotype, were established (Jordan et al., 2007, 2012). The genetic mechanisms underlying locomotion are poorly understood overall, but locomotor behaviour in D. melanogaster has been found to be underlain by genetic components including neurotransmitter secretion, neural development, nervous system and muscle development, signal transduction, chromosome segregation, and copulation (Jordan et al., 2007, and Jordan et al., 2012). If the underlying genetic mechanisms for negative geotaxis and climbing ability are different as well, which seems reasonable as selection for modification of one component of locomotion is not constant across locomotor types (Jordan et al., 2007, and Jordan et al., 2012), then it is likely that the different sensitivities to genetic background that were observed between these phenotypes result from different responses to 2nd chromosome dominant modifiers. Genes located on the second chromosome, such as muscleblind, and Ken and Barbie, as well as a block of SNPs on the 2nd chromosome, have been implicated
with variation in locomotor reactivity (Jordan et al., 2007), and negative geotaxic ability (Jordan et al., 2012), respectively.

The negative geotaxis assay was modified to improve the ability to detect small differences in performance of low cSOD activity flies, however, this modification may have limited the ability of the assay to detect small differences in performance due to sex or genetic background in the high cSOD comparison. Consistent with this idea, the negative geotaxis assay detected a smaller effect of genetic background in the high cSOD comparison, compared to that in the low cSOD comparison. Interestingly, the countercurrent assay did not detect significant variation by sex or genetic background in the low cSOD comparison. The lack of detection of modifying effects could reflect the endurance nature of the countercurrent assay (Jones and Grotewiel, 2011) in combination with the impaired locomotor performance in cSOD-null flies. However, as there were no significant background effects at either the low or high range of cSOD activity in climbing ability, this suggests the pattern reflects a real difference between the locomotor phenotypes and not a technical limitation of the countercurrent assay. In general, the locomotor assays responded differently to variation in sex and genetic background, where sensitivity of the locomotor assays to each factor varied, though the direction of change in the effect size (from low to high cSOD activity ranges) for each factor was consistent (the change in sex effect in the two traits was ~3-10x different; the change in background effect was smallest in the two traits and was ~1.5x different; the change in cSOD effect in the two traits was ~3-8x different).

Two complementary locomotor assays were performed in an attempt to more fully define the genetic background effects in locomotor ability. Climbing ability and negative
geotaxis differed in sensitivity to genetic background, consistent with previous studies finding differences exist in the genetic mechanisms underlying similar locomotor traits (Jordan et al., 2007, and Jordan et al., 2012). Both climbing ability and negative geotaxis, in this study, had a reactive component, but they differed in the duration (i.e., sprint versus endurance) of locomotion measured. Variation in sensitivity to 2nd chromosome dominant modifiers supports the hypothesis that these locomotor phenotypes have different genetic mechanisms, though similar responses to different levels of cSOD activity and sex, may suggest that some components are shared between the traits.
Figure 20: Summary of the mean ± SEM data pooled across backgrounds for negative geotaxis A) and climbing ability B) across the four levels of cSOD activity surveyed showing the trend of phenotypic response to changes in cSOD activity. Summary of the mean ± SEM background data for negative geotaxis C) and climbing ability D) across the four levels of cSOD activity surveyed showing the trend of phenotypic response to changes in cSOD activity.

4.2.3 NADP(H) Enzyme Activity

The NADP(H)/NADP⁺ pools in cells are maintained by four key cytosolic enzymes; MEN, IDH, G6PD, and 6PGD and significant interactions are present across the four (Merritt et al., 2005, 2009, Rzezniczak and Merritt, 2012) and between these enzymes and cSOD activity (Bernard et al., 2011). This last interaction is likely because NADP(H) is used by catalase and glutathione-dependent antioxidants to support scavenging of hydrogen peroxide produced by SOD (Kanzok et al., 2001). Consistent with Bernard et al. (2011), similar significant interactions were observed between cSOD activity and IDH and G6PD activity; an absence of cSOD activity was associated with reduced enzyme activity (Figure 21B and C). In contrast to this study, however, the absence of cSOD activity alone did not produce a significant difference in MEN activity, although there was a significant sex-by-cSOD activity interaction (Figure 21A), possibly reflecting the different genotypes employed here.

In both males and females, the activity of the NADP(H) enzymes responded to differences in cSOD activity, but responses differed according to sex. Similar sex-specific enzyme responses have been observed across the NADP(H) enzymes in which males seem to be more sensitive to differences in NADP(H) enzyme genotypes than do females (Merritt et al., 2009); there is also a male expression bias in MEN, IDH, and G6PD (Gnad and Parsch, 2006). The complete absence of cSOD activity is associated with changes in the activity of IDH and G6PD, but there were no differences in the activities of either of the enzymes in the 30% and 80% cSOD comparison (Figure 21B
and C), though there was a difference in MEN activity in the 30% and 80% cSOD comparison (Figure 21A). Even small amounts of cSOD activity (30%) appear to restore WT activity in the proximal phenotypes. This recovery is in contrast with results from the distal phenotypes above (longevity, viability and locomotion), in which ~50% cSOD activity was necessary to recover WT phenotypes. That different phenotypes would have different thresholds of sensitivity is completely expected, but the sensitivity of the proximal phenotypes to changes in cSOD activity and the different combinations of sex and genetic background, complicates the original expectation that distal phenotypes would be more sensitive to genetic factors than the proximal phenotypes. Observations from the NADP(H) pathway illustrating the interactions between MEN, IDH, G6PD, and 6PGD (Merritt et al., 2009, Rzezniczak and Merritt, 2012), as well as the interactions between the NADP(H) enzymes and cSOD observed in Bernard et al., (2011), and in this study suggests that the ties between cSOD and the NADP(H) enzymes may not be "simple" as expected and instead proximal phenotypes are more complex than initially expected.

Activity of the NADP(H) enzymes differed across genetic backgrounds, but responses varied between the NADP(H) enzymes and across levels of cSOD activity. G6PD was sensitive to genetic background across cSOD comparisons (Figure 21F), while MEN and IDH were only influenced by genetic background in the high cSOD comparison (Figure 21D and E). G6PD has been observed to be a major source of NADP(H) under conditions of oxidative stress (Rzezniczak and Merritt, 2012), so sensitivity of G6PD to genetic background in the low cSOD comparison may be a factor of the increased role of G6PD under conditions of stress. The differences in the
magnitude of the fold changes of the factor effects across cSOD activities for the NADP(H) enzymes, compared to the trends observed in the life history traits, and the locomotor traits, were likely related to the fact that the NADP(H) network may only be peripherally related to cSOD activity (see last section), and reflect single enzyme responses, rather than the overall response of complex mechanisms which underlie the distal traits.

MEN, IDH, and G6PD are part of an interconnected network, with changes in the activity of one enzyme, resulting in corresponding changes in the activities of the other enzymes, though the magnitudes of change vary depending on conditions (Merritt et al., 2005, 2009, Rzezniczak and Merritt, 2012). MEN, IDH, and G6PD all have decreased activities in the absence of cSOD activity (Bernard et al., 2011); however, this trend only applies with males, with sexual dimorphism in NADP(H) enzyme activity across cSOD activities. The differential sensitivities of MEN, IDH, and G6PD to genetic background at different levels of cSOD activity, and sex, indicates that while proximal phenotypes are more "simple" than distal phenotypes, they are still complex and can be affected by interactions with genetic background and sex.
Figure 21: Summary of the mean ± SEM data pooled across backgrounds for MEN activity A) IDH activity B) and G6PD activity C) across the four levels of cSOD activity surveyed showing the trend of phenotypic response to changes in cSOD activity. Summary of the mean ± SEM background data for MEN activity (units/μg protein) D) IDH activity (units/μg protein) E) and G6PD activity (units/μg protein) F) across the four levels of cSOD activity surveyed showing the trend of phenotypic response to changes in cSOD activity.

4.3 The absence of cSOD activity had the largest effect on phenotypes, distal and proximal

cSOD-null *D. melanogaster* have been observed to have altered phenotypic expression, though these changes are recovered in flies possessing 50-60% cSOD activity.
cSOD activity was the most important factor controlling phenotypic magnitude when cSOD activity was low, potentially as a result of overarching metabolic changes caused by the loss-of-function of a key antioxidant defence enzyme. Using a series of phenotypes I observed that phenotypes were generally most altered in 0% cSOD activity flies, with cSOD activity greater than 50% ameliorating these phenotypes to WT levels. The magnitudes of response varied according to phenotype, though there are predominantly large fold changes in effect size for cSOD activity, with larger phenotype effects in the low cSOD comparison.

At 50% cSOD activity, distal phenotypes were recovered to WT levels, however, across proximal phenotypes 30% cSOD activity was sufficient to recover phenotypes to WT levels (Figure 19,20,21). The differential response of the proximal and distal phenotypes assayed suggests that different threshold concentrations of cSOD activity are required to produce WT phenotypes, potentially due to different mechanisms of interaction of the phenotypes with cSOD. Multiple genes interact to generate the four distal phenotypes observed (Jones and Grotewiel, 2011, Pasyukova et al., 2000, and Spencer et al., 2003), and if cSOD is directly involved in these interactions, changes in cSOD activity could influence phenotypic magnitude by altering the interactions that occur, with 50% cSOD representing enough cSOD activity to recover WT interactions. The NADP(H) network is at least peripherally related to cSOD function, as NADP(H) is heavily relied upon to support virtually all aspects of the antioxidant defence network downstream of cSOD (Phillips et al., 1989). MEN, IDH, and G6PD activity were defined as proximal phenotypes, as enzyme activities were not considered to fall under the categorization of distal phenotypes, which result from complex downstream gene
interactions (Chari and Dworkin, 2013). However, MEN, IDH, and G6PD are part of an interconnected network, so changes in activity of one enzyme results in changes in the activities of the other enzymes (Merritt et al., 2009, Rzezniczak and Merritt, 2012). Similarly, MEN, IDH, and G6PD activities have also been shown to change in response to the absence of cSOD activity (Bernard et al., 2011). The responses of the three NADP(H) enzymes to changes in cSOD activity, and to changes in the activity of other enzymes in the NADP(H) network, suggests that the phenotypes I originally classified as (and chose to study as representative examples of) proximal phenotypes are still complex as phenotypes. Clearly, their activities are influenced by multiple genes, and possibly only indirectly by cSOD, as the results here demonstrate that NADP(H) enzyme activity is restored to WT levels with fairly low cSOD activity. My observations here suggest that the phenotypes defined as proximal are less "simple" than expected. Different cSOD activity thresholds for the defined proximal and distal phenotypes are likely the result of differences in the underlying mechanisms (i.e, the degree of network redundancy or, level of allelic polymorphism) governing the traits, and their differing relationships to cSOD activity.

The absence of cSOD activity results in overarching changes in phenotypes, proximal and distal, affirming that the cSOD-null syndrome is pervasive across genetic backgrounds. Examination of phenotypic variation across the four cSOD activities demonstrated that the proximal and distal phenotypes, used in this study, have different thresholds of cSOD activity required to restore WT phenotypes; different phenotypes interact with cSOD to different degrees. Overall, variation in cSOD activity causes pervasive changes in phenotypes of both sexes.
4.4 Genetic background effects depend on interactions with other factors and the phenotypes being examined

Eight genetic backgrounds were selected from the DGRP lines according to the performance of the parent lines in longevity, fitness, paraquat resistance, and startle response (Mackay et al., 2012), and 2nd chromosomes were extracted from these lines for use as genetic backgrounds in this study. I hypothesized that unique alleles of enhancer or suppressor loci present on the extracted 2nd chromosomes would modify expression in the observed phenotypes. However, even though the DGRP lines used were specifically selected, there was no guarantee that the phenotypes would be a function of 2nd chromosome dominant modifiers. Viability and climbing ability were not significantly affected by genetic background, while negative geotaxis and longevity were. The proximal phenotypes (NADP(H) enzyme activities) were influenced by genetic background variably depending on cSOD activity and the particular NADP(H) enzyme.

Intriguingly, the lines selected for high and low performance in startle response and longevity, did not have the same effect in the derived lines as in the parent lines. Differential phenotypic responses of the derived lines compared to the parental lines suggests the effect of the 2nd chromosome modifiers depends on whole organism genotype. The health and potential to generate damaged proteins varies within each parent DGRP line (Huang et al., 2014), and overall line health is dependent on total organism genotype. Extracting "high" and "low" line chromosomes from the parent lines alters their genetic context, and exposes them to different modifiers in the progeny than in the parent lines, presumably altering the epistatic interactions which occur. Phenotypes result from the interactions of multiple genes (Chandler, Chari, and Dworkin, 2013, and Chari and Dworkin, 2013), therefore, changing the genetic elements present alters the
interactions that may be manifest in the resultant phenotypic expression. As second chromosome heterozygotes were used I could only determine the effects of dominant modifiers, so the insensitivity of viability and climbing ability to background observed in this study may indicate that these phenotypes are insensitive to second chromosome dominant modifiers, rather than to genetic background effects overall. However, if viability and climbing ability are canalized traits, and there is low variation in the loci influencing their underlying genetic mechanisms, the phenotypes would be robust to minor changes in genetic background. Similarly, significant background effects have been observed across activity mutants from amorphs to hypermorphs (Chari and Dworkin, 2013). The lack of response to genetic background observed in some of the phenotypes in the absence of cSOD (amorph), or in the other genotypes (all hypomorphs), further supports the idea that response of phenotypes to genetic background is phenotype dependent.

None of the selected backgrounds demonstrated consistent ability to strengthen or weaken effects of the cSOD-null genotype, regardless of the phenotype they were chosen for (Table A8). Genetic backgrounds tended to perform more similarly across the proximal than the distal phenotypes, however there was no common trend of suppression or enhancement in background performance across sex and cSOD activities. Even though the genetic backgrounds did not perform as hypothesized, according to parental performance, possibly due to trade-offs (Spencer et al., 2003) in chromosome performance resulting from extraction, it was unexpected that none of the backgrounds showed consistent ability to enhance or suppress any of the cSOD-null phenotypes. Only eight genetic backgrounds were used in this study, and all of the parent DGRP lines were
derived from the same initial population in North Carolina (Mackay et al., 2012), so the number of genetic variants present in this study may have been too low for such a large effect modifier to be present. Similarly, the genetic composition of the second chromosome likely played a role in the lack of a pervasive enhancer or suppressor effects detected. For example, antioxidant enzymes might reasonably be hypothesized to suppress the effects of the cSOD-null syndrome, as these flies are under chronic oxidative stress; however, many of the major antioxidants cSOD (Flybase, 2016c), catalase (Flybase, 2016a), glutathione peroxidase (Flybase, 2016b), and thioredoxin (Flybase, 2016e), are not located on the second chromosome. Similarly, antioxidant enzymes that are present on the second chromosome, for example MnSod (Flybase, 2016d), may localize to different areas of the cell (Bafana et al., 2011, and Miller, 2012), or profound suppressor and enhancer effects may simply not have been detectable by dominant modifier screens.

Genetic background had a significant effect on most phenotypes; however, its effect varied with interactions with cSOD activity, and sex. The absence of pervasive directional modification of the phenotypes, in any of the genetic backgrounds, may be a consequence of the 'small' (single chromosome) genetic background used; however, as significant background effects were detected in many of the phenotypes surveyed, this seems unlikely. Variation in response of similar phenotypes to genetic background, and the absence of any one background with strong directional effects, likely reflects different degrees of redundancy, and allelic polymorphism in the phenotypic mechanisms. Response of phenotypes to the same genetic background is likely influenced by the differing fitness consequences for variation in each trait.
4.5 Sex had the largest effect on phenotypes, distal and proximal, in the high cSOD activity comparison

Changes in phenotypic expression associated with cSOD activity have been previously documented primarily in males (Bernard et al., 2011, Knee et al., 2013, and Parkes et al., 1998b), however sex-specific differences have been documented across various *D. melanogaster* phenotypes, including biochemical phenotypes (Jordan et al., 2007, Merritt et al., 2009, and Spencer et al., 2003). Consistent with this literature (Jordan et al., 2007, Merritt et al., 2009, and Pasyukova et al., 2000) I observed sexual dimorphism in longevity, negative geotaxis, climbing ability, and NADPH enzyme activity, but not in viability. In the distal phenotypes the magnitude of male and female response varied, but direction was constant, while in the proximal phenotypes male and female response varied in magnitude and direction.

Sex had the largest effect on phenotypes in the 30%-80% cSOD comparison (Figure 8). Males and females have the same cSOD activity in the 0%-50% cSOD flies, but have significantly different cSOD activity in the 30%-80% cSOD comparison, although the relative difference in activity was fairly consistent between males and females. One interpretation of these results is that the sex dimorphism in phenotypes observed at high cSOD activity was driven by differences in cSOD activity between males and females. Sex dimorphism in longevity has been previously noted in flies over-expressing cSOD, and one hypothesis for this phenomenon was the existence of different levels of cSOD activity between males and females (Spencer et al., 2003). While the measured cSOD activities for the 30%-80% cSOD comparison are consistent with the existence of sex dimorphism in cSOD activity, this trend was not observed in the 0%-50% cSOD comparison. Sex dimorphism was observed in longevity, negative geotaxis,
MEN activity, and G6PD activity in the 0%-50% cSOD comparison but there was no significant sex dimorphism in the actual levels of cSOD activity. There was also no sex dimorphism observed in viability at any level of cSOD activity.

An alternate hypothesis for the sexual dimorphism in cSOD activity observed in the 30%-80% cSOD comparison, but not the 0%-50% cSOD comparison, may be differential expression of the cSod transgene between male and female flies; for example, if female expression of the cSod transgene is greater than that in males (i.e. sex biased transgene expression/activity in the females). Sex differentiation between males and females, simplistically, entails the activation and suppression of different genes, as well as sex-influenced epistasis (Rhen, 2000); studies of gene expression in males and females have demonstrated that a substantial portion of the D. melanogaster transcriptome demonstrates sex-dependent regulation (Ranz et al., 2003). Differences in cSod transgene regulation between sexes would explain both the higher female cSOD activity in the 30% and 80% cSOD comparison, as well as the relatively consistent difference in 30% and 80% cSOD activity between males and females. Differential expression of the cSod transgene would also explain the lack of sexual dimorphism in cSOD activity in the 0%-50% cSOD comparison, as they do not possess the transgene. Consistent with the hypothesis of transgene driven sex dimorphism in cSOD activity, no sex bias in expression has been observed in D. melanogaster at the cSOD locus (Gnad and Parsch, 2006). Different levels of sex dimorphism across phenotypes, in the presence and absence of the cSod transgene, suggests that while sex dimorphism in actual levels of cSOD activity may exist, sensitivity to cSOD activity is likely not the only contributing factor to the sex dimorphism observed in phenotypes (Kopp et al., 2003).
Sexual dimorphism in morphology, physiology and biochemistry, and behaviour in *D. melanogaster* is thought to primarily be directed by the *dsx* controlled branch of sex differentiation (Clough *et al.*, 2014). It is hypothesized that polygenic genes, which subtly influence development and physiology, are regulated by *dsx*, which may explain the genetic background effects on some sex-related phenotypes (Clough *et al.*, 2014). Life history traits, including viability and longevity (Bonduriansky *et al.*, 2008), as well as locomotor ability (Long and Rice, 2007) contribute to the reproductive success of adult *D. melanogaster*; and longevity (Spencer *et al.*, 2003, and Pasyukova *et al.*, 2000), and locomotion (Jordan *et al.*, 2007 and Yamamoto *et al.*, 2009) are influenced by genetic background. While the complete mechanism(s) by which *dsx* controls sexually dimorphic phenotypes are not fully understood, slight differences in patterns of *dsx* regulation due to the genetic variation present across the genetic backgrounds, as well as the variation in cSOD activity, could have culminated in the variation in sexual dimorphism observed across phenotypes.

cSOD activity was the most important factor in determining phenotypic magnitude in the 0% and 50% cSOD activity comparison; however, sex was among the most important factors in the 30% and 80% cSOD comparison. The large effect of sex on phenotypes, and the novel female sex response observed in NADP(H) enzyme activity in females across cSOD activities, demonstrates the importance of including males and females in genetic analyses, as complete understanding of the genetic basis of phenotypes requires analysis of the genetic mechanisms in both sexes.
4.6 Conclusion

In the current study I provided evidence that across sex and genetic background, the absence of cSOD activity causes large responses across phenotypes, consistent with established features of the cSOD-null syndrome. Novel NADP(H) enzyme responses to the cSOD-null syndrome were also observed in female flies. My results indicate that different levels of cSOD activity influence phenotypic magnitude, though the threshold amount of cSOD activity required to revert a phenotype to what is considered the WT level differs in the proximal and distal phenotypes assessed. I provided evidence that sensitivity to genetic background depends on the phenotype being examined, and what might be perceived as similar phenotypes are not equally sensitive to genetic background, with the overall effect size of genetic background depending on interactions with other factors. Moreover, no genetic backgrounds were found that represented overarching phenotypic enhancers or suppressors of SOD-dependent phenotypes across genetic conditions. These results have shown that male and female flies can drastically differ in their phenotypic responses to the same factors, supporting the notion that utilization of both male and female animals should be encouraged in studies of biochemical/physiological processes in Drosophila. Overall, the results demonstrate that the cSOD-null syndrome is pervasive across genetic background and sex. However, as long as some cSOD activity is present most of the established cSOD-dependent phenotypes are influenced by sex and genetic background, suggesting that genetic interactions cannot be conclusively identified if they are examined in limited genetic contexts.
Chapter 5 General Conclusions and Future Work

5.1 General Conclusions

I assessed the influence of three factors; cSOD activity, sex, and second chromosome genetic background, on a range of phenotypes which have been identified as part of the cSOD-null syndrome in *Drosophila*. I found that two of the factors, cSOD activity and sex, followed consistent, and converse, trends of influence across phenotypes, with low levels of cSOD activity having the greatest influence on phenotypes, while at high cSOD activity, sex tended to have the largest influence on phenotypes. In contrast, I found that the genetic background effect was more phenotypically dependent than were the effects of cSOD activity and sex; *i.e.* whether or not genetic background had an effect varied across the phenotypes. My results demonstrate that while there is some variation in the phenotypes at 0% cSOD activity, the defined cSOD-null syndrome is pervasive and in no genetic background is this syndrome substantially reduced. Further, with the assessment of phenotypes in both male and female flies my research expands the defined cSOD-null syndrome and identifies sex-specific differences; *e.g.* that changes in NADPH enzyme activity across levels of cSOD activity differ in female and male flies. Similarly, the assessment of two locomotor phenotypes, negative geotaxis and climbing ability, demonstrates that phenotypes that seem similar do not necessarily have similar sensitivities to different factors.

Up to now, all of the cSOD studies performed in the Merritt lab, and most studies from other groups examining cSOD in *Drosophila*, have been performed using the same cSOD-null allele, *cSod*¹⁰⁸. However, the absence of a matched *cSod*⁺ control allele for the *cSod*¹⁰⁸ allele, limits the type of genetic background analyses that can be performed
using these lines. Similarly, the predominant use of a single null allele to define the cSOD-null syndrome does not take into account the possibility that some of the phenotypic differences may be a quirk of the structure of the \( cSod^{n108} \) allele. Consequently, the phenotypes observed in Chapters 3 and 4 may reflect specific epistatic interactions with the \( cSod^{n108} \) allele, or they may be reflective of the lack of cSOD activity overall. Potential future projects examining cSOD should include the generation and characterization of new \( cSod^{+/} \) matched alleles, or stocks, and further analysis of cSOD phenotypes, including chromosomal substitution, and genome-wide association studies (GWAS).

### 5.2 Project 1: Generation of cSod\(^{+/}\) Alleles

Future work in the Merritt lab will aim to generate a new series of \( cSod^{−} \) alleles which will have matched controls with 100% cSOD activity in the parent lines, allowing direct phenotypic comparisons to be made across a full range of cSOD activities (0%-100% activity). The absence of the requirement for the \( cSod \) transgene on the 2\(^{nd} \) chromosome would also allow future studies to expand on the genetic background analyses that can be performed on cSOD. Null alleles can be generated using a number of different mutagenic techniques, which vary in their effectiveness for specific gene targeting.

Forward genetic screens, such as ethyl methanesulfonate mutagenesis (EMS), generate mutations at random, and the genes that have been mutated are identified through phenotype and sequence analysis (Lin, et al., 2014). Forward genetic approaches have done a great deal for genetic studies, however, these types of approaches are untargeted, so specific genes cannot be chosen \textit{a priori} for mutagenesis, and
identification of the sites that are mutated can be labour intensive and time consuming (Bellés, 2010, and Lin, et al., 2014). Chromosomal sites may also be differentially sensitive to mutagens, which can limit the genes that are affected by forward genetic screens (Bellés, 2010, and Lin, et al., 2014).

The cSod<sup>n108</sup> allele was generated using EMS, and possesses a point mutation that causes a missense mutation, ultimately generating a non-functional (null) enzyme (Phillips et al., 1995). The cSod<sup>n108</sup> allele does not have a matched cSod<sup>+</sup> allele, as the original EMS mutated chromosome was lost subsequent to the generation of the null allele. Since the generation of the cSod<sup>n108</sup> allele by Campbell, Hilliker, and Phillips, (1986) via EMS, gene editing technologies have advanced. Reverse genetic screens, allow specific genes to be targeted for mutagenesis, meaning that new cSOD-null alleles, or cSOD null activity lines can be generated via specific gene editing.

A widespread reverse genetic screening method is RNA interference (RNAi), which takes advantage of a natural cellular process that targets messenger RNA (mRNA) for degradation (Bellés, 2010, and Hannon, 2002). In RNAi, specific sequences can be generated to target the gene of interest, and these sequence fragments bind the target mRNA to block and degrade it (Bellés, 2010, and Hannon, 2002). As the target sequences are short fragments they can potentially target multiple genes if their mRNAs share segments of uninterrupted sequences (Sharp, 2001), suggesting sequence specificity is required for targeted knock down generation. Organisms are differentially susceptible to RNAi, with less derived (closer to their 'natural' condition i.e. less lab evolved) organisms generally more sensitive to RNAi; as such, D. melanogaster, a highly derived model organism, can be poorly sensitive to RNAi (Bellés, 2010). However, cSOD
(Martin, et al., 2009) and MnSOD (Kirby et al., 2002) knockdown flies have been generated via RNAi, and mutant phenotypes in the two RNAi induced SOD lines have been identified, suggesting that RNAi can be successfully used to generate cSOD activity mutants. It should also be noted that as RNAi knocks down activity by binding to the mRNA of the gene of interest (Matzke, et al., 2001), enzyme activity in not completely knocked down by this process, so true "knockouts" are not being generated. As genes themselves are not being altered, the "knocked-out" enzyme activity is not necessarily heritable.

Clustered regularly interspaced short palindromic repeat (CRISPR) is an alternate method of reverse genetic screening, which was derived from a bacterial defence system, but has been used in Drosophila for targeted mutagenesis (Bassett and Liu, 2013). Specific genome sequences are edited, in the CRISPR system, by targeting a Cas9 complex to a specific DNA sequence to generate double strand breaks, which, when inefficiently repaired by DNA repair processes, such as non-homologous end joining (NHEJ), result in mutations that can be transmitted through the Drosophila germ-line (Bassett and Liu, 2013, and Bassett et al., 2013). CRISPR can be targeted to protein forming genes, as well as functional sites, so changes in gene activity and gene regulation can both be examined (Bassett et al., 2013). CRISPR induced mutagenesis can have a very low mutagenic rate in flies (i.e. ~5.9%; Bassett and Liu, 2013), though different methods of delivering the CRISPR components, such as injection of components (Bassett et al., 2013), or crossing of transgenic parents expressing the different components (Kondo and Ueda, 2013) can improve the mutagenic rate. Specific genome sequences can be targeted, according to the design of the sgRNA (synthetic guide RNA), but target
specificity may be low as the sgRNA recognition site is fairly small, though the small genome size in *Drosophila* means low specificity is less of an issue (Bassett and Liu, 2013). MnSOD activity has been successfully knocked down via CRISPR/Cas9 in the human HEK293T cell line (Cramer-Morales *et al.*, 2015), suggesting that it should be possible to generate cSOD mutants in *Drosophila*.

SOD alleles have been successfully edited via both RNAi and CRISPR, indicating that both methods could potentially be used to generate new cSOD*+/−* flies. As RNAi does not truly generate new "alleles", CRISPR may be a better method for long term applications, however, RNAi can be used to examine tissue specific activity knock-down. The DGRP lines are a suite of approximately 200 fully sequenced *D. melanogaster* lines, which show phenotypic variation (Mackay *et al.*, 2012), allowing the line cSod sequences to be analyzed, and new cSod alleles to be potentially generated in multiple WT cSod alleles. Generation of the cSod*−* alleles using multiple existing WT alleles from the separate DGRP lines, will allow the evaluation of the influence of allele structure on cSOD phenotypes, but will also allow the expansion of genetic background analyses on cSOD related phenotypes.

### 5.3 Project 2: Expansion of Genetic Background Influence on cSOD Phenotypes

Future work in the Merritt lab will also work to characterize the cSod*+/−* alleles generated using the DGRP lines, as well as re-characterizing the cSOD-null syndrome with the new alleles to determine whether the syndrome is consistent across disparate, 'natural', *D. melanogaster* genetic backgrounds. Chromosomal substitution lines can also
be generated using the DGRP $cSod^{+/}$ alleles to analyze the influence of zygosity on cSOD phenotypes.

Phenotypes are context dependent, meaning that genetic and environmental components influence the final expression of phenotypes (Chandler et al., 2013). The analyses performed in this study only screened for the influence of 2nd chromosome dominant modifiers, and the effect of 2nd chromosome genetic background was dependent on the phenotypes examined. Some phenotypes (e.g. viability, cSOD activity), were insensitive to changes in genetic background, while other phenotypes (e.g. longevity, G6PD activity), were sensitive to genetic background. These results, however, only indicate sensitivity to 2nd chromosome dominant modifiers, not to genetic background overall or to recessive mutations. With the T0 and T5 lines, the presence of the $cSod$ transgene on the 2nd chromosome and the $cSod$ gene on the 3rd chromosome, combined with the lack of a true $cSod^*$ control, restricts the genetic background analyses that can be performed via chromosomal substitution using these lines.

Chromosomal substitution is the replacement of one or more chromosomes with chromosomes derived from a separate source (i.e. individual, line), via a crossing scheme (Mackay, 2009). In this study I generated chromosomal substitution lines across 2nd chromosome genetic backgrounds; however, these chromosomes were introgressed into lab line derived genotypes that were essentially isogenic. cSOD activity is known to be influenced by genetic modifiers on the 2nd chromosome, as well as on the 3rd chromosome, where the structural cSOD gene is located (Graf and Ayala, 1985). This is contrary to the results from this study that indicate that cSOD activity was not influenced
by 2\textsuperscript{nd} chromosome genetic background, though this insensitivity may have been due to the isogenic nature of the genotypes other than the 2\textsuperscript{nd} chromosome.

The \textit{cSod}\textsuperscript{+} and \textit{cSod}\textsuperscript{-} alleles generated in the DGRP lines (previous section) could be used to generate chromosomal substitutions that would vary not only in the zygosity of the allele (\textit{i.e.} homozygous recessive, heterozygous, homozygous dominant) but also in the zygosity of the \textit{cSod} allele present (\textit{i.e.} \textit{cSod}\textsuperscript{1+/1+}, \textit{cSod}\textsuperscript{1+/2+}, or \textit{cSod}\textsuperscript{2+/2+}), as well as in the zygosity for the chromosomes present (\textit{i.e.} \textit{X}/\textit{X}, \textit{2}/\textit{2}; \textit{3}/\textit{3}). These substitution lines can then be used to explore a range of questions including to what degree the phenotypes in the introgressed line(s) differ from the parental lines, how the cSOD-null syndrome is influenced when the substitution line is heterozygous for null alleles (\textit{i.e.} the alleles are derived from different DGRP lines), as well as identifying the distribution of the phenotypic modifiers across the X, 2\textsuperscript{nd} and 3\textsuperscript{rd} chromosomes.

As a model system, \textit{D. melanogaster} is highly amenable to studies of chromosomal substitution, since there are only three chromosome pairs to track during genetic crosses. Generation of large scale cSOD substitution lines, and subsequent phenotypic analysis of these lines will further knowledge towards not only defining a comprehensive cSOD-null syndrome, but will also contribute towards establishing which types of phenotypes are canalized, and as such are robust to genetic background. Aside from contributing towards defining existing variation in phenotypes, cSOD chromosome substitution lines will also allow epistatic interactions to be mapped via fine scale genetic mapping techniques, such as GWAS.

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5.4 Project 3: GWAS on cSOD Phenotypes

Future work in the Merritt lab could also use the \(cSod^{+/}\) alleles generated in the DGRP lines, as well as the derived chromosome substitution lines, to determine the genetic locations contributing to epistatic effects at different levels of cSOD activity, and how they vary across DGRP\textit{i} backgrounds. Mapping epistatic interactions associated with cSOD will contribute to existing knowledge on the genetic regions that cSOD is known to interact with, as well as providing novel information on how epistatic effects vary across levels of cSOD activity in \textit{D. melanogaster}.

GWAS are a statistical method used to connect phenotypes to genotypes by examining the complete genomes of the sample organisms and identifying genetic variants that contribute to the phenotypes, including genetic loci and regulatory regions (Flint, 2013, and Nuzhdin, \textit{et al.}, 2012). GWAS primarily relate single nucleotide polymorphisms (SNPs) to the phenotype or condition being analyzed (Pearson and Manolio, 2008). GWAS are usually untargeted studies as specific regions of the genome are not targeted for exploration; rather, this method is used to identify genetic variants contributing to a trait regardless of where they occur (Flint, 2013). Disease phenotypes are among the most common types of phenotypes analyzed using GWAS, but they can also be used to analyze variation contributing to quantitative traits (polygenic traits with quantifiable variation \textit{e.g.} longevity; Flint, 2013, and Pearson and Manolio, 2008). GWAS are better able to detect when common genetic variants contribute to phenotypes, so they are limited in their ability to detect rare genetic variants that contribute the majority of variation in the phenotype of interest (Flint, 2013). Similarly, samples that vary greatly in their haplotype structure (\textit{i.e.} derived from vastly different populations),
can confound variant detection by GWAS (Flint, 2013), as different haplotype architectures will not share the same "common" genetic variants. However, the option to input variables (Pearson and Manolio, 2008), such as sex, age, and geographic background in GWAS can help to prevent confounding results by controlling for their cause.

The nature of the DGRP lines makes them amenable to GWAS for several reasons. First, as the lines were all derived from the same natural population of *D. melanogaster* in North Carolina, the haplotype structure of the population should be relatively consistent, which reduces confounding effects during the analyses (Mackay *et al.*, 2012). Similarly, the DGPR line genomes are fully sequenced, with the sequences and phenotypic data available online (Huang *et al.*, 2014, and Mackay *et al.*, 2012), which increases the power of detection in GWAS as the data from the DGRP lines not used directly in the study can still contribute to the reference database, improving the power of the analyses.

The chromosomal substitution lines developed for the previous project (previous section), localising phenotype modifiers to the different chromosomes, can similarly be used in GWAS to specifically map enhancers and suppressors of cSOD phenotypes, and how they vary, or not, across genetic backgrounds. GWAS on the *cSod*<sup>+/−</sup> substitution lines could also be used to corroborate, or oppose, results from the literature which link cSOD with loci such as *Wwox* (O'Keefe *et al.*, 2011), *MnSod* (Lim *et al.*, 2014), and *Trxr-1* (Missirlis, Phillips, and Jäckle, 2001), as well as exploring differences in epistatic effects between the *cSod* alozymes.
As discussed in the introduction, the \textit{cSod} gene has two allozymes, \textit{cSod-fast} and \textit{cSod-slow}, and cSOD studies examining cSOD-null phenotypes have been performed predominantly with the \textit{cSod-fast} allozyme (Bernard \textit{et al.}, 2011, Knee \textit{et al.}, 2013, Martin, Jones, and Grotewiel, 2009, Parkes \textit{et al.}, 1998b, Phillips \textit{et al.}, 1995, Phillips \textit{et al.}, 1989, Sun and Tower, 1999, and Woodruff \textit{et al.}, 2004) as it was the allozyme the \textit{cSod}^{n108} null allele was generated from (Campbell \textit{et al.}, 1986), and is the more common allozyme (Ayala, \textit{et al.}, 2002). Due to the predominant use of the \textit{cSod-fast} allozyme in cSOD studies, or the failure to clarify the allozyme used, there is less information on how the \textit{cSod-slow} and \textit{cSod-fast} allozymes differ in phenotypes related to the cSOD-null syndrome, and epistatically. GWAS comparing the two \textit{cSod} allozymes would work to elucidate epistatic differences between them.

Previous GWAS studies in the DGRP lines have examined phenotypes such as sensitivity to chronic oxidative stress (Jordan \textit{et al.}, 2012), resistance to ROS-induced locomotor decline (Jordan \textit{et al.}, 2012), and longevity (Ivanov \textit{et al.}, 2015) and variation has been found in the DGRP lines for these traits. Sensitivity to chronic oxidative stress, locomotor decline, and longevity are all phenotypes which can also be influenced by changes in cSOD activity (Martin, \textit{et al.}, 2009 and, Phillips \textit{et al.}, 1995). Although variation in \textit{Sod} was not found to influence all the traits examined by GWAS (Jordan \textit{et al.}, 2012), this may simply be indicative of a lack of variation in the \textit{Sod} loci in the DGRP lines, or the presence of rare alleles. However, as the traits to be examined are related to the \textit{cSod}^{+/−} state, variation in \textit{Sod} that is introduced as part of the phenotype, which will circumvent this potential limitation.
5.5 Conclusions

The results from my research describe the variable effects of changes in cSOD activity on phenotypes across genetic backgrounds and between sexes. My results suggest that the defined cSOD-null syndrome is pervasive, even across genetic backgrounds. Interestingly, my results also suggest that at higher cSOD activity more variation in phenotypes is attributable to sex and genetic background. Novel sex specific responses to different genetic backgrounds and cSOD activities demonstrate that male and female response can vary not only in the magnitude of the response, but also in the direction of the response. However, results from this study were limited by the genetic architecture of the T0 and T5 lines, which restricted genetic background analyses to 2nd chromosome dominant modifiers. Generation of novel cSod<sup>+/−</sup> alleles in the DGRP lines would create matched cSOD-null and cSOD control lines for use in further analyses, including corroborating the defined cSOD-null syndrome with novel cSod alleles, as well as expanding cSOD genetic background analyses on phenotypes and identification of genetic variants interacting with the cSod locus under various genetic conditions.
References


http://dgrp2.gnets.ncsu.edu/


Appendices

Figure A1: Summary of the mean ± SEM background responses for cSOD activity showing the trend of cSOD activity response to changes in genotype, with cSOD activity measured standardized by protein concentration (units/μg protein). 0% and 50% cSOD Activity: cSOD Activity - $F_{1,127} = 84.1$, $p < 0.0001$, $\eta^2_p = 0.40$. 30% and 80% cSOD Activity: cSOD Activity - $F_{1,124} = 158$, $p < 0.0001$, $\eta^2_p = 0.56$; Sex - $F_{1,124} = 9.65$, $p = 0.0023$, $\eta^2_p = 0.072$. 
Table A1: Raw longevity ANOVA outputs indicating significance of factors and their interactions, as well as the effect sizes of individual factors and interactions. Bolded values denote significant factors in longevity determination.

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Table A2: Raw viability ANOVA outputs indicating significance of factors and their interactions, as well as the effect sizes of individual factors and interactions. Bolded values denote significant factors in viability determination.

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Table A3: Raw negative geotaxis ANOVA outputs indicating significance of factors and their interactions, as well as the effect sizes of individual factors and interactions. Bolded values denote significant factors in negative geotaxis determination.

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<td>Sex</td>
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<td>0.19889</td>
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<td>2.30</td>
<td>0.132</td>
<td>0.0145</td>
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<td>0.20362</td>
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<td>&lt;.0001</td>
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<td>Error</td>
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<td>0.04113</td>
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</tr>
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</table>

| **T5 - 30% and 80% cSOD Activity Cross** | | | | | |
| Sex                                    | 1    | 0.43415 | 8.80   | **0.0038** | 0.0809 |
| Background                             | 7    | 0.08201 | 1.66   | 0.127    | 0.104 |
| cSOD Activity                          | 1    | 0.36130 | 7.33   | **0.008** | 0.0683 |
| Sex X Background                       | 7    | 0.14886 | 3.02   | **0.0064** | 0.174 |
| Background X cSOD Activity             | 7    | 0.07907 | 1.60   | 0.143    | 0.101 |
| Sex X cSOD Activity                    | 1    | 0.08857 | 1.80   | 0.183    | 0.0176 |
| Sex X Background X cSOD Activity       | 7    | 0.10012 | 2.03   | 0.0585   | 0.124 |
| Error                                  | 269  | 0.04931 |        |         |     |

Table A4: Raw countercurrent ANOVA outputs indicating significance of factors and their interactions, as well as the effect sizes of individual factors and interactions. Bolded values denote significant factors in climbing ability determination.

<table>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>1</td>
<td>0.20444</td>
<td>3.55</td>
<td>0.0606</td>
<td>0.0130</td>
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<tr>
<td>Background</td>
<td>7</td>
<td>0.04590</td>
<td>0.797</td>
<td>0.590</td>
<td>0.0203</td>
</tr>
<tr>
<td>cSOD Activity</td>
<td>1</td>
<td>28.84678</td>
<td>501</td>
<td>&lt;.0001</td>
<td>0.651</td>
</tr>
<tr>
<td>Sex X Background</td>
<td>7</td>
<td>0.02552</td>
<td>0.443</td>
<td>0.874</td>
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</tr>
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<td>0.935 2.46E-05</td>
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</tr>
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<td>Sex X Background X cSOD Activity</td>
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<td>0.038725</td>
<td>0.673</td>
<td>0.695</td>
<td>0.0172</td>
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<td>Error</td>
<td>269</td>
<td>0.05758</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| **T5 - 30% and 80% cSOD Activity Cross** | | | | | |
| Sex                                    | 1    | 1.59670 | 20.8   | **<.0001** | 0.106 |
| Background                             | 7    | 0.06956 | 0.906  | 0.503   | 0.0350 |
| cSOD Activity                          | 1    | 4.728976 | 61.6   | **<.0001** | 0.260 |
| Sex X Background                       | 7    | 0.123302 | 1.61   | 0.136   | 0.0604 |
| Background X cSOD Activity             | 7    | 0.028009 | 0.365  | 0.922   | 0.0144 |
| Sex X cSOD Activity                    | 1    | 0.274622 | 3.58   | 0.0602  | 0.0200 |
| Sex X Background X cSOD Activity       | 7    | 0.128282 | 1.67   | 0.119   | 0.0627 |
| Error                                  | 175  | 0.076765 |        |         |     |
Table A5: Raw MEN activity ANOVA outputs indicating significance of factors and their interactions, as well as the effect sizes of individual factors and interactions. Bolded values denote significant factors in MEN activity determination.

<table>
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<th>η²</th>
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<tbody>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Sex</td>
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<td>2.81E-05</td>
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<td>0.00850</td>
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<td>1.27E-05</td>
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<th>η²</th>
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Table A6: Raw IDH activity ANOVA outputs indicating significance of factors and their interactions, as well as the effect sizes of individual factors and interactions. Bolded values denote significant factors in IDH activity determination.

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</table>
**Table A7**: Raw G6PD activity ANOVA outputs indicating significance of factors and their interactions, as well as the effect sizes of individual factors and interactions. Bolded values denote significant factors in G6PD activity determination.

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<td>2.65</td>
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</tr>
</tbody>
</table>
Table A8: Line effects for longevity, viability, negative geotaxis, countercurrent (climbing ability), malic enzyme (MEN), isocitrate dehydrogenase (IDH), and glucise-6-phosphate dehydrogenase (G6PD). Bolded lines reflect phenotypes that demonstrated significant line effects.

<table>
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<th>Longevity</th>
<th>Viability</th>
<th>Negative Geotaxis</th>
<th>Climbing Ability</th>
<th>MEN Activity</th>
<th>IDH Activity</th>
<th>G6PD Activity</th>
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<td>307</td>
<td>517</td>
<td>517</td>
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</table>
Table A9: Maximum and minimum values, pooled across genetic background, for longevity, viability, negative geotaxis, countercurrent (climbing ability), malic enzyme (MEN), isocitrate dehydrogenase (IDH), and glucise-6-phosphate dehydrogenase (G6PD). Bolded lines reflect phenotypes that did not demonstrate significant cSOD activity effects.

<table>
<thead>
<tr>
<th>cSOD Activity</th>
<th>Sex</th>
<th>Units/ug</th>
<th>cSOD Activity</th>
<th>(PI)</th>
<th>(CF)</th>
<th>MEN Activity</th>
<th>IDH Activity</th>
<th>G6PD Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>Male</td>
<td>0.000192±0.000048</td>
<td>5.7±0.1</td>
<td>0.211±0.012</td>
<td>0.41±0.05</td>
<td>0.30±0.03</td>
<td>0.0231±0.0004</td>
<td>0.00706±0.00015</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>0.000106±0.000016</td>
<td>8.5±0.2</td>
<td>0.195±0.014</td>
<td>0.38±0.05</td>
<td>0.23±0.03</td>
<td>0.0234±0.0006</td>
<td>0.00827±0.00024</td>
</tr>
<tr>
<td>50%</td>
<td>Male</td>
<td>0.000875±0.000010</td>
<td>57.1±0.5</td>
<td>0.339±0.012</td>
<td>0.84±0.02</td>
<td>0.93±0.02</td>
<td>0.0252±0.0004</td>
<td>0.00974±0.00024</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>0.000883±0.000012</td>
<td>66.6±0.8</td>
<td>0.330±0.012</td>
<td>0.73±0.03</td>
<td>0.88±0.03</td>
<td>0.0200±0.0004</td>
<td>0.00874±0.00022</td>
</tr>
<tr>
<td>30%</td>
<td>Male</td>
<td>0.000408±0.000045</td>
<td>48.2±0.7</td>
<td>0.237±0.014</td>
<td>0.79±0.06</td>
<td>0.69±0.05</td>
<td>0.0278±0.0005</td>
<td>0.00976±0.00025</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>0.000546±0.000069</td>
<td>55.9±0.8</td>
<td>0.261±0.017</td>
<td>0.63±0.06</td>
<td>0.47±0.05</td>
<td>0.0214±0.0007</td>
<td>0.00915±0.00034</td>
</tr>
<tr>
<td>80%</td>
<td>Male</td>
<td>0.00184±0.000015</td>
<td>54.4±0.8</td>
<td>0.299±0.017</td>
<td>0.86±0.03</td>
<td>0.95±0.01</td>
<td>0.0245±0.0005</td>
<td>0.01017±0.00035</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>0.00253±0.000021</td>
<td>61.7±1.1</td>
<td>0.318±0.020</td>
<td>0.79±0.03</td>
<td>0.85±0.04</td>
<td>0.0227±0.0007</td>
<td>0.00923±0.00027</td>
</tr>
</tbody>
</table>
Table A10: Maximum and minimum line values for longevity, viability, negative geotaxis, countercurrent (climbing ability), malic enzyme (MEN), isocitrate dehydrogenase (IDH), and glucise-6-phosphate dehydrogenase (G6PD). Bolded lines reflect phenotypes that demonstrated significant line effects.

<table>
<thead>
<tr>
<th>cSOD Activity</th>
<th>Sex</th>
<th>Status</th>
<th>Longevity (Days)</th>
<th>Viability (Frequency)</th>
<th>Negative Geotaxis (PL)</th>
<th>Climbing Ability (CF)</th>
<th>MEN Activity Units/ug</th>
<th>IDH Activity Units/ug</th>
<th>G6PD Activity Units/ug</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>Male</td>
<td>Low Line</td>
<td>4.75±0.22</td>
<td>0.186±0.046</td>
<td>0</td>
<td>0.151±0.069</td>
<td>0.0218±0.0015</td>
<td>0.00600±0.00037</td>
<td>0.00393±0.00031</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>High Line</td>
<td>8.38±0.53</td>
<td>0.245±0.025</td>
<td>0.778±0.062</td>
<td>0.391±0.11</td>
<td>0.0241±0.0011</td>
<td>0.00797±0.00062</td>
<td>0.00568±0.00048</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>Low Line</td>
<td>6.58±0.38</td>
<td>0.126±0.029</td>
<td>0.175±0.094</td>
<td>0.153±0.059</td>
<td>0.0214±0.0017</td>
<td>0.00741±0.00043</td>
<td>0.00456±0.00047</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>High Line</td>
<td>13.4±0.62</td>
<td>0.266±0.054</td>
<td>0.702±0.098</td>
<td>0.317±0.21</td>
<td>0.0250±0.0015</td>
<td>0.00896±0.00071</td>
<td>0.00551±0.00030</td>
</tr>
<tr>
<td>50%</td>
<td>Male</td>
<td>Low Line</td>
<td>50.8±2.3</td>
<td>0.306±0.039</td>
<td>0.772±0.052</td>
<td>0.867±0.11</td>
<td>0.0213±0.00096</td>
<td>0.00835±0.00026</td>
<td>0.00518±0.00019</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>High Line</td>
<td>64.5±1.2</td>
<td>0.389±0.025</td>
<td>0.905±0.030</td>
<td>0.990±0.010</td>
<td>0.0277±0.0016</td>
<td>0.0110±0.00073</td>
<td>0.00840±0.00043</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>Low Line</td>
<td>58.7±1.8</td>
<td>0.293±0.015</td>
<td>0.507±0.074</td>
<td>0.798±0.097</td>
<td>0.0184±0.0011</td>
<td>0.00801±0.00045</td>
<td>0.00483±0.00039</td>
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<tr>
<td></td>
<td>Female</td>
<td>High Line</td>
<td>74.4±2.1</td>
<td>0.366±0.022</td>
<td>0.846±0.050</td>
<td>0.950±0.050</td>
<td>0.0225±0.00070</td>
<td>0.0104±0.00074</td>
<td>0.00637±0.00038</td>
</tr>
<tr>
<td>30%</td>
<td>Male</td>
<td>Low Line</td>
<td>42.4±4.2</td>
<td>0.189±0.052</td>
<td>0.500±0.29</td>
<td>0.463±0.20</td>
<td>0.0252±0.0014</td>
<td>0.00830±0.00033</td>
<td>0.00758±0.00065</td>
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<tr>
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<td>High Line</td>
<td>54.8±1.4</td>
<td>0.307±0.044</td>
<td>1</td>
<td>0.902±0.077</td>
<td>0.0301±0.00072</td>
<td>0.0109±0.0010</td>
<td>0.00922±0.00036</td>
</tr>
<tr>
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<td>Low Line</td>
<td>41.7±3.3</td>
<td>0.207±0.029</td>
<td>0.125±0.12</td>
<td>0.206±0.15</td>
<td>0.0164±0.00069</td>
<td>0.00692±0.00042</td>
<td>0.00508±0.00038</td>
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<tr>
<td></td>
<td>Female</td>
<td>High Line</td>
<td>60.8±1.5</td>
<td>0.304±0.053</td>
<td>0.938±0.063</td>
<td>0.740±0.080</td>
<td>0.0269±0.0042</td>
<td>0.0115±0.0024</td>
<td>0.00728±0.0010</td>
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<tr>
<td>80%</td>
<td>Male</td>
<td>Low Line</td>
<td>41.4±3.0</td>
<td>0.236±0.035</td>
<td>0.724±0.12</td>
<td>0.900±0.10</td>
<td>0.0215±0.0016</td>
<td>0.00822±0.00076</td>
<td>0.00662±0.00047</td>
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<tr>
<td></td>
<td>Male</td>
<td>High Line</td>
<td>62.4±2.1</td>
<td>0.337±0.060</td>
<td>0.957±0.043</td>
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<td>0.0273±0.0016</td>
<td>0.0127±0.00092</td>
<td>0.00971±0.00060</td>
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<tr>
<td></td>
<td>Female</td>
<td>Low Line</td>
<td>55.9±4.0</td>
<td>0.272±0.047</td>
<td>0.698±0.026</td>
<td>0.667±0.23</td>
<td>0.0194±0.0022</td>
<td>0.00843±0.00045</td>
<td>0.00541±0.00036</td>
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<tr>
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<td>Female</td>
<td>High Line</td>
<td>65.1±2.8</td>
<td>0.362±0.010</td>
<td>0.892±0.10</td>
<td>0.984±0.016</td>
<td>0.0249±0.0021</td>
<td>0.00993±0.00088</td>
<td>0.00724±0.00055</td>
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