ALTERED GENE EXPRESSION AND BEHAVIOUR IN A DROSOPHILA MODEL FOR CHRONIC OXIDATIVE STRESS

by

Andrea Huston

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Cell and Systems Biology
University of Toronto

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Master of Science Thesis 2011  
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University of Toronto

**Abstract**

Reactive oxygen species (ROS) are a by-product of aerobic metabolism and have been implicated in cancer, artherosclerosis, diabetes and aging. Antioxidant enzymes, such as superoxide dismutase (SOD), work to neutralize ROS and oxidative stress occurs when the antioxidant capacity of the cell is overwhelmed. Using a *Drosophila* mutant with defective cytoplasmic SOD function (cSOD<sup>n108</sup>), we are able to study the consequences of excess ROS on gene expression. Microarray experiments indicate gene expression changes associated with immune response, heat shock, detoxification, proteolysis, carbohydrate metabolism, lipid metabolism and behaviour. Behavioural and physiological assays investigated possible phenotypes predicted by changes in gene expression. We found that cSOD<sup>n108</sup> mutants feed less yet demonstrate a remarkable resistance to starvation. In addition, cSOD<sup>n108</sup> mutants show a reduced response to sucrose, odorants and decreased locomotor activity. These phenotypes correlate with observed gene expression changes and suggest a potentially altered energy metabolism in response to chronic oxidative stress.
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<tr>
<td>Acp</td>
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<tr>
<td>ALS</td>
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<tr>
<td>AP-1</td>
<td>activating protein 1</td>
</tr>
<tr>
<td>AP-2</td>
<td>activating protein 2</td>
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<td>BP</td>
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<td>corrected probability</td>
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<td>DAM</td>
<td><em>Drosophila</em> activity monitor</td>
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<td>extracellular superoxide dismutase</td>
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CHAPTER 1

General Introduction
As a consequence of aerobic metabolism, organisms must cope with the endogenous production of reactive oxygen species (ROS). ROS include superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and the hydroxyl radical (OH). Mitochondria, the site of oxidative phosphorylation, produce the majority of reactive oxygen via the univalent reduction of molecular oxygen (Turrens, 2003). Univalent reduction of oxygen initially produces superoxide. Several sites along the electron transport chain are able to leak electrons to oxygen, causing superoxide formation. Approximately 4% of all molecular oxygen utilized undergoes this transformation (Turrens, 2003).

Superoxide precedes the formation of almost all other forms of ROS. Although considered a relatively weak oxidizing agent, superoxide can dismutate either spontaneously or enzymatically to produce more damaging oxygen species. Dismutation of superoxide generates hydrogen peroxide. Hydrogen peroxide can react via the Fenton reaction to produce the highly reactive hydroxyl radical. The hydroxyl radical is considered one of the strongest oxidants in nature and is thought to cause the majority of ROS-associated damage.

Cells are not without means to counteract the deleterious effects of ROS. Antioxidant enzymes form a defense network that exists as a component of the cellular environment. These enzymes include the superoxide dismutases (SODs), catalase (CAT), glutathione reductase (GR) and thioredoxin reductase (TrxR) (Landis et al., 2004).

Cellular antioxidants not only functions to neutralize ROS damage, but also to maintain ROS homeostasis. Despite the intrinsically destructive nature of reactive oxygen, cells have evolved to utilize ROS in a variety of processes such as cellular signaling and immune response (Scandalios, 2002). The maintenance of low steady-state
levels of superoxide (~$10^{-10}$ M$^{-1}$) has been argued to exert beneficial effects as superoxide may react to neutralize more deleterious radicals and thus terminate further propagation (Imlay and Fridovich, 1991; Liochev and Fridovich, 2007).

The superoxide dismutase family of enzymes remains critical in the maintenance of cellular ROS homeostasis. It has been estimated that cytosolic superoxide dismutase accounts for 0.4% of all soluble protein in *Drosophila* adult tissue (Lee et al., 1981). Superoxide dismutases catalyze the dismutation of superoxide to form hydrogen peroxide which is further broken down into water and oxygen by catalase and peroxidases (Zelko et al., 2002). The conversion of superoxide to hydrogen peroxide occurs as follows (McCord and Fridovich, 1969):

$$O_2^- + O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$$

Although superoxide can spontaneously dismutate to form hydrogen peroxide at a rate of $\sim 10^5$ M$^{-1}$ s$^{-1}$, superoxide dismutase increases the speed of this reaction to a diffusion-limited rate of $\sim 10^9$ M$^{-1}$ s$^{-1}$ (Liochev and Fridovich, 2007).

**The SOD family of enzymes**

Several different forms of superoxide dismutases (SODs) exist. All superoxide dismutases perform the same function and contain a catalytic metal atom in the active site (Fridovich, 1999). This metal co-factor can vary depending on the class of SOD enzyme. Molecular weight, subunit assembly, chaperone requirement and cellular localization can also vary between different SOD enzymes (Miao and St. Clair, 2009).

In eukaryotes, three forms of SOD occur: MnSOD and two distinct forms of CuZnSOD. Eukaryotic MnSOD (SOD2) is found exclusively in the mitochondrial matrix,
has a manganese atom at the active site and forms a homotetramer. Eukaryotic CuZnSOD exists as a homodimer with a copper and a zinc atom in the active site. The copper atom is required for catalytic activity and the zinc atom functions to stabilize protein structure (Miao and St. Clair, 2009). CuZnSOD was originally thought to localize to the cytosol; however, evidence shows that CuZnSOD also localizes to the intermembrane space of the mitochondria, the nucleus and to lysosomes (Zelko et. al., 2002). Despite the occurrence of CuZnSOD in a variety of subcellular structures, CuZnSOD is still frequently referred to as “cytoplasmic” CuZnSOD and commonly abbreviated as SOD1.

Another, more recently-discovered form of CuZnSOD localizes to the extracellular space. Extracellular CuZnSOD (EC-SOD or SOD3) is the least characterized of the eukaryotic SODs (Fridovich, 1999). SOD3 forms a homotetramer, becomes glycosylated and contains a signal sequence targeting the enzyme for secretion (Fridovich, 1999).

**SOD1**

The SOD family of enzymes has been characterized in terms of gene structure, regulation, expression and protein activity. For the purpose of this thesis, further discussion will focus on SOD1.

**Gene structure**

SOD1 gene structure varies among different organisms. In mammals, the SOD1 gene contains five exons and four introns (Miao and St. Clair, 2009). In *Drosophila*, there
are two exons separated by a single intron. SOD1 gene structures are shown for bovine, mouse, human and fly (Figure 1.1).

**Gene regulation:**

SOD1 is constitutively and stably expressed in the cytosol (Zelko et. al., 2002). However, mRNA levels fluctuate depending on physiological conditions. The SOD1 promoter region is GC-rich and contains a TATA and CCAAT box (Miao and St. Clair, 2009). Both promoter and intron regions are important in SOD1 gene regulation as they contain binding sites for various regulatory factors. Binding sites for the following transcription factors have been identified in mammals: NF-κB, AP-1, AP-2, Sp1, and CCAAT-enhancer-binding proteins (C/EBP) (Miao and St. Clair, 2009).

Epigenetic modification is not considered a common mechanism of SOD1 gene regulation as promoter methylation patterns were found unchanged regardless of SOD1 activity (Oates and Pamphlett, 2007). However, epigenetic silencing of SOD2 has been reported, particularly in cases of human breast cancer (Huang et. al., 1999; Hitchler et. al., 2006; Hitchler et. al., 2008). Post-transcriptional regulation, such as mRNA processing and microRNA modulation, has not been a widely investigated regulatory mechanism (Miao and St. Clair, 2009). Currently, modification of 3’UTR length is the only known post-transcriptional regulation of SOD1 that occurs. Altering 3’UTR length was found to affect SOD1 enzyme activity and aberrant 3’UTRs have been associated with motor neuron dysfunction (Kilk, et. al., 1995; Shaw, et. al., 1997).
**Bos taurus**

**Mus musculus**

**Homo sapien**

**Drosophila melanogaster**

Figure 1.1: SOD1 gene structures shown for *Bos taurus*, *Mus musculus*, *Homo sapien* and *Drosophila melanogaster*. The mammalian SOD1 gene typically contains 5 exons and 4 introns, whereas Drosophila SOD1 contains 2 exons separated by a single intron. Images were obtained from the Ensembl Genome Brower ([www.Ensembl.org](http://www.Ensembl.org)).
**Post-translational regulation:**

The SOD1 protein requires post-translational modification to become active. Activation involves insertion of a metal cofactor and formation of an intramolecular disulfide bond. Two pathways of SOD1 activation have been identified: The copper chaperone (CCS)-dependent pathway and the poorly understood CCS-independent pathway (Kirby et. al., 2008). CCS catalyzes the insertion of copper and the oxidation of the SOD1 disulfide bond leading to enzyme activation and stabilization (Kirby et. al., 2008). The pathway utilized for SOD1 activation varies between different organisms. *Drosophila* relies primarily on the CCS-dependent pathway but some evidence suggests that CCS-independent activation is partially utilized (Kirby et. al., 2008).

**Drosophila SOD1**

*Drosophila* SOD1 constitutes 0.4% of total soluble protein in adult tissue and functions as a critical regulator of ROS homeostasis (Lee et. al., 1981). The *Drosophila* SOD1 protein exists as a homodimer of 32 kilodaltons and has two naturally occurring allozyme variants (SODF and SODS). These variants differ in electrophoretic mobility, thermostability and specific activity (Campbell et. al., 1986; Ayala et. al., 1971; Lee et. al., 1981).

The *Drosophila* SOD1 gene spans 1.8-kb, contains two exons and a single 712-bp intron. The *Drosophila* SOD1 promoter is not well characterized; however, regions important for spatial and temporal expression have been determined. An 1140-bp fragment encompassing the intron and exon 2 is required for normal SOD1 transcript and
protein expression. The pattern of transcript and protein expression also varies with developmental stage (Radyuk et. al., 2004).

Embryonic SOD1 transcripts are maternally derived (Missirlis et. al., 2001). Protein and mRNA expression remains ubiquitous during embryogenesis but falls slightly during the larval and pupal stages with expression adopting more discrete patterns during the three larval stages (Radyuk et. al., 2004). First instar larvae show relatively low SOD1 mRNA and protein abundance with some localization to salivary glands. Second instar larvae have SOD1 mRNA and protein localization to the salivary glands and gut epithelium. Third instar localization occurs in the salivary glands, gut epithelium, oenocytes, malpighian tubules, ring gland and reproductive organs. In pupae, SOD1 mRNA and protein is initially seen in the malpighian tubules and gut; however, after 12 hours localization to the fat bodies and peripheral nervous system occurs. Adult flies show high SOD1 mRNA and protein expression in the gut with patterns similar to late larval development (Radyuk et. al., 2004).

**SOD enzymes in disease and aging**

ROS-associated damage has been implicated in various pathologies including cancer (Wiseman and Halliwell; 1996, Schumacker, 2006), artherosclerosis (Lusis, 2000), diabetes (Droge, 2002), and neurodegenerative disorders (Noor et. al., 2002). Neurodegenerative disorders such as Huntington’s, Parkinson’s, Alzheimer’s and Amyotrophic lateral sclerosis (ALS) are each associated with ROS, suggesting that neural tissue forms a vulnerable target. SOD1 mutations have been specifically linked to ALS (Rosen et. al., 1993; Rosenblum et. al., 1996; Maier and Chan, 2002).
Familial Amyotrophic Lateral Sclerosis (fALS), a heritable form of ALS, remains one of the most widely studied diseases associated with SOD enzyme dysfunction. ALS affects the nervous system, causing progressive and fatal degradation of motor neurons in the corticospinal tracts and brain stem (Maier and Chan, 2002). Currently more than 150 different mutations within the SOD1 coding region are linked to fALS disease progression (Rosen et. al., 1993; Siddique and Deng, 1996; Turner and Talbot, 2008). The most common occurring ALS mutation in humans is Ala4Val (exon 1) (Rosen et. al., 1993).

Although SOD1 mutation only accounts for a relatively small percentage of overall ALS cases (2%) and 15-20% of fALS cases, the discovery of a direct cause of ALS proved critical in understanding the underlying mechanism of pathology (Rosen et. al., 1993; Ince et. al., 1998). Perhaps most surprising was the finding that fALS mutations are dominant and cause a “gain-of-function” in which the SOD1 enzyme remains active yet altered in catalytic specificity. It was previously believed that a loss of SOD1 enzyme activity caused ALS. This notion seems intuitive given evidence that a loss of SOD1 function can result in defective locomotor activity and retinal degradation (Phillips et. al, 1995).

In addition, SOD enzymes have been investigated as potential modulators of aging. ROS are considered central to the aging process and remain at the foundation of the free radical theory of aging originally proposed by Denham Harman (Harman, 1956). The free radical theory of aging, later re-named the oxidative stress theory of aging to encompass non-radical toxic oxygen intermediates, remains supported to date by a large body of evidence (Zou et. al., 2000).
According to the oxidative stress theory of aging, the accumulation of oxidative damage eventually overwhelms the capacity of cellular defense and repair networks, leading to senescence (Beckman and Ames, 1998). Mitochondria produce the majority of \( \text{O}_2^-/\text{H}_2\text{O}_2 \) that causes oxidative damage and the generation of mitochondrial \( \text{O}_2^-/\text{H}_2\text{O}_2 \) has been found to be inversely proportional to maximum lifespan (Ku et. al., 1993; Barja and Herrero, 2000). The accumulation of oxidatively damaged macromolecules has also been shown to increase exponentially with age (Sohal and Weindruch, 1996). Damage, such as the cross-linking of proteins and lipids, can disrupt the normal function of cells and ultimately affect lifespan. For example, protein carbonyl content in flies is inversely proportional to maximum lifespan and organisms with increased longevity generally incur relatively lower amounts of oxidized lipids, protein, and DNA (Sohal et. al., 1993; Sohal et. al., 1995; Agarwal and Sohal, 1996).

Currently, SOD2 mutation is associated with Progeria, a disease with a devastating range of symptoms that include limited growth, frailty, cardiovascular problems and accelerated aging (Rosenblum et. al., 1996). Altered SOD1 activity can either increase or decrease lifespan. However, transgenic modification of SOD1 as a means to attenuate aging has produced mixed results (Phillips et. al., 1989; Parkes et. al., 1998, Sun and Tower, 1999). These studies will be briefly discussed later in this chapter.

**Drosophila studies of SOD1**

*Drosophila* mutants with defective SOD1 function have been generated to study the effects of disrupted ROS homeostasis (Phillips et. al., 1989). One of the *Drosophila* SOD1 mutations generated (via EMS) is known as cSOD\(^{n108}\). The cSOD\(^{n108}\) mutation
disrupts the ability of SOD1 to effectively dimerize, resulting in a severe hypomorph (Phillips et. al., 1989). The cSOD\textsuperscript{n108} allele was subsequently recombined with the larval marker red (Phillips et. al., 1989). The resulting chromosome was balanced over TM3, conferring the visible phenotypes Stubble and Serrate (Sb, Ser).

Flies homozygous for the cSOD\textsuperscript{n108} allele have dramatically decreased lifespan (11.8-day mean adult lifespan; median lifespan 10\% of wild type). The early mortality and onset of senescence observed in cSOD\textsuperscript{n108} mutants mimics premature aging (Rogina and Helfand, 2000). Mutants appear relatively normal as embryos and larvae; however, a high mortality rate is associated with late pupae and early adults (Phillips et. al., 1989).

Other phenotypes include frequent eclosion lethality (~27\% eclose), male sterility and female semi-sterility, sensitivity to paraquat, hypersensitivity to ionizing radiation and hypersensitivity to glutathione depletion (Phillips et. al., 1989; Parkes et. al., 1998; Missirlis et. al., 2001). Increased DNA damage has also been observed in cSOD\textsuperscript{n108} flies. The negative charge associated with DNA attracts positively charged transition metals, creating an environment for the local generation of hydroxyl radicals. This leads to an increased rate of spontaneous somatic and germ line mutation, with mutations occurring preferentially in somatic cells (Phillips et. al., 1995; Woodruff et. al., 2004). The importance of SOD1 in maintaining genomic integrity has also been noted in other organisms, particularly in microbes (Storz et. al., 1990; Gralla and Kosman, 1992).

Loss of cytosolic aconitase activity is also known to occur in cSOD\textsuperscript{n108} mutant flies (Woodruff et. al., 2004; Kirby et. al., 2008). Aconitase contains a 4Fe-4S cluster which forms a preferential superoxide target (D’Autréaux and Toledano, 2007).
Oxidation of $4\text{Fe}-4\text{S}$ converts cytoplasmic aconitase to iron-regulatory protein-1 (IRP-1), possibly leading to changes in gene expression (Missirlis et. al., 2003).

**Manipulation of SOD1 expression to extend lifespan and rescue $c\text{SOD}^{n108}$ mutant phenotypes**

Manipulation of SOD1 expression has been shown to rescue deleterious phenotypes in $c\text{SOD}^{n108}$ mutant flies and to extend lifespan in wild type flies.

Three different groups overexpressing SOD1 in wild type flies reported an increase in resistance to oxidative stress and limited lifespan extension (Orr and Sohal, 1994; Parkes et. al., 1998; Sun and Tower, 1999). Sun and Tower obtained a 12% increase in mean lifespan using the FLP binary transgenic system to induce adult-specific SOD1 overexpression. Orr and Sohal were able to increase mean lifespan by 34% when SOD1 and catalase were overexpressed simultaneously. This study was later criticized due to a lack of adequate controls and failure to account for insertion position effects (Sohal et. al., 2002; Orr and Sohal, 2003).

Other attempts to increase lifespan in wild type flies via SOD1 overexpression using a native promoter or a constitutively expressed actin5C promoter produced little to no effect, or even a deleterious effect on lifespan (Reveillaud et. al., 1991; Seto et. al., 1990; Orr and Sohal, 1993).

Further work by Parkes et. al. investigated the concept that a particular tissue or cell-type can limit longevity. Motor neurons became of interest due to their vulnerability to ROS damage, lack of detectable SOD1 activity, and role in ALS disease progression (Klichko et. al., 1999). Human SOD1 was overexpressed in the motor neurons of wild
type flies, causing a remarkable 40% extension of lifespan (Parkes et. al., 1998). Expression of human SOD1 in the motor neurons of cSOD\textsuperscript{n108} mutant flies rescued all deleterious phenotypes providing intriguing evidence that neural tissue can mediate the effects of ROS damage (Parkes et. al., 1998).

The concept that a particular tissue could limit lifespan has generated considerable interest. It remains possible that the vulnerability of the nervous system to ROS damage can induce senescence for the entire organism. In the absence of predation, death of laboratory Drosophila may be caused by failure of the nervous system (Parkes et. al., 1998). Another possibility remains that alteration of ROS equilibrium in neural tissue alters synaptic transmission, leading to organism-wide changes in physiology and behaviour, thus modulating lifespan (Parkes et. al., 1998). Indeed, a prominent role for neuroendocrine signaling has been reported as a regulator of lifespan in several organisms. In Caenorhabditis elegans, the daf-2 and age-1 insulin/IGF signaling mutants produce a long-lived phenotype with lifespan extension up to 200% (Kimura et. al., 1997; Morris et. al., 1996). The Drosophila insulin/IGF signaling mutant, chico, extends female maximum lifespan by 41% (Clancy et. al., 2001). These findings have led to the investigation of the insulin signaling pathway as a central regulator of stress resistance and lifespan.

Other attempts to rescue deleterious phenotypes associated with SOD1-deficiency have included hypoxia and vitamin supplementation treatment. Vitamin supplementation in food media partially rescues the shortened lifespan of SOD1-defecient flies. Supplementation with α-tocopherol (25 I.U./mL) produced the most significant effect, extending the lifespan of SOD1-deficient flies to a maximum of ~32 days from ~18 days.
Retinol supplementation (1000 I.U./mL) extended lifespan to a maximum of ~26 days and ascorbic acid supplementation was found to produce no effect on lifespan (Bahadorani et. al., 2008).

Chronic hypoxia (5% O₂) was also shown to rescue the shortened lifespan associated with SOD-deficiency (Wicks et. al., 2009). SOD1- and SOD2-deficient Drosophila mutants maintained under chronic hypoxia had a dramatic increase in lifespan (~fourfold) as compared to mutants maintained under normoxia (21% O₂). Remarkably, SOD2-deficient mutants initially raised under normoxia and then transferred to hypoxia were able to recover normal lifespan. In contrast, this treatment failed to recover lifespan in SOD1-deficient mutants indicating a compartment-specific mechanism of oxidative damage that is not reversible in the intermembrane space of the mitochondria and cytosol (Wicks et. al., 2009). Given this result, it becomes apparent that further study is needed to elucidate how oxidative damage is managed.

Transgenic rescue of SOD1 function via insertion of a genomic copy of Drosophila cSOD restores all deleterious phenotypes associated with the cSODⁿ¹⁰⁸ allele and provides a suitable control for further genetic characterization of cSOD-null mutants. Transgenic flies are identical in background to cSODⁿ¹⁰⁸ mutants with the exception of a P element insertion containing a 1.8-kb genomic region of the cSOD gene that includes the endogenous promoter. This insertion consists of approximately 340-bp of upstream promoter sequence, a 413-bp 5’UTR, 247-bp of 3’UTR, and the 712-bp intron (Seto et. al., 1990). The amount of upstream non-coding sequence included in the insert is limited by the close proximity of other genes to cSOD in the Drosophila genome.
The generation of a transgenic rescue not only provides an appropriate control for gene expression, behavioural, and other studies, but also demonstrates that cSOD\textsuperscript{n108} phenotypes arise from SOD1 mutation alone. Interestingly, the transgene restores approximately 40% of SOD1 enzymatic activity yet fully recovers viability and fertility (Parkes et al., 1998). This is consistent with the suggestion that only a small fraction of available SOD1 is required to maintain organism viability while the bulk of SOD1 is used as defense against oxidative stress challenges (Kirby et al., 2008).

**Understanding the oxidative stress response**

The oxidative stress response has been largely studied in prokaryotes (Storz and Imlay, 1999). In bacteria, oxidative insult is sensed through specialized regulatory proteins that directly detect levels of superoxide and hydrogen peroxide: SoxR/S and OxyR (Wu and Weiss, 1991; Zheng and Storz, 2000). SoxR/S and OxyR function as transcription factors, inducing the expression of genes that provide immediate antioxidant protection: for example NADH-ferredoxin oxidoreductase and manganese-superoxide dismutase (Zheng et al., 2001).

Eukaryotes have no known homologs of SoxR/S and OxyR and instead utilize a much larger network of regulators (Scandalios, 2002). Oxidative stress in eukaryotes has been primarily investigated using yeast and *Arabidopsis* models (Gasch et al., 2000; Desikan et al., 2001). Initial genomic studies indicate that a much larger portion of the eukaryotic genome is utilized in the oxidative stress response that previously assumed. For example, nearly 1/3 of the yeast genome was found to respond to oxidative injury induced by H\textsubscript{2}O\textsubscript{2} (Causton et al., 2001; Gasch et al., 2000). In *Arabidopsis*, oxidative
stress not only induced genes with antioxidant function, but also genes involved in the defense response, cell signaling, transcription, and cell rescue (Desikan et. al., 2001).

In higher eukaryotes, such as *Drosophila*, the oxidative stress response has only recently begun to be understood. The majority of studies rely on the administration of \( \text{H}_2\text{O}_2 \) or paraquat to induce acute stress. Exposure to paraquat induces gene changes in the following functional categories: peptidases, peptidase inhibitors, lipases, glutathione transferases and oxidoreductases (Girardot et. al., 2004). \( \text{H}_2\text{O}_2 \) produces similar gene changes, suggesting a general pattern for an acute oxidative stress response in higher eukaryotes (Girardot et. al., 2004).

Conditions of chronic oxidative stress have not been extensively explored at the genomic level in *Drosophila*. Acute oxidative stress is often associated with changes in gene expression that lead to the immediate repair and elimination of damage. Chronic oxidative stress, as thought to occur in cSOD^{n108} mutant *Drosophila*, may initiate long-term physiological programs to cope with prolonged injury. These programs potentially involve neuroendocrine mechanisms, resulting in altered physiology and behavioural patterns. Examining cSOD^{n108} mutants could provide insight into conditions associated with chronic inflammation, such as metabolic disorder, cancer, neurodegenerative disease and aging.

*Research Question 1: What are the changes in gene expression associated with the cSOD^{n108} Drosophila mutant?*

Our goal is to examine the global gene expression changes associated with chronic oxidative stress using the cSOD^{n108} *Drosophila* mutant. ROS are known to
modulate gene expression via the following signaling pathways: Ras/RAF/MEK/ERK, p38, JNK (Allen, 1998; Fuh et. al., 2002). The potential of ROS to modulate signal transduction likely results in an altered transcriptional profile.

To characterize gene expression changes resulting from cSOD\textsuperscript{n108} mutation, NimbleGen arrays were used to examine the transcriptional profiles of 2-3-day old males, 5-6-day-old males and 2-3-day old females for cSOD\textsuperscript{n108} mutants as compared to transgenic controls. Functional analysis was performed to determine significantly enriched GO Ontology terms, allowing us to gain biological meaning from lists of altered transcripts showing 2-fold or greater change (P<0.05).

**Research Question 2: Does SOD\textsuperscript{n108} mutation alter feeding-related behaviours?**

Preliminary microarray analysis of cSOD\textsuperscript{n108} mutants indicated gene changes associated with a defense response, proteases, lipases, carbohydrate metabolism, gustatory perception and odorant perception (C. Mahon and T. Westwood, unpublished). Altered expression of genes involved in carbohydrate and lipid metabolism may function to adapt SOD\textsuperscript{n108} mutants to the possibility of prolonged food inaccessibility. Metabolic adaptation to stress is known to be regulated via JNK signaling (Karpac et. al., 2009), which works to suppress insulin signaling and the mobilization of energy stores (Hull-Thompson et. al., 2009). Changes in gustatory and odorant perception may be related to an adaptive metabolic stress response, leading us to question how feeding-related behaviours may be altered.
To determine whether gene expression changes are reflected in feeding-related behaviours, we examined food intake, starvation stress resistance, odorant perception, sucrose response, and locomotor activity.

This thesis combines genomic techniques with behavioural and physiological assays in order to present a holistic view of oxidative stress. Research Question 1 will be the focus of Chapter 2 and Research Question 2 will be the focus of Chapter 3.

Using a range of assays, my aim is to provide insight into how transcriptional programs translate into output.
CHAPTER 2

Oxidative Stress Induces Transcriptional Changes in

Superoxide Dismutase Drosophila Mutants
Abstract

Oxidative stress results from an imbalance in ROS generation and the mechanisms that work to neutralize ROS and ROS-associated damage. Neutralization of ROS occurs through the action of antioxidant enzymes such as the superoxide dismutases, catalase and glutathione peroxidase. Disruption of ROS homeostasis leads to altered signal transduction and transcription factor activity, potentially affecting transcriptional profiles. Genomic studies of the Drosophila oxidative stress response have largely relied on acute induction of oxidative stress which does not necessarily reflect the chronic oxidative condition associated with ROS-associated pathologies. Using a Drosophila mutant with defective superoxide dismutase function (cSODn108) and a transgenic rescue control, we are able to study the consequences of excess ROS on gene expression using microarray technology. Transcriptional profiles were determined for 2-3-day old males, 5-6-day old males and 2-3-day old females. Results show that a large part of the transcriptome is altered in all three groups, with the direction and magnitude of changes being similar between groups. Altered transcripts are associated with the processes of immune response, heat shock, detoxification, proteolysis, iron-homeostasis, carbohydrate metabolism, lipid metabolism and behaviour.

We are able to conclude that chronic oxidative stress induces transcriptional changes associated with a range of different processes. Some of these processes may lead to altered organism physiology and behaviour. Further characterization of cSODn108 mutant phenotypes may provide insight into how transcriptional alterations relate to physiological and behavioural phenotypes.
Introduction

Destructive intermediates derived from oxygen are known as reactive oxygen species (ROS). These include the superoxide anion ($\text{O}_2^-$), hydrogen peroxide ($\text{H}_2\text{O}_2$), and the highly reactive hydroxyl radical (‘OH) (Fridovich, 1999). ROS are generated in cells as a consequence of aerobic energy metabolism and through the activity various enzymes such as the cytochrome P450s (Zangar et. al., 2004), NADPH oxidases (Bedard and Krause, 2007), cyclooxygenases (Versteilen et. al., 2010), and lipoxygenases (Kim et. al., 2008). Cellular antioxidant enzymes exist to counteract ROS and include the superoxide dismutases (SODs), catalase (CAT), glutathione peroxidase, thioredoxin, and peroxiredoxins (Miao and St. Clair, 2009). Cells also possess non-enzymatic means to neutralize ROS. Small molecule antioxidants, such as α-tocopherol, β-carotene, ascorbate and glutathione, compete with other biological molecules as substrates for oxidation (Droge, 2002).

The balance between the rate of ROS production and the rate of ROS clearance determines ROS homeostasis (Droge, 2002). Oxidative stress occurs when this balance is disrupted, either through depletion of antioxidants or through increases in ROS. Oxidative stress can lead to the accumulation of macromolecular damage, altered protein function and the initiation of apoptosis (Stadtman, 2004; Evans et. al., 2004).

Oxidative stress has been implicated in various pathologies including cancer (Wiseman and Halliwell; 1996, Schumacker, 2006), artherosclerosis (Lusis, 2000), diabetes (Droge, 2002), and neurodegenerative disorders (Noor et. al., 2002). In addition, oxidative stress remains a major mechanism of aging (Zou, 2000).
All cells are capable of sensing and responding to oxidative stress (Scandalios, 2002). Cells can gauge ROS levels using the ratio of NADPH to NADP+ (Liochev and Fridovich, 1992a). In addition, proteins susceptible to oxidation can adopt regulatory roles. Superoxide is highly reactive with proteins containing iron-sulphur clusters [Fe-S] (D’Autréaux and Toledano, 2007). Oxidation of [Fe-S]-containing SoxR in bacteria activates the SoxR-bound DNA operator and results in transcription of manganese-superoxide dismutase and fumarase C (Liochev and Fridovich, 1992b). Oxidation of aconitase, a tricarboxylic acid cycle enzyme containing [4Fe-4S], converts cytoplasmic aconitase to iron regulatory factor-1 (IR1). IR1 induces expression of genes involved in iron homeostasis, allowing cells to reduce hydroxyl radical generation (Missirlis et. al., 2003). Hydrogen peroxide reacts preferentially with the thiol side chain of cysteine residues, allowing specific proteins to function as regulatory cysteine switches (Paulsen and Carroll, 2010). ROS are also known to alter various signal transduction pathways: Ras/RAF/MEK/ERK, p38, JNK (Fuh, et. al., 2002). The downstream effects of signaling cascades often involves alteration of gene transcription, leading us to question the extent of global transcriptional changes in response to ROS imbalance.

The transcriptional profiles associated with oxidative stress have largely been defined using prokaryotic models (Scandalios, 2002). Transcription of genes that protect against further oxidative damage, such as the ROS scavengers glutathione and thioredoxin, characterize the prokaryotic response (Wu and Weiss, 1991). This response is rapid and highly dynamic, primarily involving transcription of antioxidant enzymes (Liochev and Fridovich, 1992c).
The global transcriptional changes associated with oxidative stress have only recently begun to be understood in higher eukaryotes (Scandalios, 2002). Higher eukaryotes lack the fast, dynamic response observed in single-celled organisms. Instead, transcriptional programs integrate multiple responses, which may lead to various physiological adaptations (Hull-Thompson et. al., 2009; Karpac et. al., 2009; Berdichevsky et. al., 2010).

To date, genomic studies of oxidative stress in Drosophila have relied on oxidizing agents, such as paraquat or hydrogen peroxide, to induce acute stress (Girardot et. al., 2004; Landis et. al., 2004, Landis and Tower, 2005). Acute oxidative stress presents an imperfect model for understanding the chronic stress analogous to ROS-related pathologies such as neurodegeneration, cancer, diabetes and aging.

In order to study chronic oxidative stress, a Drosophila mutant with defective Cu, Zn-superoxide dismutase (SOD1) function was generated via treatment with ethyl methanesulfonate (EMS) (Campbell et. al., 1986; Phillips et. al., 1989). The resulting allele, cSOD\textsuperscript{n108}, represents a severe hypomorph with almost no detectable SOD1 activity (Phillips et. al., 1989). Mutants lack the ability to effectively scavenge superoxide, producing “enfeebled” flies with severely reduced viability. Associated phenotypes include a high eclosion lethality, decreased adult lifespan, sensitivity to oxidizing agents, male sterility and female semi-sterility (Phillips et. al., 1989, Parkes et. al., 1998).

Several additional phenotypes have been more recently identified in cSOD\textsuperscript{n108} flies. These phenotypes include behavioural defects such as a reduced response to odorants and sucrose, decreased feeding and reduced locomotor activity (see Chapter 3).
Chronically stressed cSOD\textsuperscript{n108} flies also show a surprising resistance to starvation and contain significantly more lipid stores than controls (see Chapter 3).

Characterizing the gene expression changes associated with the cSOD\textsuperscript{n108} condition may elucidate physiological and behavioural adaptations initiated in response to chronic oxidative stress. In this Chapter, we aim to characterize gene expression changes for cSOD\textsuperscript{n108} mutants using a transgenic rescue as the control. Transcriptional alterations will be examined in 2-3-day old males, 5-6-day old males and 2-3-day old females and compared. Functional analysis of transcripts showing altered expression allows the identification of categories of enriched GO terms which in turn will help determine the biological significance of observed gene changes. This analysis may identify novel gene networks activated under conditions of chronic oxidative stress and provide rationales for the phenotypes observed in cSOD\textsuperscript{n108} mutants.
Materials and Methods

**Fly Lines and Handling**

cSOD\textsuperscript{n108} red

The cSOD\textsuperscript{n108} allele is carried on the third chromosome (3L; 68A) and marked with the visible larval/adult marker, red, which confers red malpighian tubules in larvae and red-brown eye colour in adults (Phillips et al. 1989). Homozygous cSod\textsuperscript{n108} flies, referred to as “T0”, are sterile and unable to produce progeny. In order to propagate, cSod\textsuperscript{n108} flies are maintained as a balanced stock: cSOD\textsuperscript{n108} red/TM3 [In (3LR) TM3 Sb Ser ri p\textsuperscript{p} sep bx\textsuperscript{34e} e\textsuperscript{e}] or cSOD\textsuperscript{n108} red/TM3. Heterozygous cSOD\textsuperscript{n108} red/TM3 flies produce progeny and display Stubble (Sb) bristles and Serrate (Ser) wings.

Transgenic Rescue of cSOD\textsuperscript{n108} red

To provide an adequate control, a transgenic rescue was generated containing the Drosophila SOD1 gene and its endogenous promoter region. The transgene contains a 1.8-kb genomic fragment encompassing the coding region, the single 725-bp intron, 413-bp of 5’ untranslated region and 247-bp of 3’ flanking DNA (Seto et. al., 1990). Transgenic flies, T5/T5, cSOD\textsuperscript{n108}red/TM3 or “T5”, share an identical background to cSOD\textsuperscript{n108} red mutants with the exception of the transgene. The transgene was shown to fully restore all deleterious phenotypes originally characterized in cSOD\textsuperscript{n108} red flies (Parkes et. al, 1998).
**Fly Rearing**

All strains were maintained at 25°C on a 12-hour light/dark cycle (lights on at 9am) and standard fly media (recipe available from the Bloomington Drosophila Stock Center at Indiana University: http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/bloomfood.htm). Propionic acid was added to fly media (4.5mL/L of media) to limit growth of microorganisms. Flies were transferred to bottles (6oz polypropylene square bottom, available at: http://www.flystuff.com/bottles.php) containing fresh media every 2 weeks or as needed. Bottle density was controlled with approximately 100 adult flies per bottle allowed to lay eggs on fresh media for 2 days and then discarded or transferred to another set of fresh bottles.

**Microarray Experimental Procedure**

**Sample collection**

Homozygous cSOD<sup>n108</sup> red flies were collected from a balanced stock (cSOD<sup>n108</sup> red/TM3). Only ~10% of the stock population consists of homozygous cSOD<sup>n108</sup> red mutants due to high eclosion lethality. A minimum of 32 bottles were required in order to obtain adequate yields of experimental flies. Collection entailed clearing bottles of adults and allowing 24-hours for pupae to eclose. Flies 0-24-hours old were collected and transferred to fresh bottles and reared at 25°C on a 12-hour light/dark cycle for an additional 48-hours (2-3-day old cohort) or an additional 120-hours (5-6-day old cohort). Flies 2-3-days or 5-6-days old were then briefly anesthetized (<5min) using CO<sub>2</sub> and sorted by genotype and sex. Groups of 50-60 flies were collected in 2mL screw-cap tubes and immediately snap-frozen in liquid nitrogen and stored at -80°C until RNA
extraction was performed. Mutant SOD\textsuperscript{n108} fly samples (T0) and transgenic control samples (T5) were collected in parallel. Triplicate biological samples, for each sample group, were prepared.

RNA isolation and labeling

RNA was isolated according to CDMC protocol with minor modifications (www.flyarrays.com). Frozen samples consisting of 50-60 male or female flies were homogenized in 1mL of TRIzol Reagent (Invitrogen, cat. #15596-018) and incubated at room temperature for 5 minutes. 200μL of chloroform was added, followed by 5 minutes of incubation at room temperature and centrifugation for 15 minutes at 4°C and 12 000g. Approximately 400μL of the aqueous phase was transferred to a new tube and an equal volume of ice-cold isopropanol was added followed by incubation at room temperature for 10 minutes. Samples were then centrifuged at 12 000g for 10 minutes. The resulting RNA pellet was washed twice with 75% ethanol. After the second ethanol wash, RNA was centrifuged at maximum speed for 30 seconds and remaining ethanol was removed using a pasture pipette. RNA pellets were then allowed to air dry for approximately 5 minutes or until the edges of the pellet appeared translucent. Pellets were re-suspended in 50μL nuclease-free water. RNA samples had a resulting concentration of approximately 2000-3000ng/μL.

Aliquots of extracted RNA were run on a glyoxylated RNA gel to determine RNA integrity (CDMC protocol available at www.flyarrays.com). An absorbance reading (OD) of each RNA sample was obtained using a NanoDrop ND-1000 UV-Vis Spectrophotometer. OD 260/280 and 260/230 readings were generally >2.00 indicating
that extracted RNA was clean (no contamination from chaotropic salts, phenol or protein) and required no additional purification.

Indirect fluorescent labeling of cDNA was performed according to CDMC protocol with minor modifications (www.flyarrays.com). RNA was reverse transcribed using 15μg of total RNA. RNA was incubated (65°C for 5 minutes followed by 42°C for 5 minutes) with a master mix containing 5X Superscript II buffer (Invitrogen, cat. # 18064-014), AncT primer (100μM), dNTP-dTTP mix (20mM), dTTP (2mM), AA-dUTP (4mM) and 0.1 M DTT (Invitrogen, cat. # 18064-014). 2.0μL of Superscript II RT (Invitrogen, cat. # 18064-022) was then added and the reaction was incubated for 3 hours at 42°C. Resulting cDNA samples are amino-modified due to the incorporation of amino-modified dUTP (AA-dUTP) and dATP during synthesis. Template RNA was degraded using alkaline hydrolysis (3M NaOH). The reaction was neutralized with the equivalent concentration of hydrochloric acid (3M HCl) and 65mM TRIS pH=7.5. Unincorporated nucleotides, primers, and buffers were removed using the Purelink purification kit (Invitrogen, cat. # K3100-01; protocol available at: http://products.invitrogen.com/ivgn/product/K310001). The wash solution provided in the kit was substituted by 80% ethanol since it has been found by our lab to inhibit conjugation of aa-UTP substituted cDNA with Alexa dyes. cDNA was eluted twice with 50μL of nuclease-free water and precipitated by one volume of ice-cold isopropanol in the presence of 0.3M sodium acetate and 40μg glycogen. cDNA was left to precipitate overnight at -20°C.

Precipitated cDNA was centrifuged at maximum speed for 30 minutes at room temperature. Resulting cDNA pellets were washed with 75% ethanol and re-suspended in
6μL nuclease-free water. A 1μL aliquot of each cDNA sample was removed in order to assess spectrometric curves (NanoDrop ND-1000 UV-Vis Spectrophotometer). Modified cDNA was coupled with Alexa Fluor 555 and 647 succinimidyl esters (Invitrogen, cat. # L1014-06). Alexa647 was added to cDNA from T0 flies and Alexa555 was added to cDNA from T5 flies. Incorporation of the fluorescent dyes was allowed to occur for 1 hour at room temperature in the dark. OD readings verified the incorporation of Alexa dyes (NanoDrop ND-1000 UV-Vis Spectrophotometer). Matched T0 and T5 labeled cDNA was combined and unincorporated dye was removed using the Purelink purification kit as previously described. The resulting pellets were re-suspended in hybridization solution containing 2X Hybridization Buffer, Hybridization Component A and Alignment Oligo (kit available from Roche-NimbleGen, cat. # 05583683001). (This hybridization solution is not described by CDMC protocol). Labeled cDNA was then loaded onto NimbleGen microarray slides (slides described below) using an X4 Mixer (Roche-NimbleGen, cat. # 05223733001) and hybridization was allowed to occur for 16-20 hours using the Hybridization System 4 instrument from NimbleGen. Following hybridization, slides were washed using the NimbleGen Wash Buffer Kit (cat. # 05584507001) according to protocol: (http://www.nimblegen.com/products/kits/processing/wash-buffer/index.html).

After washing, slides were dried briefly (2-3 seconds) using nitrogen gas and scanned as described below.
**The NimbleGen Array Platform and Acquisition of Microarray Data**

Custom NimbleGen arrays for Drosophila were used (OID18792). Each array contains a total of 72,000 probes covering 15,473 target genes (Dm 4.3). Each microarray slide holds a total of 4 arrays. Slides were scanned using an Axon 4000B scanner and GenePix Pro 6.0 scanning software. Scanning was done using 33% laser power and several scans for each array were done at PMT settings between 600-620 for Alexa647 (red) and 500-530 for Alexa555 (green). Histograms for corresponding red and green curves at different PMT settings were examined in GenePix Pro to determine which scans showed the appropriate dynamic range. Only red and green scans with highly similar overall intensities were used for analysis. The selected scans were loaded into NimbleScan software with the following design file: 081202_DM_TW_exp.ndf. Contrast and brightness was optimized for each image using default settings. Each array was aligned and gridded automatically using NimbleScan software and NimbleScan call and paired files were generated.

**Analysis of Microarray Data**

**Generation of Transcript Lists**

NimbleScan paired reports for the three biological replicates of each sample group were loaded into ArrayStar3.0 (DNAStar). Data was normalized using the Robust Multichip Average (RMA). Raw fluorescence data from each probe was averaged and a fold-change value was generated for the corresponding transcript. Only fold change values within the 95% confidence interval as determined by a moderated t-test were considered significant. Each transcript ID (FBtr) was annotated with a corresponding
gene ID (CG) and gene name using FlyBase (Tweedie et. al., 2009; available at: http://flybase.org/).

Lists of transcripts with a 2-fold or greater change in T0 versus T5 flies were generated for: 2-3-day old males, 5-6-day old males and 2-3-day old females. Biological replicates within each experimental group had high correlation coefficients between microarrays (data not shown). Correlation coefficients for the biological replicate samples generally had $R^2$ values between 0.96-0.98.

The top 20 up- and down-regulated transcripts (with the largest fold changes) were identified for each of the three groups. These lists were combined in order to compare top changers between groups.

*Hierarchical Clustering*

Normalized fold-change data for approximately 72,000 transcripts for each of the three groups was loaded into a MultiExperiment Viewer, TIGR MeV 4.6.2 (Saeed et. al., 2006; Saeed et. al., 2003). To reduce saturation, the colour scale limits were adjusted to a lower limit value of -5, a midpoint value of 0, and an upper limit value of 5. Hierarchical clustering was performed using the Pearson Uncentered algorithm, average linkage, optimized gene leaf order and optimized sample leaf order. Hierarchical clustering allows us to divide whole genome transcriptional changes into similar clusters. Clusters generated from multiple experiments are aligned in order to compare similarities and differences between different groups. 2-3-day old males and 2-3-day old females were compared; 2-3-day old males and 5-6-day old males were compared; and 2-3-day old males, 5-6-day old males and 2-3-day old females were compared.
**Generation of Venn Diagrams**

Genes occurring in common between groups were examined using VENNY to compare lists and generate Venn Diagrams (Oliveros, 2007; available at: 
[http://bioinfo.ogp.cn.3c.ic.es/tools/venny/index.html](http://bioinfo.ogp.cn.3c.ic.es/tools/venny/index.html)). 2-fold or greater up- or down-regulated gene lists (CG) were entered into VENNY. Venn Diagrams show how up-regulated and down-regulated gene lists overlap between 2-3-day old males, 5-6-day old males and 2-3-day old females. Genes found to be in common between groups were extracted from VENNY and further analyzed.

**Functional Analysis**

Biological information was extracted from transcript lists using the DAVID Bioinformatics Resource (Huang, Sherman and Lempicki, 2009; available at: [http://david.abcc.nci.3cr.gov/](http://david.abcc.nci.3cr.gov/)). To uncover functional categories in each data set, transcript lists (2-fold or greater changers, P<0.05) were searched and annotated against a whole-genome background for *Drosophila*. Lists of up-regulated and down-regulated transcripts (FBtr) were searched independently in order to identify functional categories specifically over- or under-represented. Transcripts were annotated with terms belonging to the following processes: GO Biological Process (level 5), GO Molecular Function (level 5), INTERPRO, and KEGG. Significantly enriched functional categories were determined based on the frequency of annotated transcripts and the probability of a given transcript occurring within that category. Only functional categories with P <0.05 were considered significant.
**GO Graph Analysis and Generation of Annotation Tables**

In order to visualize the relationship between functional categories, GO Graphs were created using the GO Graph Viewer from Babelomics 4 (Medina et. al., 2010; available at: [http://babelomics.bioinfo.cipf.es/](http://babelomics.bioinfo.cipf.es/)). Functional categories were mapped according to either GO Biological Process (BP) or GO Molecular Function (MF). Categories mapping to the same process were identified and the most descriptive functional category was selected to represent the corresponding process. Annotation tables were generated to summarize GO BP, GO MF, INTERPRO and KEGG functional categories for 2-3-day old males, 5-6-day old males and 2-3-day old females.
Results

**Oxidative stress induces global transcriptional changes that are similar between different experimental groups**

The oxidative stress response has been shown to alter a significant portion of the transcriptome in eukaryotes with up to one-third of transcripts in yeast being affected by hydrogen peroxide exposure (Causton et. al., 2001; Gasch et. al., 2000). Using a *Drosophila* model for oxidative stress, we wanted to visualize global transcriptional changes for approximately 15,500 genes of the genome represented by the 72,000 probes on NimbleGen arrays. Transcriptional changes associated with a lack of functional SOD1 were examined using flies homozygous for the cSOD$^{n108\text{red}}$ mutation (“T0”). Control flies are identical in background to T0 except for a P-element insertion containing functional SOD1 under the native promoter (“T5”). The T5 transgenic rescue has been previously shown to restore all deleterious phenotypes associated with cSOD$^{n108\text{red}}$ mutation (Parkes et. al., 1998).

Using T5 as a control, we are able to measure transcriptional changes resulting solely from lack of SOD1 activity. Fluorescence data from each probe was used to determine a fold-change value for the corresponding transcript. Normalized fold-change data was loaded into a multi-experiment viewer (TIGR MeV 4.6.2) to generate heat maps representing whole genome transcriptional changes. Heat maps were aligned by hierarchical clustering (Pearson Uncentered, optimized, average linkage).

Clustered heat maps show that a significant portion of the transcriptome is altered in T0 as compared to T5 (Figure 2.1). In general, the majority of transcript changers, in terms of direction and magnitude, are similar between the three groups examined (2-3-
day old males, 5-6-day old males and 2-3-day old females). More transcriptional similarities occur between 2-3-day old males and 5-6-day old males than between 2-3-day old males and 2-3-day old females. Although the two male groups are highly similar, some divergence in transcript expression was observed and is represented by Clusters A, B and C (Figure 2.2). Transcripts found in Cluster A correspond to long-chain fatty acid transporter activity and metal ion binding. Cluster B includes transcripts associated with fatty acid synthase activity and chitin-based cuticle formation. Cluster C contains transcripts associated with oxidation reduction, lipid metabolic process, carbohydrate metabolic process, nutrient reservoir activity, amino acid biosynthetic process, proteolysis, cell death and reproduction. The majority of transcripts belonging to Cluster C are altered in terms of magnitude but not direction, with 5-6-day old males demonstrating higher fold changes than 2-3-day old males (Appendix A, Table S1).

Transcript changers are less similar between 2-3-day old males and 2-3-day old females, with a larger number of diverging changers occurring. These discrepancies are represented by Clusters A, B, C, and D (Figure 2.3). Transcripts belonging to Cluster A are similar in terms of direction but not magnitude of change (Appendix A, Table S2). These transcripts are associated with lipase activity, the DNA damage response, courtship behaviour, signal transduction, transcription factor activity, antibacterial humoral response, proteolysis and response to heat. Transcripts belonging to Cluster A are more highly induced in 2-3-day old males than in 2-3-day old females. Cluster B contains transcripts altered in both magnitude and direction. These transcripts are associated with proteosome function, proteolysis, oxidation reduction, the Wnt receptor signaling pathway, sensory perception of chemical stimulus, mating behaviour, lipid metabolic
process and carbohydrate metabolism. Clusters C and D both contain transcriptional changes diverging in direction. Transcripts unique to these clusters are involved in protein import into mitochondrial inner membrane, eggshell chorion assembly, mRNA binding and mitotic metaphase.

Although differences in transcript expression occur between groups, the majority of the transcriptome is altered in a similar pattern in cSOD\(^{108}\) mutants with the majority of up- and down-regulated transcripts in alignment. In general, \textit{Drosophila cSOD}\(^{108}\) mutants tend to mount a genome-wide response and have a larger portion of the transcriptome down-regulated rather than up-regulated.

\textbf{Common responses to oxidative stress}

A common response to oxidative stress has been suggested to occur regardless of the parameters used to induce stress (Girardot et. al., 2004; Landis et. al., 2004). To elucidate a general mechanism of oxidative stress response in cSOD\(^{108}\) mutants, significantly altered transcripts (FBtr) were annotated with the corresponding gene ID (CG) and genes in common between groups were identified. Since a given gene can represent more than one transcript, lists of unique gene changers were shorter than the original transcript lists representing 2-fold or greater changers (P<0.05).

The number of significant transcript changers was 235 for 2-3-day old males, 340 for 5-6-day old males and 229 for 2-3-day old females. The number of altered genes was 223 for 2-3-day old males, 332 for 5-6-day old males and 224 for 2-3-day old females. Of these 95, 123, and 158 were up-regulated and 128, 209, and 66 were down-regulated for 2-3-old males, 5-6-day old males and 2-3-day old females respectively.
**Figure 2.1**: Heat map visualizing whole-genome transcriptional changes in T0 versus T5 flies for 2-3-day old males, 5-6-day old males and 2-3-day old females. Hierarchical clustering was performed using an optimized Pearson Uncentered algorithm. Red indicates up-regulated transcripts; green indicates down-regulated transcripts. The scale bar shows the magnitude of differential expression.
Figure 2.2: Heat map visualizing whole-genome transcriptional changes for 2-3-day old males and 5-6-day old males; T0 versus T5. Hierarchical clustering was performed using an optimized Pearson Uncentered algorithm. Clusters of transcripts (FBtr) that differ in terms of magnitude and/or direction of fold change are labeled A, B, and C.
Figure 2.3: Heat map visualizing whole-genome transcriptional changes for 2-3-day old males and 2-3-day old females; T0 versus T5. Hierarchical clustering was performed using an optimized Pearson Uncentered algorithm. Clusters of transcripts (FBtr) that differ in terms of magnitude and/or direction of fold change are labeled A, B, C, and D.
To examine which 2-fold or greater gene changers occur in common between groups, Venn Diagrams were generated using VENNY. A total of 21 up-regulated genes and 24 down-regulated genes were shared between 2-3-old males, 5-6-day old males and 2-3-day old females (Figure 2.4).

Up-regulated genes shared between all three groups were found to be associated with oxidation reduction, serine-type endopeptidase activity, serine-type endopeptidase inhibitor activity, metal ion transmembrane transporter activity, microtubule binding, and lipid/carbohydrate homeostasis (Appendix A, Table S3).

A gene for microtubule associated protein 205 (map205) is highly up-regulated with a 26.74-fold change occurring in 2-3-day old males, a 68.60-fold change in 5-6-day old males and a 120.24-fold change in 2-3-day old females. Microtubule binding proteins promote stability by association with microtubules (Bulinski and Borisy, 1980; Olmsted, 1986; Aizawa et. al., 1990). Map205 is specifically associated with mitosis and cytokinesis events (Archambault et. al., 2008). Quantitative real time PCR confirmed induction of this gene (A., Soltyk, A., Huston, data not shown).

Down-regulated genes common to all three groups were found to be associated with serine-type endopeptidase activity, serine-type endopeptidase inhibitor activity, carbohydrate metabolic process, proteolysis, redox regulation, and haemolymph juvenile hormone binding (Appendix A, Table S4). Transcripts belonging to the amylase gene family were prominently down-regulated. Quantitative real time PCR confirmed repression of the amylase distal gene transcript (A. Soltyk, A., Huston, data not shown).

More transcripts occur in common between 2-3-day old males and 5-6-day old males than between 2-3-day old males and 2-3-day old females, with 46 genes up-
regulated and 88 genes down-regulated between young and old males (Figure 2.4). Up-regulated transcripts in males are associated with lipase activity, oxidation reduction, microtubule binding, apoptosis/DNA damage response, polyamine biosynthetic process, unfolded protein response, nervous system development and phospholipase activity (Appendix A, Table S5).

Down-regulated between male groups were transcripts involved in reproductive function and odorant perception. These transcripts correspond to accessory gland peptides (acp) and odorant binding proteins (obp). Quantitative real time PCR confirmed repression of obp57a (A., Soltyk, A., Huston, data not shown).

Other down-regulated transcripts between males were associated with electron carrier activity, phospholipid metabolic process, carbohydrate metabolic process, triglyceride lipase activity and other lipases (Appendix A, Table S6).

Males and females have fewer transcripts in common, with 38 genes up-regulated and 28 genes down-regulated (Figure 2.4). Many of the up-regulated transcripts in common between 2-3-day old males and 2-3-day old females are involved in the defense response (Appendix A, Table S7). Down-regulated transcripts are largely involved in proteolysis, carbohydrate metabolism and haemolymph juvenile hormone binding (Appendix A, Table S8).
Figure 2.4: Lists of up- or down-regulated 2-fold or greater gene changers (CG, P<0.05) were entered into VENNY, an interactive tool for generating Venn Diagrams. Genes in common between 2-3-day old males, 5-6-day old males and 2-3-day old females were determined.
**Group specific responses to oxidative stress**

Many of the gene changers in common between groups do not necessarily represent the most significantly altered transcripts. In order to compare highly altered transcripts, a list of top 20 up-regulated and top 20 down-regulated transcripts was generated for each group. These lists were combined and redundant transcripts removed, allowing us to compare the magnitude and direction of change for top transcript changers between groups (Table 2.1 and Table 2.2). Top transcript changers are represented by their corresponding gene ID.

The top up-regulated transcript, a lipase, shows a 100.93-fold change in 2-3-day old males, a 59.93-fold change in 5-6-day old males but no change in 2-3-day old females. This lipase functions to regulate the activity of a male-specific reproductive peptide: Acp70A (Pilpel and Nezer, 2008). The low induction of this gene in females is consistent with the sex-specific role of this peptide in male *Drosophila* reproduction.

Transcripts associated with oxidation reduction and the unfolded protein response were found more strongly induced in females. Interestingly, a marker gene for DNA damage and apoptosis, Companion of reaper (Corp), was not significantly induced in females as compared to males. Up-regulation of Corp in males increases with age (9.64-fold in 2-3-day old males versus 18.76-fold in 5-6-day old males), while no change is seen in 2-3-day old females.

Many transcripts tended to have a higher magnitude of fold-change in older cSODn108 flies. For example, transcripts associated with proteolysis and carbohydrate metabolism become more prominently down-regulated in the 5-6-day old males as compared to 2-3-day old males.
Table 2.1: Most highly induced gene transcripts in cSOD\textsuperscript{n108} mutant flies as compared to transgenic rescue control for 2-3-day old males, 5-6-day old males and 2-3-day old females.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Fold Change</th>
<th>Gene Name</th>
<th>Gene Function</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Dmel_CG18284</td>
<td>lipase activity</td>
</tr>
<tr>
<td>CG7203</td>
<td>44.86</td>
<td>Dmel_CG7203</td>
<td>Unknown</td>
</tr>
<tr>
<td>CG8864</td>
<td>34.58</td>
<td>Cyp28a5</td>
<td>electron carrier activity; P450</td>
</tr>
<tr>
<td>CG1483</td>
<td>26.74</td>
<td>Map 205</td>
<td>mitotic cell cycle</td>
</tr>
<tr>
<td>CG4650</td>
<td>24.49</td>
<td>Dmel.CG4650</td>
<td>serine-type endopeptidase activity</td>
</tr>
<tr>
<td>CG14579</td>
<td>18.85</td>
<td>Dmel.CG14579</td>
<td>Unknown</td>
</tr>
<tr>
<td>CG4740</td>
<td>14.54*</td>
<td>Attacin-C</td>
<td>antibacterial humoral response</td>
</tr>
<tr>
<td>CG34276</td>
<td>10.77*</td>
<td>Dmel.CG34276</td>
<td>Unknown</td>
</tr>
<tr>
<td>CG31832</td>
<td>9.83</td>
<td>Dmel.CG31832</td>
<td>receptor binding; signal transduction</td>
</tr>
<tr>
<td>CG10965</td>
<td>9.64</td>
<td>Companion of reaper</td>
<td>DNA damage response; induction of apoptosis</td>
</tr>
<tr>
<td>CG30272</td>
<td>9.02</td>
<td>Dmel.CG30272</td>
<td>transmembrane transport</td>
</tr>
<tr>
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<td>antibacterial humoral response</td>
</tr>
<tr>
<td>CG1385</td>
<td>7.38</td>
<td>Defensin</td>
<td>antibacterial humoral response</td>
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<td>Dipterin B</td>
<td>antibacterial humoral response</td>
</tr>
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<td>CG30091</td>
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</tr>
<tr>
<td>CG14934</td>
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<td>alpha-glucosidase activity</td>
</tr>
<tr>
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<td>antibacterial humoral response</td>
</tr>
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</tr>
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<td>technical knockout</td>
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<td>polyamine biosynthetic process</td>
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<td>Transporter</td>
<td>neurotransmitter transport</td>
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<td>Dmel.CG10102</td>
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<tr>
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<td>Jonah 99Fi</td>
<td>serine-type endopeptidase activity</td>
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<tr>
<td>CG33521</td>
<td>3.24</td>
<td>Dmel.CG33521</td>
<td>zinc ion binding; zinc finger</td>
</tr>
<tr>
<td>CG31764</td>
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<td>defense response to virus</td>
</tr>
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<td>2.49</td>
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<td>Unknown</td>
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<tr>
<td>CG17470</td>
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<td>Dmel.CG17470</td>
<td>mesoderm development</td>
</tr>
<tr>
<td>CG10814</td>
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<td>oxidation reduction</td>
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<td>1.36</td>
<td>Heat shock protein 68</td>
<td>unfolded protein binding</td>
</tr>
<tr>
<td>CG4830</td>
<td>-1.20</td>
<td>Dmel.CG4830</td>
<td>long-chain fatty acid transporter activity</td>
</tr>
</tbody>
</table>

*Fold change value did not meet the P<0.05 cut-off criteria
Table 2.2: Most repressed gene transcripts in cSOD\textsuperscript{n108} mutant flies as compared to transgenic rescue control for 2-3-day old males, 5-6-day old males and 2-3-day old females.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>2-3d M</th>
<th>5-6d M</th>
<th>2-3d F</th>
<th>Gene Name</th>
<th>Gene Function</th>
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<tr>
<td>CG1304</td>
<td>-83.83</td>
<td>-116.18</td>
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<td>serine-type endopeptidase activity</td>
</tr>
<tr>
<td>CG32986</td>
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<td>-22.80</td>
<td>-1.09</td>
<td>Dmel_CG32986</td>
<td>Unknown</td>
</tr>
<tr>
<td>CG32751</td>
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<td>-7.97</td>
<td>-23.03</td>
<td>Vanin-like protein 2</td>
<td>nitrogen compound metabolic process</td>
</tr>
<tr>
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<td>-18.09</td>
<td>-46.05</td>
<td>-11.11</td>
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<td>serine-type endopeptidase inhibitor activity</td>
</tr>
<tr>
<td>CG30360</td>
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<td>-16.88</td>
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<td>carbohydrate metabolic process</td>
</tr>
<tr>
<td>CG11784</td>
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<td>-11.43</td>
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<td>Dmel_CG11784</td>
<td>glutathione transferase activity</td>
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<td>CG2071</td>
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<td>serine-type peptidase activity</td>
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<td>CG17876</td>
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<td>-12.02</td>
<td>-6.88</td>
<td>Amylase distal</td>
<td>carbohydrate metabolic process</td>
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<td>CG18730</td>
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<td>-11.45</td>
<td>-6.94</td>
<td>Amylase proximal</td>
<td>carbohydrate metabolic process</td>
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<td>-1.34</td>
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<td>CG13349</td>
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<td>-1.15</td>
<td>Proteasomal ubiquitin</td>
<td>proteasome assembly</td>
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<td>dipeptidyl-peptidase activity; proteolysis</td>
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<td>CG8221</td>
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<td>-1.07</td>
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<td>gram-negative bacterial cell surface binding</td>
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<td>lectin</td>
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<td>CG12275</td>
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<td>-1.53</td>
<td>-7.51</td>
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<td>5.12</td>
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</tr>
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</tr>
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<td></td>
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<td>dehydrogenase</td>
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<td>-13.10</td>
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<td>-12.17</td>
<td>Dmel_CG9650</td>
<td>zinc ion binding; nucleic acid binding</td>
</tr>
</tbody>
</table>

*Fold change value did not meet the P<0.05 cut-off criteria
**Functional categories of genes enriched during oxidative stress**

Observation of individual gene changers provides limited insight into which processes are significantly altered in cSOD<sup>n108</sup> mutants. To extract biologically meaningful information, significantly enriched functional categories were determined. Gene lists were annotated and functional characterization was performed using the DAVID bioinformatics tool ([http://david.abcc.ncifcrf.gov/](http://david.abcc.ncifcrf.gov/)).

Gene Ontology was used to annotate genes using the following GO categories: Biological Process, Molecular Function, INTERPRO and KEGG. Enriched categories for up- or down-regulated transcripts were identified and summarized (Table 2.3). Many of the functional categories generated tended to occur within the same biological pathway.

In order to generate concise annotation tables, redundant functional categories were identified and the most descriptive category was chosen to represent the corresponding biological pathway. To view relationships between categories, GO graphs were generated in order to map functional categories relative to one another (Appendix A, Figures S10- S15).

An example of a typical GO graph is represented in Figure 2.5.

Assigned node scores are based on the number of transcripts annotated within a particular category. Categories with higher node scores contain a greater number of transcripts and tend to be more descriptive.

Using GO graphs to identify functional categories with high node scores, concise annotation tables were generated for 2-3-day old males (Table 2.4), 5-6-day old males (Table 2.5), and 2-3-day old females (Table 2.6).
The immune response was found to be the most significant up-regulated functional category for all three groups. Cytochrome P450 enzyme function was also highly up-regulated in 2-3-day old males and 2-3-day old females but not 5-6-day old males. Interestingly, cytochrome P450 enzyme function was also simultaneously down-regulated in 2-3-day old males. However, enrichment of up-regulated and down-regulated cytochrome P450 function occurred as a result of a different set of transcripts exclusive for each direction.

Processes associated with heat shock and serine-type endopeptidase activity were found up-regulated in males but not in females. Unique to 5-6-day old male flies was the up-regulation of processes associated with metal ion homeostasis and nervous system remodeling. Unique to female flies was up-regulation of the biogenic amine biosynthetic process and the phenylalanine metabolic process. Females also had functional enrichment of lipocalin-related proteins, with transcripts corresponding to Neural Lazarillo (NLaz) and Glial Lazarillo (GLaz). Remarkably, NLaz occurs as a highly up-regulated transcript for 2-3-day old males and 5-6-day old males; however, enrichment of the corresponding functional category was not observed for either male group given our selected level of stringency for DAVID functional analysis.

In general, males had more functional categories shown to be down-regulated than up-regulated. Females demonstrated the reverse, with a higher number of functional categories found to be up-regulated. Males, but not females, specifically down-regulated processes associated with triacylglycerol lipase activity, accessory gland-specific peptide 53Ea (acp53Ea) and GNS1/SUR4. Additional processes associated with behaviour were also down-regulated in 2-3-day old males and 2-3-day old females, but not in 5-6-day old
males. Enrichment of behavioural processes was due to the occurrence of transcripts annotated with odorant binding protein and haemolymph juvenile hormone binding protein function.

Common to all three groups was the prominent down-regulation of proteolysis and carbohydrate metabolism. Proteolysis was consistently found to be the most significantly down-regulated process containing a relatively large number of repressed protease transcripts. Transcripts annotated within the category of carbohydrate metabolism were consistently found to belong to the amylase family of enzymes.
Figure 2.5: GO graph showing down-regulated transcripts annotated with GO Molecular Function in cSOD<sup>n108</sup> mutant flies as compared to transgenic rescue controls for 2-3-day old males. GO graph generated using the Babelomics 4 analysis tool. Node scores indicate enrichment with higher node scores reflecting that more transcripts are annotated within that node (category).
Table 2.3: Summary of all functional categories generated for cSOD$^{n108}$ mutant flies as compared to transgenic rescue controls for the following groups: 2-3-day old males, 5-6-day old males and 2-3-day old females. DAVID was used to generate GO Ontology terms corresponding to GO Biological Process (BP, level 5), Molecular Function (MF, level 5), INTERPRO and KEGG (P<0.05). Symbols indicate the presence (+) or absence (-) of significantly altered categories.

<table>
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Table 2.4: A) Functional classes of up-regulated transcripts with 2-fold or greater induction (P<0.05) for 2-3-day old male cSOD*108 mutants as compared to transgenic control.

<table>
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<tr>
<th>Category</th>
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<th>Count</th>
<th>%</th>
<th>P-Value</th>
<th>Genes</th>
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<td>CG30091, CG30289, CG4650, CG6639, CG9733, deltaTrypsin, gammaTrypsin</td>
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</table>

*Metabolism of Terpenoids and Polyketides

Table 2.4: B) Functional classes of down-regulated transcripts with 2-fold or greater induction (P<0.05) for 2-3-day old male cSOD*108 mutants as compared to transgenic control.

<table>
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<td>3.70E-02</td>
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Table 2.5: A) Functional classes of up-regulated transcripts with 2-fold or greater induction (P<0.05) for 5-6-day old male cSOD<sup>nut108</sup> mutants as compared to transgenic control.

<table>
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<th>Category</th>
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Table 2.5: B) Functional classes of down-regulated transcripts with 2-fold or greater induction (P<0.05) for 5-6-day old male cSOD\textsuperscript{108} mutants as compared to transgenic control.

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<td>Cellular retinaldehyde binding/α-tocopherol transport</td>
<td>IPR001071</td>
<td>3</td>
<td>1.6</td>
<td>0.028</td>
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<tr>
<td>MF 5</td>
<td>Pantetheine hydrolase activity</td>
<td>GO:0017159</td>
<td>2</td>
<td>1.1</td>
<td>0.043</td>
<td>Vanin-like protein 2, Vanin-like protein 3</td>
</tr>
<tr>
<td>INTERPRO</td>
<td>Drosophila ACP53EA</td>
<td>IPR009392</td>
<td>2</td>
<td>1.1</td>
<td>0.043</td>
<td></td>
</tr>
<tr>
<td>INTERPRO</td>
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<td>IPR013766</td>
<td>3</td>
<td>1.6</td>
<td>0.047</td>
<td></td>
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Table 2.6: A) Functional classes of up-regulated transcripts with 2-fold or greater induction (P<0.05) for 2-3 day old female cSOD^{c108} mutants as compared to transgenic control.

<table>
<thead>
<tr>
<th>Category</th>
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<th>Genes</th>
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<td>DIM</td>
<td>IPR013172</td>
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<td>CG15065, Immune induced molecule 1, Immune induced molecule 2, Immune induced molecule 3</td>
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<tr>
<td></td>
<td></td>
<td>Inmune-induced_DIM</td>
<td></td>
<td></td>
<td></td>
<td>CG12896, CG17470, CG8250, Myosin alkali light chain 1, Paramyosin, goliath</td>
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<tr>
<td>BP 5</td>
<td>Mesoderm development</td>
<td>GO:0007498</td>
<td>6</td>
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<td>6.00E-04</td>
<td>CG8250, Scavenger receptor class C, type I, Scavenger receptor class C, type IV</td>
</tr>
<tr>
<td>INTERPRO</td>
<td>Mam</td>
<td>IPR000998</td>
<td>3</td>
<td>1.9</td>
<td>1.30E-03</td>
<td>Alcohol dehydrogenase; Adh-related,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mam_dom</td>
<td></td>
<td></td>
<td></td>
<td>CG11236, CG12116, CG13091, CG1441,</td>
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<tr>
<td></td>
<td></td>
<td>IPR016040</td>
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<td>5.1</td>
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<td></td>
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<td>NAD(P)-Bd_dom</td>
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<td></td>
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<td></td>
<td></td>
<td>GO:0042742</td>
<td>5</td>
<td>3.2</td>
<td>1.80E-03</td>
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<tr>
<td>INTERPRO</td>
<td>Peroxiredoxin, C-terminal</td>
<td>IPR019479</td>
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<td>3.50E-03</td>
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<tr>
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<td>Hemocyanin/insect LSP</td>
<td>IPR013788</td>
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<td>4.50E-03</td>
<td>Cytochrome P450 6g1, Probable cytochrome P450 28a5, Probable cytochrome P450 28d1, Probable cytochrome P450 309a2, Probable cytochrome P450 4d21, Probable cytochrome P450 4p3</td>
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<tr>
<td>KEGG</td>
<td>Limonene and pinene degradation</td>
<td>dme00903</td>
<td>6</td>
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<td>14-3-3 protein zeta, Henna, Ornithine decarboxylase 2</td>
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<tr>
<td>MF 5</td>
<td>Serine-type endopeptidase inhibitor activity</td>
<td>GO:0004867</td>
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<td>7.60E-03</td>
<td>CG10031, CG31778, CG34276, CG3604, Serine protease inhibitor 1</td>
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<td>GO:0042401</td>
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<td>1.9</td>
<td>8.00E-03</td>
<td>Alcohol dehydrogenase; Adh-related,</td>
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<td>Starch and sucrose metabolism</td>
<td>dme00500</td>
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<td>9.00E-03</td>
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<tr>
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<td>Metabolism of Xenobiotics by cytochrome P450</td>
<td>dme00980</td>
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<td>3.2</td>
<td>1.10E-02</td>
<td>Alcohol dehydrogenase; Adh-related,</td>
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<td>INTERPRO</td>
<td>CHK kinase-like</td>
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<td>2.6</td>
<td>2.00E-02</td>
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<tr>
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<td>GO:0006026</td>
<td>3</td>
<td>1.9</td>
<td>2.40E-02</td>
<td>Imaginal disc growth factor 1, Imaginal disc growth factor 5, Peptidoglycan-recognition protein SC1a/b</td>
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<tr>
<td>INTERPRO</td>
<td>Lipocalin-related protein and Bos/Can/Equ allergen</td>
<td>IPR000566</td>
<td>2</td>
<td>1.3</td>
<td>3.40E-02</td>
<td>Glial Lazarillo, Neural Lazarillo</td>
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<td>KEGG</td>
<td>Phenylalanine metabolism</td>
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<td>1.9</td>
<td>3.70E-02</td>
<td>CG11796, Henna, Peroxiredoxin 2540</td>
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Table 2.6: B) Functional classes of down-regulated transcripts with 2-fold or greater induction (P<0.05) for 2-3-day old female eSOD<sup>−/−</sup> mutants as compared to transgenic control.

<table>
<thead>
<tr>
<th>Category</th>
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<th>ID</th>
<th>Count</th>
<th>%</th>
<th>P-Value</th>
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<tr>
<td>BP 5</td>
<td>Proteolysis</td>
<td>GO:0006508</td>
<td>11</td>
<td>17.2</td>
<td>8.30E-05</td>
<td>CG11034, CG11941, CG1304, CG13095, CG17012, CG30083, CG9377, Jonah 25Biii, Jonah 99Ci, Jonah 99Cii; Jonah 99Ciii, Serine protease 6</td>
</tr>
<tr>
<td>INTERPRO</td>
<td>Obp/ Haemolymph juvenile hormone binding</td>
<td>IPR004272</td>
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<td>4.7</td>
<td>7.10E-03</td>
<td>CG33306, CG7916, CG8997</td>
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<td>MF 5</td>
<td>Amylase activity</td>
<td>GO:0016160</td>
<td>2</td>
<td>3.1</td>
<td>1.50E-02</td>
<td>Amylase distal, Amylase proximal</td>
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<tr>
<td>KEGG</td>
<td>Glutathione metabolism</td>
<td>dme00480</td>
<td>3</td>
<td>4.7</td>
<td>3.60E-02</td>
<td>CG11784, Glucose-6-phosphate 1-dehydrogenase, Glutathione S transferase E10</td>
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<td>INTERPRO</td>
<td>RWD</td>
<td>IPR006575</td>
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<td>3.1</td>
<td>4.20E-02</td>
<td>CG13344, CG17260</td>
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</tbody>
</table>
Discussion

The oxidative stress response in cSOD\textsuperscript{n108} Drosophila represents a chronic condition similar to other oxidative stresses and aging

The transcriptional changes associated with exposure to either paraquat or hydrogen peroxide include the induction of peptidases, lipases, glutathione transferases, oxidoreductases and proteins that bind iron, such as the monooxygenases (Landis et. al., 2004; Girardot et. al., 2004). It has been suggested that the induction of these enzymes forms the basis of a general response to oxidative stress in Drosophila (Landis et. al., 2004; Girardot et. al., 2004). The up-regulation of serine proteases has also been observed in other oxidative stress studies using paraquat exposure to induce acute stress (Zou et. al., 2000; Zou et. al., 2010).

Consistent with this suggestion, we observe transcripts corresponding to these categories among our top gene changers. However, peptidase transcripts are both prominently up- and down-regulated across all three groups, presenting an unusual trend unique to cSOD\textsuperscript{n108} mutants. Several possibilities exist to explain this observed phenomenon.

Induction of peptidase gene transcripts may be due to an increase in the degradation of oxidized proteins consistent with increased levels of ROS. Proteins and free amino acids act to buffer cells from the effects of ROS (Droge, 2002). Although amino acids and proteins have no specific antioxidant activity, they are present at high concentrations. Tyrosine, tryptophan, histidine and cytosine form particularly vulnerable ROS targets (Droge, 2002). Since free amino acids have more scavenging activity than
intact proteins, the enhancement of proteolysis may be a response to oxidative stress in order to restore ROS homeostasis (Droge, 2002).

Repression of protease gene transcripts may be related to decreased activity of proteosomes during oxidative stress. Oxidized proteins are normally degraded via the 26S-proteosomal pathway (Shringarpure et. al., 2001; Grune et. al., 2001; Linton et. al., 2001). However, excessive protein damage has been shown to inhibit proteosome function (Grune and Davies, 2003). Several studies have shown that inhibition of the proteosome occurs in aging, resulting in the induction of apoptosis. Transcripts associated with apoptosis (corp and reaper) are significantly induced in eSOD^108 mutants as compared to the transgenic control, with a greater induction occurring in older males.

More prominent, and perhaps less expected, was the induction of the innate immune response. Studies of oxidative stress and aging have previously noted induction of immune response genes in aging flies and flies exposed to paraquat or 100% O_2 (Landis et. al., 2004; Girardot et. al., 2004). Oxidative stress potentially damages the barriers that normally prevent the entry of pathogens through the gut or tracheoles (Girardot et. al., 2004). Also possible is that stress signaling, such JNK, utilizes conserved pathways that overlap with signaling pathways involved in immune response. For instance, JNK activation has been found to occur downstream of lipopolysaccharide (LPS) induction (Boutros et. al., 2002). Oenocytes in *Drosophila* function to regulate both immune response and metabolism, indicating that regulation of these processes share common ancestral origin (Hotamisligil, 2006).

Consistent with other oxidative stress and aging studies was the induction of transcripts corresponding to heat shock proteins and cytochrome P450 enzymes.
Cytochrome P450s act as electron transporters and function in detoxification (Wickramasinghe, 1975). Induction of cytochrome P450 transcripts has been observed in *Drosophila* following exposure to paraquat (Girardot et. al., 2004). Up-regulation of hsp70 and hsp22 transcripts are observed in flies exposed to 100% oxygen and in aged flies, with the strong induction of hsp22 serving as a marker for aging (Landis et. al., 2004). Transcripts corresponding to hsp70 were significantly induced in cSOD$^{n108}$ mutants; however hsp22 transcripts were only weakly induced. This result illustrates that our model for oxidative stress represents a unique condition that does not occur in other oxidative stress studies.

*The oxidative stress response in cSOD$^{n108}$ Drosophila is associated with several novel functional categories*

Oxidative challenge can be expected to mount a response that will protect an organism from further injury. The induction of enzymes involved in the neutralization of toxic compounds and the repair of damage forms the basis of a stress response. However, only a few of the functional categories observed in cSOD$^{n108}$ mutants were clearly associated with this role. For example, electron carrier activity (cytochrome P450s) and chaperone binding (heat shock proteins) were the processes identified with a known function in stress biology.

The induction of transcripts annotated with nervous system remodeling (reaper and spinster) was observed in 5-6-day old male cSOD$^{n108}$ mutants. Spinster has been found to be involved in the clearance of lipofuscin-like material from nerve cells (Nakano et. al., 2001). Lipofuscin results from the accumulation of oxidized lipids in lysosomes.
(Nakano et. al., 2001). Induction of genes that function in nervous system remodeling potentially reflects a repair process associated with increased neural damage in older cSOD\textsuperscript{n108} mutants.

Unexpected was a prominent down-regulation of transcripts belonging to the cytochrome P450 family of enzymes. Concurrent up-regulation of cytochrome P450 transcripts also occurred, similar to what was observed for peptidase transcripts. This result can be clarified given the large size and diversity of the cytochrome P450 family. In addition to detoxification, cytochrome P450s function in biosynthesis, growth, development, lipid and hormone synthesis, and behavioural phenotypes related to aggression, mating and feeding (Chung et. al., 2009).

Cytochrome P450s are also prominent regulators of metabolic gene expression (Riddiford, 1993). Thus, it remains likely that many of the altered cytochrome P450s transcripts in cSOD\textsuperscript{n108} mutants play a role in metabolism rather than ROS detoxification. Supporting this suggestion, Cyp4g1, one of the cytochrome P450 transcripts found repressed, has been shown to localize to oenocytes and regulates triacylglycerol composition (Gutierrez et. al., 2006).

In addition, many of the cytochrome P450 transcripts altered in cSOD\textsuperscript{n108} mutants potentially function in regulating feeding behaviour. Repressed cytochrome P450 transcripts, Cyp4e3, Cyp6a2 and Cyp310a1, have each been associated with the induction of feeding in larvae (Chung et. al., 2009). Specifically, Cyp301a1 is expressed the hindgut, Cyp4e3 in the midgut and Cyp6a2 in the midgut and Malpighian tubules of feeding, but not wandering, third instar larvae (Chung et. al., 2009).
Several other transcripts with a known function in modulating organism behaviour were also found altered in cSOD$^{n108}$ mutants. These transcripts are associated with male reproductive behaviour (accessory gland peptides), odorant and sensory perception (odorant binding proteins and juvenile hormone binding proteins), feeding and the anticipation of food availability (takeout).

**cSOD$^{n108}$ mutants may induce adaptations in order to cope with chronic stress**

Chronic exposure to oxidative insult may initiate transcriptional programs that ultimately affect the behaviour and physiology of the organism. The induction of NLaz and GLaz in cSOD$^{n108}$ mutants, two lipocalin-related protein transcripts, provides evidence of activated JNK signaling. Lipocalin-related proteins are known to function in the regulation of energy stores and can modulate oxidative stress resistance, starvation stress resistance and lifespan in *Drosophila* (Karpac et. al., 2009, Hull-Thompson et. al., 2009).

The reallocation of energy stores may serve to adapt an organism experiencing stress to anticipate food shortage as stressed animals may have decreased foraging capability. Repression of transcripts associated with triacylglycerol lipase activity was observed in cSOD$^{n108}$ mutants and may indicate increased lipid storage. Glycogen also acts as an important energy reservoir in flies (Clark, 1989). Amylase-related enzyme transcripts were found down-regulated in cSOD$^{n108}$ mutants with alpha-amylase (distal and proximal) prominently down-regulated for all three groups. Alpha-amylase hydrolyzes α-1-4 glycoside bonds in polysaccharides and is required in *Drosophila* to
metabolize glycogen (Prigent et. al., 1998). Increased glycogen storage is also consistent with activated JNK signaling (Hull-Thompson et. al., 2009).

In addition, NLaz suppresses insulin-signaling which may lead to modified olfactory behaviour and taste perception. Insulin-signaling has been observed to have a regulatory role in the peripheral sensory system, particularly in neurons involved in taste and odorant perception (Cornils et. al., 2011). Down-regulation of odorant binding protein transcripts in cSOD\textsuperscript{n108} mutants may modulate chemosensory perception in response to altered insulin-signaling. Suppression of insulin-signaling may also result altered neuroendocrine signaling (Ruaud and Thummel, 2008). The induction of biogenic amine biosynthesis in female cSOD\textsuperscript{n108} mutants is consistent with this suggestion. Biogenic amines function as neurotransmitters, neuromodulators and neurohormones in the \textit{Drosophila} central nervous system (Monastirioti, 1999). Biogenic amines can regulate circadian rhythms, endocrine secretion and learning and memory (Blenau and Baumann, 2001).

Further work to characterize behaviour and physiology in cSOD\textsuperscript{n108} mutants is needed to ascertain how observed changes in gene expression correlate with feeding-related phenotypes. The characterization of feeding behaviour, starvation stress tolerance, locomotor activity, and response to odorants/tastants will be addressed in Chapter 3.
CHAPTER 3

Feeding-Related Behavioural Phenotypes in Superoxide Dismutase Drosophila Mutants

Several of the assays presented were performed in conjunction with Umar Farooq (Figures 3.1-3.2) and Karen Pacheco (Figures 3.4-3.6).
Abstract

Reactive oxygen species (ROS) cause damage to cellular components and excess ROS result in oxidative stress. Oxidative stress has been implicated in aging and diseases such as cancer, diabetes and amyotrophic lateral sclerosis. Organisms have adapted enzymatic mechanisms to cope with oxidative stress. The superoxide dismutase enzyme family (SOD, EC 1.15.1.1) neutralizes the superoxide anion, one of the most prevalent ROS generated by aerobic metabolism. We previously investigated gene expression changes associated with oxidative stress using a *Drosophila* model. This model carries a mutation in the cytoplasmic form of superoxide dismutase which confers a severe hypomorph (cSOD\textsuperscript{n108}). Gene expression changes indicate an altered metabolic strategy, leading us to investigate feeding-related behavioural phenotypes in cSOD\textsuperscript{n108} mutants. Experiments performed assess feeding, sucrose response, olfaction, starvation stress resistance and locomotor activity. Results show that cSOD\textsuperscript{n108} mutants feed less yet demonstrate a remarkable resistance to starvation. In addition, cSOD\textsuperscript{n108} mutants are less responsive to odorants, sucrose stimulation and show decreased locomotor activity. These results support a correlation between observed changes in gene expression and phenotypes relating to feeding indicating metabolic adaptation in response to chronic oxidative stress.
**Introduction**

Reactive oxygen species (ROS) result as a by-product of normal aerobic metabolism. ROS cause damage to proteins, lipids and DNA and have been implicated in various age-related pathologies. Cells possess antioxidant enzymes to help neutralize ROS which include the superoxide dismutases (SODs), catalase (CAT) and thioredoxin reductase (TrxR). Oxidative stress results from an imbalance in the generation of ROS and the mechanisms that function in ROS attenuation (Droge, 2002).

Microarray analysis of a SOD1-defective *Drosophila* mutant (cSOD\(^{n108}\)) confirms that excess ROS alter transcription with prominent gene changes in the processes of immune response, heat shock response, oxidation reduction, proteolysis, lipid metabolism and carbohydrate catabolism. These transcript changes suggest an altered metabolic strategy in cSOD\(^{n108}\) mutants. In addition, transcripts associated with feeding and chemosensory perception are shown to be significantly altered, leading us to examine feeding-related phenotypes.

Feeding behaviour is a complex and conserved trait regulated by energy and nutrient status. Neuropeptides, such as mammalian *neuromedin U* and the fly homolog *hugin*, regulate food intake in response to nutrient signals (Melcher and Pankratz, 2005). *Drosophila* neurons expressing *hugin* are found in the subesophageal ganglion (SOG) of central nervous system (CNS) (Meng et al., 2002; Melcher and Pankratz, 2005). These neurons lie in close proximity to the axon terminals of gustatory sensory neurons, possibly influencing taste perception (Melcher and Pankratz, 2005; Amrein and Thorne, 2005).
Quantifying food intake in Drosophila is a difficult aspect of feeding behaviour to measure and no available method is without drawback (Wong et. al., 2008). The CAFE assay, a method based on using capillary tubes to present food in liquid form (Ja et. al., 2007), is considered the most accurate for quantifying intake but has been shown to reduce egg-laying and lifespan in flies (Wong et. al., 2008). Alternatively, food intake can be measured using dye-labeled food coupled with simultaneous observation of proboscis extension events (Wong et. al., 2009).

The sensory system is critical to feeding behaviour as flies rely on olfactory and gustatory cues to identify potential nutrient sources (Brummel et. al., 2004; Carvalho et. al., 2005). Assessment of olfaction in Drosophila is typically performed using a choice assay. Olfactory choice can be measured using either a Y- or T-shaped maze (Devaud, 2003). In the T-maze assay, flies are placed directly at the choice point with controlled air flow used to deliver the odor (Devaud, 2003). Results are typically presented as a performance index based on the mean proportion of flies making a particular choice (Devaud, 2003).

Perception of taste can be measured by observing an “all-or-nothing response” such as in the proboscis extension response (PER) assay. The PER assay assesses both gustatory perception and motivation to feed (Vaysse and Médioni, 1973; Rodrigues and Siddiqi, 1981; Shiraiwa and Carlson, 2007). This assay relies on the presentation of sucrose to immobilized flies by touching a solution to sensilla located on the tarsus or labellum. Extension of the proboscis is recorded as a positive response and results are presented as a response index (Shiraiwa and Carlson, 2007).
In addition to olfactory and gustatory behaviour, locomotor activity can affect feeding in *Drosophila* as locomotion influences almost all complex behaviours. The locomotor centre in the fly brain is solicited for courtship, circadian rhythm and learning and memory (Martin, 2003). Locomotor activity in the context of feeding is observed in foraging behaviour which can be defined as the locomotor pattern associated with the search for food (Osborne et. al., 1997). Change in locomotor activity can indicate the overall status of the fly. Electronic devices, such as the Trikinetics *Drosophila* Activity Monitor (DAM), measure activity over prolonged periods of time with experiments typically running 1 to 2 weeks (Klarsfeld et. al., 2003). Individual flies are enclosed in glass tubes and an infra-red light beam detects activity. Each time the fly crosses the light beam, an activity count is recorded (Konopka and Benzer, 1971; Hamblen et al., 1986).

In this chapter, we aim to assess food-intake, olfaction, sucrose response, starvation stress resistance and locomotor activity in cSOD\textsuperscript{n108} mutants. Experiments include a blue-dye feeding assay, observation of proboscis extension events during feeding, a proboscis extension response assay, a T-maze assay to test repellents (3-Octanol and MCH) and attractants (1% yeast), a starvation stress assay and assessment of locomotor activity using *Drosophila* Activity Monitors (DAM).
Materials and Methods

Fly Lines and Handling

Flies were reared on standard cornmeal media (Bloomington recipe) on a 12-hour Light/Dark cycle at 25°C (described in Chapter 2). For all behavioural experiments, Oregon-R was used as our wild-type strain. Oregon-R was the original background in which the cSOD\textsuperscript{n108} mutation was isolated (Parkes et. al., 1998). An additional control line with one copy of the cSOD\textsuperscript{n108} mutation over a wild-type chromosome was crossed from cSOD\textsuperscript{n108} red/TM3 males and Oregon-R virgin females. Only F1 progeny with the genotype +/+, cSOD\textsuperscript{n108} red/+ (or T0/Oregon-R) were used for experiments. The cSOD\textsuperscript{n108} red mutant (T0) and the corresponding transgenic rescue (T5) have been previously described (see Chapter 2).

Behavioural Experimental Procedures

Activity assay

Locomotor activity was measured using Trikinetics Drosophila Activity Monitors (Krupp et. al., 2008). For each genotype, individual 0-24-hour old male and female flies were placed in glass tubes containing 5% sucrose and 1% agar. This media provided humidity and nourishment over the course of the experiment. Tubes were inserted into activity monitors and flies were entrained for 3 days on a Light/Dark cycle. Activity monitors generate an infrared beam through the centre of each tube and measure activity by counting the number of infrared beam breaks each minute. Locomotion activity was measured under conditions of constant darkness (Dark/Dark cycle).
Blue dye feeding assay and proboscis observation

Quantification of food intake was performed using a protocol adapted from the Partridge lab (Wong et. al., 2009). Mated male and female flies were sorted using light CO₂ anesthesia and placed into vials containing 1% non-nutrient agar for 24-hours prior to feeding. After the 24-hour food deprivation period, 2-3-days old flies were transferred to feeding vials consisting of 0.05% erioglaucine (FD & C blue no. 1; Sigma), 5% sucrose and 1% agar. A control vial was set up per replicate for each group. Control vials consisted of 5% sucrose and 1% agar. Flies were allowed to feed for 30 minutes in same-sex groups of 5 flies per vial. A total of 3 replicates for each genotype were performed each day for a total of 5 days (n=15, 75 individuals per group).

Visual observations of fly proboscis extension events onto the food surface were taken during the 30 minute feeding period. Vials were randomized and observations were performed blind. Scores for each vial were recorded approximately every 2-3 minutes for 2-3 seconds. The total number of proboscis extension events was tallied for each genotype and expressed as a proportion. The proportion was determined by dividing the sum of PE events by the number of feeding opportunities (number of flies per vial x number of vials x number of times vial was observed).

After the 30 minute feeding and observation period, flies were transferred to microfuge tubes and snap-frozen in liquid nitrogen. Flies were weighed and then homogenized in 500μL PBS (pH 7.2). An additional 200μL of ethanol (50%) was added and samples were centrifuged for 10 minutes at maximum speed (13, 000g). A 500μL aliquot of supernatant was transferred to a fresh tube and absorbance (OD) readings were
recorded at 630nm (NanoDrop ND-1000 UV-Vis Spectrophotometer). OD readings from control vials (with no dye) were used to subtract background.

**Olfactory Assays**

Olfactory response for both attractive and repellent odors was measured using a T-maze apparatus as previously described (Devaud et al., 2001; Devaud, 2003). Mated flies (1-2-days old) were sorted by sex and genotype using light CO₂ anesthesia (<5min) and allowed to recover for 24-hours before testing. Groups of 30 flies, 2-3-days old, were transferred to test vials via aspiration and allowed to acclimatize for 30 minutes. Flies were tested in an environmental chamber set at 25°C and 60% humidity. Barometric pressure was carefully monitored during testing. A fresh aliquot of each odor to be tested was obtained on the day of testing.

**The following repellent odors were tested:**

- 3-methyl-2-cyclohexene-1-one (MCH) \(\rightarrow\) undiluted + diluted (1/1000)
- 3-octanol (3-OCT) \(\rightarrow\) undiluted

These particular repellent odors were chosen because they have been extensively utilized in numerous learning assays (Devaud et al., 2001; Devaud, 2003).

**The following attractive odors were tested:**

- yeast (1% yeast solution)

Yeast solution was prepared by slowly dissolving 1g of inactivated yeast into 99mL of ddH₂O. The solution was mixed using a stir bar and heated slightly using the lowest setting on a hot plate. Yeast was determined to be dissolved when the solution appeared relatively homogeneous.
An air flow (1.5 liters per minute) delivered odorants to flies placed at the choice point in the T-maze. Flies were allowed 1 minute to decide between the arm containing the odor and the arm containing air (control). The number of flies choosing a given arm was tallied and a “corrected probability” (COR) was determined. COR accounts for the natural tendency of flies to distribute equally between the two arms. The performance index was calculated using the following formulas:

For COR for repellents

\[ \text{COR}_{\text{odor}} = \frac{\text{control arm}}{\text{odor arm} + \text{control arm}} \]

For COR for attractants

\[ \text{COR}_{\text{odor}} = \frac{\text{odor arm}}{\text{odor arm} + \text{control arm}} \]

The performance index (PI)

\[ \text{PI}_{\text{odor}} = \frac{(\text{COR}_{\text{odor}} - 0.5) \times 100}{0.5} \]

Proboscis extension response (PER) assay

The proboscis extension response assay has been previously described and a video protocol demonstrating the technique can be obtained from JOVE (Shiraiwa and Carlson, 2007). Prior to the assay, flies are food deprived for 24-hours in isolation vials (1% agar). In order to provide adequate hydration, a 2mL tube filled with water and plugged with cotton was embedded in the agar. Proper hydration of flies reduces the occurrence of false positives during testing.

After food deprivation, flies were isolated using an aspirator. Individual flies were inserted into a modified P100 pipette tip, resulting in immobilization of the fly. Flies were positioned in a prone position, with the head protruding from the pipette tip and the
body firmly held in place using a plaster plug. This technique was practiced extensively prior to data collection and performed as gently as possible in order to minimize stress from excessive handling.

All groups were tested on the same day with the order of groups randomized. Testing was performed blind and consisted of presenting sucrose solution to immobilized flies using a wick. Wicks were obtained by pulling threads from a KimWipe with sharp tweezers. The following sucrose solutions were presented in randomized order: 0.1%, 0.3%, 1%, 3%, 10%, and 30% (m/v). Solution saturated wicks were gently touched to the sensory sensilla of the labellum. A positive response was counted as a full and sustained extension of the proboscis. Each concentration of sucrose was presented with intervening water presentation. Individuals that had a positive response to water were recorded as a “false positives” and further testing was not attempted as this indicates dehydration.

Starvation stress resistance assay

Starvation stress assays have been previously described (Djawdan et. al., 1998). Flies from each line were sorted by sex and genotype using light CO₂ anesthesia (<5 minutes) and placed in groups of 10 on vials containing fresh food media. After a recovery period of 48-hours, flies (2-3-days old) were transferred to food deprivation vials (1% non-nutrient agar). A total of 5 vials, with 10 flies per vial, were set up per group. Observations were taken every 4 hours (round the clock) to tally the number of dead flies. Survivor curves were generated and analyzed using Kaplan-Meier Survival Analysis.
Results

**Drosophila cSOD\(^{a108}\) mutants have altered food intake**

We were able to quantify food intake in *Drosophila* using media containing sucrose and non-toxic blue dye (erioglaucine). The composition of media has been found to affect feeding with sucrose producing a stimulatory effect (Edgecombe et. al., 1994). Prior to the assay, flies are food deprived for 24-hours which also stimulates feeding. Observations of proboscis extension (PE) events were taken during the assay. The proportion of PE events indicates the relative amount of time flies spend feeding.

For Figures 3.1 to 3.6, data were analyzed for statistical significance using various implementations of ANOVA, e.g. Ranks: Dunn’s or Tukey method, etc. For Figures 3.7 and 3.8, survival curves were generated using Kaplan-Meier Survival Analysis (Gehan-Breslow) and pairwise multiple comparison procedures were performed using the Holm-Sidak method. (See the legend for each figure for the exact statistical tests used).

Results show that both T0 mutant males and females consume less food than controls as measured by absorbance at 630nm (Appendix B, Figure S16). However, since the weight of flies can vary between genotypes, with T0 and T5 males being statistically different (Appendix B, Figure S17), we wanted to adjust absorbance to account for larger flies having a higher capacity for food intake. Adjusted absorbance readings also show that T0 mutant males and females consume significantly less food than controls (Figure 3.1).

PE events, defined as proboscis extension onto food media, further verify that T0 mutants feed less with T0 males and females having significantly fewer proboscis extension events than controls (Figure 3.2).
Lower food intake correlates with decreased proboscis extension response (PER)

Perception of taste allows an animal to distinguish between edible and toxic compounds and gustatory stimuli can produce reflexive behaviour toward food. To further test food-related behaviour, and to control for possible locomotor biases in the previous assay, a proboscis extension response assay was performed on individual isolated flies in order to gage motivation to feed and the ability to detect different sucrose concentrations. Flies were food deprived for 24-hours prior to testing and immobilized via aspiration into a modified pipette tip. Hydrated flies were presented with randomized concentrations of sucrose using a thin wick to stimulate sensilla on the labellum corresponding to sucrose perception. The PER assay shows that T0 males are significantly less responsive to sucrose stimulation than controls and T0 females are slightly less sensitive but not significantly so (Figure 3.3).

Olfactory response of T0 mutants to repellents and attractants

To further investigate possible sensory defects in T0 mutant flies, we assessed olfactory performance using a T-maze apparatus to measure response to both repellents and attractants. Olfaction is crucial to the perception of food and remains an important feeding-related behaviour. The T-maze apparatus has typically been used to test learning and memory in Drosophila by observing the avoidance response to repellents MCH and 3-Octanol being measured (Devaud, 2003). We investigated the avoidance response of T0 mutants to both 3-Octanol and MCH without a learning and memory component. Results show that T0 mutant males and females tend to have decreased sensitivity to both
undiluted MCH (Figure 3.4) and undiluted 3-Octanol (Appendix B, S18). However, this decrease is generally not statistically significant.

To assess olfactory sensitivity, we next tested diluted MCH (1/1000). Results indicate that T0 mutants follow the same trend observed for undiluted repellents, with T0 males and females having a decreased response which is generally not found to be statistically significant (Figure 3.5).

The ability to respond to repellents indicates that T0 mutants do not demonstrate a severe general olfactory defect, as would be conferred by loss of a broadly expressed odorant receptor such as Or83b (Libert et al., 2007). However, measuring avoidance to repellents does not necessarily elucidate the ability of T0 mutants to detect food-related odors. In order to assess the response of T0 mutants to attractive odors, we used a T-maze apparatus to test a 1% yeast solution. *Drosophila* normally feed on the fermentation products of yeast, and yeast has been shown to be a highly attractive odor to flies (Poon et. al., 2010).

Similar to what was observed for repellent odors, both T0 male and female mutants show less sensitivity to yeast odor but with no statistical significance (Appendix B, Figure S19). Overall, males and females of all genotypes did not show a strong response to yeast. Given the relatively short period (1 minute) flies are allotted to make a decision in the T-maze, a poor response to yeast may reflect the lack of motivation of well-fed flies to move towards a potential food source. We repeated the assay using flies previously food deprived for 24 hours. Food deprived flies were found to be generally more responsive to yeast than non-food deprived flies. Food deprived T0 male and
female mutants tended to be less attracted to yeast odor than controls, but not significantly so (Figure 3.6).

A trap assay performed verified that T0 mutants are less responsive to food odor (K. Pacheco, A., Huston, data not shown). However, the Trap assay requires motility in order for flies to reach the food source. This presents experimental bias, rendering interpretation of results problematic. Interestingly, T0 mutants were observed capable of detecting the food source and entering traps, however, they were delayed in entering traps compared to controls (K. Pacheco, A. Huston, data not shown).

**T0 mutants are significantly more starvation stress resistant than controls**

Starvation stress tolerance can indicate the physiological status of the fly. Given that T0 mutants show decreased food intake, a decreased proboscis extension response and a decreased response to odorants, we wanted to test starvation stress tolerance in T0 mutant flies. Starvation stress was measured by maintaining flies on non-nutrient agar. Survival curves were generated by counting the number of deceased flies every 4 hours after an initial period of 24 hours during which no deaths occurred. The Kaplan-Meier Survival Analysis was used to determine statistical significance. This method accounts for later time points being less statistically reliable given a decrease in population over time.

Results contradicted our predicted hypothesis that T0 mutants would be more sensitive to starvation. T0 males were significantly more starvation stress resistant than controls (Figures 3.7). T0 females were significantly more starvation stress resistant than
the transgenic rescue and showed no difference compared to wild type (Figure 3.8). This result indicates that despite feeding less, T0 mutant flies are not food deprived.

**T0 mutant males are less active than controls but still rhythmic**

Activity is an important food-related behaviour shown to be under circadian control (Ja et. al., 2008). Locomotor activity and the strength of circadian cycles can also indicate fly status and motivation. In addition, this behaviour is of interest given the observation that food deprived animals show increased activity (Martin, 2003).

Using a Trikinetics *Drosophila* Activity Monitor (DAM) and standard beam-crossing assay, we were able to measure overall activity. Results indicate that T0 mutant males have significantly less overall activity than controls (Figure 3.9a). Surprisingly, T0 mutant males remain rhythmic (Appendix B, Figure S20). Female T0 mutants show no decline in activity compared to controls until 9-10 days of age (Figure 3.9b). T0 females also retain locomotor rhythmicity (Appendix B, Figure S21).
Figure 3.1: Blue dye-feeding assay. A) Average absorbance (630 nm) readings adjusted by weight for male flies. B) Average absorbance (630 nm) readings adjusted by weight for female flies.
Figure 3.1: Blue-dye feeding assay. N=15 with 5 flies per sample (total of 75 flies for each group). Blue-dye samples were paired with non-dye controls. Absorbance values from non-dye controls were averaged and subtracted from the equivalent blue-dye value in order to account for background. Absorbance values are adjusted by the average weight (mg) of male or female flies for each genotype. Statistical tests were performed using ANOVA on Ranks: Dunn’s Method. A) Male blue-dye feeding assay. Average absorbance (630nm +/-SEM) was 0.837 (+/-0.252), 13.023 (+/-0.695), 8.521 (+/-0.534) and 10.153 (+/-1.009) for T0, T5, Oregon-R and T0/Oregon-R male flies respectively. **T0 absorbance readings were lower than T5, Oregon-R and T0/Oregon-R (P<0.05). **T5 absorbance readings were higher than Oregon-R (P<0.05). No statistical difference in absorbance was found between Oregon-R and T0/Oregon-R. B) Female blue-dye feeding assay. Average absorbance (630nm +/-SEM) is 1.313 (+/-0.371), 10.804 (+/-0.788), 8.341(+/-0.446) and 9.386 (+/-0.513) for T0, T5, Oregon-R and T0/Oregon-R female flies respectively. **T0 has significantly lower absorbance than T5, Oregon-R and T0/Oregon-R (P<0.05). T5 absorbance readings were higher than Oregon-R (P<0.05). There is no significant difference between T5, Oregon-R and T0/Oregon-R.
Figure 3.2: Observations of proboscis extension (PE) events during 30 minute blue-dye feeding assay. A) Proportion of male proboscis extension events. B) Proportion of female proboscis extension events.
Figure 3.2: Observations of proboscis extension (PE) events during 30 minute blue-dye feeding assay. N=21 with 5 flies per vial (total of 105 flies for each group). Food deprived flies were placed in vials containing 5% sucrose, 0.05% blue-dye and 1% agar. Vials were randomized and each vial was observed for PE events every 2-3 minutes for 2-3 seconds during the 30 minute feeding assay. The total number of proboscis extension events was tallied for each genotype and expressed as a proportion. The proportion was determined by dividing the sum of PE events by the number of feeding opportunities (number of flies per vial x number of vials x number of times vial was observed). A) Proportion of male PE events. Proportions (+/-SEM) were 0.0158 (+/-0.00588), 0.118 (+/-0.0187), 0.158 (+/-0.0197) and 0.113 (+/-0.0265) for T0, T5, Oregon-R and T0/Oregon-R male flies respectively. **T0 males have a fewer proboscis extension events as compared to T5, Oregon-R and T0/Oregon-R (P<0.05, ANOVA on Ranks: Tukey Test). No difference in proboscis extension events was found between T5, Oregon-R and T0/Oregon-R. B) Proportion of female PE events. Proportions (+/-SEM) were 0.0304 (+/-0.00573), 0.105 (+/-0.0132), 0.0920 (+/-0.0113) and 0.0781 (+/-0.00752) for T0, T5, Oregon-R and T0/Oregon-R females respectively. **T0 females have fewer proboscis extension events as compared to T5, Oregon-R and T0/Oregon-R (P<0.05, ANOVA on Ranks: Dunn’s Method). No difference in proboscis extension events was found between T5, Oregon-R and T0/Oregon-R.
Figure 3.3: Proboscis extension response assay. A) Average male sucrose response. B) Average female sucrose response.
Figure 3.3: Proboscis extension response assay measuring mean sucrose response.

Immobilized flies were presented with the following concentrations of sucrose in randomized order: 0.1, 0.3, 1.0, 3.0, 10, 30 % (w/v). Positive responses were scored for each concentration for each group and average scores were combined and represented as a response index. A response index of 6 indicates a positive response to all 6 concentrations of sucrose. A) Male proboscis extension response assay. N=24, 23, 15 and 18 for T0, T5, Oregon-R and T0/Oregon-R respectively. **T0 is significantly less responsive to sucrose as compared to T5, Oregon-R and T0/Oregon-R (P<0.05, ANOVA on Ranks: Dunn’s Method). T5 is significantly less responsive to sucrose as compared to Oregon-R (P<0.05, ANOVA on Ranks: Dunn’s Method). Oregon-R and T0/Oregon-R are not statistically different. B) Female proboscis extension response assay. N=17, 19, 17 and 17 for T0, T5, Oregon-R and T0/Oregon-R respectively. **T0 and T5 are significantly less responsive to sucrose compared to Oregon-R and T0/Oregon-R (P<0.05, ANOVA on Ranks: Dunn’s Method). No statistical difference in sucrose response between T0 and T5 or Oregon-R and T0/Oregon-R (P<0.05, ANOVA on Ranks: Dunn’s Method).
Figure 3.4: Olfactory response to MCH (undiluted) as measured in a T-maze assay. Performance index (PI) indicates the proportion of flies making a choice between the odour and the control. A) Male response to undiluted MCH. B) Female response to undiluted MCH.
**Figure 3.4:** Olfactory response to MCH (undiluted) as measured in a T-maze assay.

Performance index (PI) indicates the proportion of flies making a choice between the odour and the control. N=9 for T0, T5, Oregon-R and T0/Oregon-R. Male or female flies (2-3-days old) were tested in groups of 30 (total of 540 flies tested for each genotype). Flies were transferred to empty vials via aspiration for 30 minutes prior to testing. Flies were then placed at the choice point between the two arms of the T-maze and allowed 1 minute to decide between the odour and the control. The performance index was calculated as previously described (see Materials and Methods). Statistical tests were performed using one-way ANOVA and Tukey for all pairwise multiple comparisons. **A)** Male response to undiluted MCH. The PI (+/-SEM) was 75.9 (+/-5.8), 87.9 (+/-4.7), 98.8 (+/-1.2) and 94.4 (+/-2.4) for T0, T5, Oregon-R and T0/Oregon-R male flies respectively. No statistical difference was found between T0 and T5. T0 are significantly less sensitive to undiluted MCH than Oregon-R (P=0.002) and T0/Oregon-R (P = 0.012). **B)** Female response to undiluted MCH. The PI (+/-SEM) was 79.8 (+/-6.7), 93.9 (+/-3.5), 93.9 (+/-3.0) and 91.1 (+/-4.3) for T0, T5, Oregon-R and T0/Oregon-R female flies respectively. No statistical difference was found between groups.
**Figure 3.5:** Olfactory response to MCH (diluted 1/1000) as measured in a T-maze assay. Performance index (PI) indicates the proportion of flies making a choice between the odour and the control. **A)** Male response to diluted MCH. **B)** Female response to diluted MCH.
**Figure 3.5**: Olfactory response to MCH (diluted 1/1000) as measured in a T-maze assay. Assay was conducted as described in the legend of Figure 3.4. A) Male response to diluted MCH. The PI (+/-SEM) was 14.6 (+/-3.1), 18.3 (+/-3.0), 18.2 (+/-3.4) and 27.0 (+/-3.8) for T0, T5, Oregon-R and T0/Oregon-R male flies respectively. No statistical difference was found between groups. B) Female response to undiluted MCH. The PI (+/-SEM) was 11.2 (+/-5.5), 15.3 (+/-4.9), 28.3 (+/-5.9) and 36.7 (+/-6.8) for T0, T5, Oregon-R and T0/Oregon-R female flies respectively. T0 and T0/Oregon-R are statistically different (P=0.021). No statistical difference was found between remaining groups.
Figure 3.6: Olfactory response to yeast (1%) for food deprived flies as measured in a T-maze assay. Performance index (PI) indicates the proportion of flies making a choice between the odour and the control. A) Male response to yeast (1%). B) Female response to yeast (1%).
Figure 3.6: Olfactory response to yeast (1%) for food deprived flies as measured in a T-maze assay. Assay was conducted as described in the legend of Figure 3.4 with the exception that flies were food deprived for 24 hours prior to testing and the performance index was calculated using the COR for attractants (see Materials and Methods). A) Male response to yeast (1%). The PI (+/-SEM) was 13.2 (+/-2.8), 19.3 (+/-1.7), 24.2 (+/-2.6) and 18.9 (+/-2.5) for T0, T5, Oregon-R and T0/Oregon-R male flies respectively. T0 and Oregon-R are statistically different (P=0.016, one-way ANOVA: Tukey). No statistical difference was found between remaining groups. B) Female response to yeast (1%). The PI (+/-SEM) was 10.7 (+/-0.8), 15.6 (+/-1.0), 19.6 (+/-0.8) and 14.0 (+/-2.2) for T0, T5, Oregon-R and T0/Oregon-R female flies respectively. T0 and Oregon-R are statistically different; Oregon-R and T0/Oregon-R are statistically different (P<0.05; ANOVA on Ranks: Tukey). No statistical difference was found between remaining groups.
Figure 3.7: Male starvation stress resistance assay. Survival curves are shown for T0, T5, Oregon-R and T0/Oregon-R male flies.
**Figure 3.7:** Male starvation stress resistance. N=5 (10 flies per vial; 50 flies total per group). Flies were placed into food deprivation vials containing non-nutrient agar and a hydration source (as described for PER assay). After an initial 24 hours, observations were taken every 4 hours to score the number of dead flies. Survival curves were generated using Kaplan-Meier Survival Analysis: Gehan-Breslow. All pairwise multiple comparison procedures were performed using the Holm-Sidak method. T0 are more starvation stress resistant than T5 (P=9.38E-010). T0 are more starvation stress resistant than Oregon-R (P=2.864E-014). T0 are more starvation stress resistant than T0/Oregon-R (P=7.38E-08). The remaining genotypes are not statistically different from one another.
Figures 3.8: Female starvation stress resistance assay. Survival curves are shown for T0, T5, Oregon-R and T0/Oregon-R female flies.
Figure 3.8: Female starvation stress resistance. Assay was conducted and analyzed as described in the legend of Figure 3.7. T0 females are more starvation stress resistant than T5 females ($P=1.376E-011$). T5 females less starvation stress resistant than Oregon-R and T0/Oregon-R females ($P=6.661E-016$ and $1.665E-015$ respectively). The remaining genotypes are not statistically different.
Figure 3.9: Average daily activity as measured using *Drosophila* Activity Monitors. A) Average daily male activity. B) Average daily female activity.
Figure 3.9: Average daily activity as measured using *Drosophila* Activity Monitors.

Locomotor activity was measured in constant darkness (Dark/Dark). Flies were previously entrained for 3 days (L/D). Activity counts were generated using the number of infrared beam breaks detected by monitors. Counts were averaged for each hour. Average daily activity represents the average activity of all hours in a 24-hour Dark/Dark cycle. A) Average daily male activity. B) Average daily female activity.
Discussion

Flies normally feed on microorganisms found on the surface of fruit and rely on chemosensory cues to identify potential food sources (Brummel et. al., 2004; Carvalho et. al., 2005). Hence quantification of food intake provides an incomplete assessment of Drosophila feeding behaviour as many factors are involved in the decision to feed. To gain a more complete view of feeding behaviour, we performed a range of assays. Assays revealed several novel phenotypes associated with T0 mutants. Mutants were shown to feed less, are less responsive to sucrose stimulation and show a trend of decreased sensitivity to both repellent and attractive odors. Remarkably, T0 mutants are more starvation stress resistant than controls. Locomotor activity is also significantly decreased in T0 mutant males while remaining relatively normal in T0 mutant females until approximately 9-10-days of age; afterward female activity begins to sharply decline.

These phenotypes correlate with observed changes in gene expression (see Chapter 2). Functional processes associated with proteolysis, carbohydrate and lipid metabolism were prominently down-regulated in T0 mutant flies indicating a metabolic alteration that may be reflected in feeding behaviours. In addition, transcripts specifically implicated in feeding (Cyp4e3, Cyp6a2 and Cyp310a1), olfaction (odp56a, odp56e, odp57a, odp99b) and energy homeostasis (LNaz and GLaz) were significantly altered.

ROS have been previously noted to cause damage to neural tissue (Kerr et. al., 2009). Oxidative damage in neurons may alter neuron activity which potentially leads to organism-wide changes in physiology and behaviour. Oxidation of K+ channels for example, is associated with decreased gustatory neuron excitability and decreased taste perception in aging C.elegans (Sesti et. al., 2010). It therefore remains possible that
damage to the peripheral nervous system could induce sensory defects leading to decreased odorant and taste perception in T0 mutant flies. However, T0 mutants remain capable of an odorant response for the three odorants tested. This indicates that sensory neurons likely remain functional in T0 mutant flies.

The ability of T0 mutants to demonstrate a relatively robust response to both repellent and attractive odors seems counter-intuitive given the observed down-regulation of several odorant binding protein transcripts in the microarray experiments. However, odorant binding proteins lack a defined function and are speculated to act as general transporters in the hemolymph fluid surrounding odorant receptors in sensilla (Vosshall, 2000). It has also been suggested that odorant binding proteins are required for odor discrimination rather than odor detection (Wang et. al., 2007). Olfactory assays performed did not evaluate odor discrimination in T0 mutants as odorants belonging to each chemical class were not tested. Thus it remains unclear whether odor discrimination is affected in T0 mutants. While the T0 mutants had slightly weaker, but not statistically different, responses to the various odors compared to control flies, this observed trend may be due to decreased locomotor activity in T0 flies.

_Altered feeding behaviour and sensory perception may optimize survival of Drosophila experiencing oxidative stress_

Nutrient status has been shown to modulate fecundity, stress resistance and lifespan through interaction with intracellular signaling pathways such as TOR and insulin (Partridge et. al., 2005; Grandison et. al., 2009). This suggests that feeding-behaviour can potentially modulate the overall fitness of an animal. Reduced feeding in
T0 mutants may reflect an adaptation to cope with increased oxidative stress. Stressed flies have been shown capable of self-regulating nutrient consumption (Ayres and Schneider, 2009). This phenomenon has also been noted in other insects and spiders (Mayntz et al., 2005).

The effect of dietary restriction on lifespan and stress resistance has been well established (Partridge et. al., 2005). Dietary restriction (where intake is reduced to 60% of normal), extends lifespan in a range of species from nematodes (Braeckman et al, 2001) to flies (Pletcher et al, 2002) to mammals (Masoro, 2005).

It remains possible that T0 mutants self-regulate nutrient intake in order to promote survival under conditions of chronic oxidative stress. Sucrose response has been suggested to indicate the current metabolic status in the fly (Swithers and Davidson, 2008). Thus decreased sucrose response in T0 mutants could also reflect an altered metabolic strategy that results in decreased food intake.

The decreased response to odorants in T0 mutants is also consistent with this suggestion. Sensory signals originating from sensory structures are communicated through neuroendocrine circuits (Libert et al 2007). Evidence indicates that olfaction influences global energy balance, exerting a regulatory effect similar to what is observed for dietary restriction (Libert et. al., 2007). For example, Drosophila mutants lacking functional Or83b (an odorant receptor required for general olfaction) demonstrate olfactory defects, altered metabolism, increased stress resistance and extended lifespan (Libert et al, 2007).
Increased starvation stress tolerance reflects an altered metabolic strategy in T0 mutants

Energy homeostasis in *Drosophila* uses at least two different lipocatabolic systems with one relying on adipokinetin hormone (Gronke et. al., 2007) and the other on the adipose TAG lipase homolog brummer (Gronke et. al., 2005). In addition, nutrient-specific signaling pathways such as IGF/insulin-signaling have been shown to modulate lipid stores (Tu and Tatar, 2003). T0 mutants demonstrate a remarkable resistance to starvation yet increased lipid content (A. Huston, data not shown) leading us to question the mechanisms through which this occurs. Lipocalin-related proteins, such as NLaz, function in the JNK signaling cascade and have been found induced in T0 mutants. JNK works to antagonize insulin signaling and increases stress resistance and starvation tolerance (Hull-Thompson et. al., 2009). Phenotypes observed in T0 mutants are consistent with the JNK activation. However, additional experiments are required to explore this possibility. The implication of JNK activation in T0 mutants will be further discussed in Chapter 4.
CHAPTER 4

General Discussion
The cSOD\textsuperscript{n108} mutation confers a complex condition demonstrating a range of transcriptional changes and phenotypes. Various processes appear altered in cSOD\textsuperscript{n108} mutant *Drosophila* making biological interpretation challenging. The complexity of the cSOD\textsuperscript{n108} model reflects the indiscriminate nature of reactive oxygen species (ROS). ROS damage to DNA, proteins, and lipids can propagate randomly and leads to global effects that alter cellular redox status (López-Mirabal and Winther, 2007). Altered redox status can modify the function of enzymes, regulatory proteins and transcription factors, further disrupting cell homeostasis.

ROS damage elicits various repair responses. Oxidative damaged to DNA initiates the base excision repair (BER) pathway (Barzilai and Yamamoto, 2004; Fensgård et. al., 2010). Oxidized proteins are degraded through the 26-S proteasomal pathway (Davies, 2001) or via lysosomes (Chondrogianni et. al., 2002). Oxidization of membrane phospholipids, a vulnerable ROS target, causes a conformational change targeting damaged phospholipids for hydrolysis by phospholipases (van den Berg et. al., 1993). Repair of macromolecules helps maintain the normal function of the cell. However, once repair capacity is overwhelmed, and damage reaches a critical state, apoptotic pathways are initiated (Roos and Kaina, 2006).

**Analysis of the cSOD\textsuperscript{n108} mutant transcriptome**

Microarray experiments performed on cSOD\textsuperscript{n108} mutants reflect widespread damage to macromolecules. Transcriptional changes indicate a DNA damage response as well as the induction of apoptosis. In addition, induction of lipase and protease transcripts may reflect increased turnover of damaged lipids and proteins. Heat shock protein and
cytochromeP450 transcripts are up-regulated, further indicating attempts to repair and attenuate oxidative damage in cSOD_{n108} mutants. Other genomic studies of oxidative stress in *Drosophila* have reported similar observations (Girardot et. al., 2004; Landis et. al., 2004).

However, many of the transcriptional changes observed in cSOD_{n108} mutants are not intuitive to a predicted oxidative stress response. Transcripts associated with innate immunity were abundantly induced. Previous oxidative stress studies have also noted induction of the innate immune response (see Chapter 2 Discussion). Carbohydrate catabolism was repressed with the down-regulation of amylase transcripts and lipid catabolism was repressed with the down-regulation of triacylglycerol lipase transcripts. Transcripts associated with proteolysis were also altered. These transcriptional changes suggest an altered metabolism in cSOD_{n108} mutants. The induction of lipocalin-related protein transcripts further supports this suggestion.

The repression of transcripts involved in behavioural processes forms another interesting result novel to cSOD_{n108} mutants. These down-regulated transcripts correspond to accessory gland peptides (36DE, 53C14a/b/c, 62F, 63F, 76A, 98AB), cytochrome P450s (4e3, 6a2, 310a1), odorant binding proteins (56a, 56e, 57a, 99b), haemolymph juvenile hormone binding proteins and Takeout. The corresponding peptides have been implicated in reproduction, feeding behaviour and odorant perception (Chapman et. al., 2000; Chung et. al., 2009; Wang et. al., 2007).
Feeding-related behavioural phenotypes in cSOD<sup>n108</sup> mutants

Many of the transcriptional changes in cSOD<sup>n108</sup> mutants are associated with metabolism, feeding and odorant perception, leading us to examine behavioural phenotypes. Behavioural assays measured food intake, sucrose response, odorant response, starvation stress resistance and locomotor activity.

Results indicate that cSOD<sup>n108</sup> mutants feed less, have fewer proboscis extension events, and are less responsive to sucrose and odorants. In addition, cSOD<sup>n108</sup> mutants are more starvation stress resistant and preliminary assessment of lipid stores indicates higher lipid content than controls. These behavioural phenotypes, in the context of the observed transcriptional changes, reflect a potential adaptive strategy in response to chronic oxidative stress in cSOD<sup>n108</sup> mutants.

Behavioral phenotypes reflect a potential adaptive strategy in cSOD<sup>n108</sup> mutants

The generation of superoxide largely occurs as a by-product of oxidative phosphorylation. Increased cellular demand for ATP metabolizes a greater volume of oxygen via the electron transport chain resulting in increased superoxide production (St-Pierre et. al., 2006). Production of mitochondrial superoxide correlates inversely with maximum lifespan and the amount of oxidized protein, lipid and DNA (Ku et. al., 1993; Barja and Herrero, 2000; Sohal et. al., 1993; Agarwal and Sohal, 1996).

Reduced feeding, sucrose and odorant response and locomotor activity in cSOD<sup>n108</sup> mutants may be induced as a mechanism to attenuate ROS generation by reducing ATP demand. Dietary restriction exerts cellular protection from a variety of stress conditions ranging from chemotherapy in humans (Lee and Longo, 2011) to
hypoxic injury in *Drosophila* (Vigne et al., 2009). According to the “rate-of-living” hypothesis, dietary restriction should decrease metabolic rate and therefore production of mitochondrial ROS (Szatrowski and Nathan, 1991). However, the mechanisms underlying the protective effects of dietary restriction remain poorly understood. Currently, no experimental evidence exists to support a correlation between metabolic rate, ROS production, oxidative damage and longevity (Miwa et al., 2004; Hulbert et al., 2004).

Alternatively, ROS-damage to sensory and motor neurons presents a direct physiological cause for observed feeding-related phenotypes. Neural tissue has been noted as a vulnerable ROS target (Metodiewa et al., 1999). Motor neurons have been suggested to form a lifespan determining “rate-limiting” tissue in *Drosophila* (Parkes et al., 1998) and ROS-associated oxidation of neuronal K+ channels is implicated in the loss of gustatory perception in aging *C. elegans* (Sesti et al., 2009). Decreased proboscis extension, locomotor activity and odorant and sucrose response may reflect neural degeneration to both sensory and motor neurons. However, the neurophysiological basis of these behavioural phenotypes has not been characterized, and future work in this area could include electrophysiological and morphological investigations of cSOD\textsuperscript{n108} mutant peripheral nervous system.

**Evidence of activated JNK signaling in cSOD\textsuperscript{n108} mutants**

Increased starvation stress resistance and decreased feeding confounds interpretation of the cSOD\textsuperscript{n108} mutant condition. Intuitively, we predicted that decreased feeding would translate into decreased starvation stress resistance. However, the contrary
was observed with mutants displaying an impressive resistance to starvation and potentially increased energy reserves. Activation of the c-Jun N-terminal kinase (JNK) signaling pathway provides a possible explanation for these observations. Activation of JNK signaling has been shown to increase paraquat resistance, starvation stress resistance and longevity in *Drosophila* (Hull-Thompson et. al., 2009). *Drosophila* mutants over-expressing NLaz, a critical down-stream effector molecule required for JNK activation, demonstrate higher triacylglyceride and glycogen stores than controls (Hull-Thompson et. al., 2009). Microarray data suggests JNK activation in cSOD\textsuperscript{n108} mutants with NLaz transcripts induced 5.21-, 4.10-, and 9.07-fold in 2-3-day old males, 5-6-day old males and 2-3-day old females respectively. In addition, one of the most prominently up-regulated transcripts, map205, has a putative function in JNK signaling (Archambault et. al., 2008). Repression of triacylglycerol lipase and amylase transcripts in cSOD\textsuperscript{n108} mutants is also consistent with reported JNK-mediated increases in lipid and glycogen stores (Hull-Thompson et. al., 2009).

**The Energy Paradox**

The response to oxidative stress can be predicted to be an energetically costly process. Both repair and initiation of apoptosis requires ATP for various enzymatic reactions (Zamaraeva et. al., 2005). The catalytic mechanism of the proteosome, for example, depends on ATP hydrolysis to break peptide bonds (Benaroudj et. al., 2003).

We observe that cSOD\textsuperscript{n108} mutants feed less, have potentially higher energy stores and fail to demonstrate significant growth defects or differences in weight compared to controls. These observations in the context of an energetically demanding oxidative stress
response lead us to question how cSOD\textsuperscript{n108} mutants meet basic cellular energy requirements.

Several possibilities exist to explain this apparent paradox. Mutants demonstrate decreased reproductive function as indicated by the down-regulation of transcripts associated with accessory gland peptides. Additionally, cSOD\textsuperscript{n108} males are completely infertile and females are semi-infertile (Parkes et. al., 1998). Reproduction has been shown to require a substantial allocation of energy resources (Sibly and Curnow, 1993). Decreased reproduction potentially compensates for decreased feeding in order to maintain energy balance in cSOD\textsuperscript{n108} mutants.

Decline in locomotor activity presents an additional mechanism that serves to conserve energy output. Assessment of locomotor activity reveals that cSOD\textsuperscript{n108} males are significantly less active than controls. Activity is also decreased in females, but this decrease is delayed until about 9 days of age.

The regulation of locomotor behaviour is highly complex and known to be under circadian control (Allada and Chung, 2010). Interestingly, cSOD\textsuperscript{n108} mutants demonstrate no evidence of disrupted circadian rhythm making dysfunction of internal clocks an unlikely explanation for observed decreases in activity.

**The origin of the cSOD\textsuperscript{n108} mutant phenotype**

The design of this work follows the premise that changes in gene expression exert a causal effect leading to changes in behaviour. Examination of behavioural phenotypes followed the observation of relevant transcriptional changes. But given the complexity of behavioural-genetic interaction, we must question the basis of this paradigm. Are the
behaviours in cSOD^{n108} mutants resulting from initiation of transcriptional programs responding to increased cellular ROS? Or alternatively, does ROS exert an effect on the nervous system leading to altered behaviour and subsequent transcriptional adjustment? The possibility that cSOD^{n108} mutant phenotypes operate downstream of stress-induced signaling pathways presents an intriguing concept that may help elucidate candidate genes implicated in feeding-related behaviour. However, further work is required to investigate the origin of observed cSOD^{n108} mutant phenotypes. Additional experiments should also address other questions arisen throughout this work. These experiments are discussed below.

**Future Directions**

Further assessment of physiological phenotypes is required to determine how nutrient storage is affected under conditions of chronic oxidative stress. Experiments should include assays to measure glycogen stores, triglyceride stores and total protein content. In addition, hemolymph characterization (to measure circulating carbohydrates) would provide insight into the energy status of cSOD^{n108} mutants. Assessment of metabolic rate is required to characterize cellular respiration in cSOD^{n108} mutants. Examining rate of oxygen consumption per unit of mass has previously been used to measure respiration in *Drosophila* (Hulbert et. al., 2004). Further verification of cellular energy status can be determined using well-established protocols to assess the ratio of ATP:ADP (Nagel et. al., 2010).

Additional behavioural experiments are required to assess phenotypes relating to courtship behaviour. We have examined feeding on the basis that transcripts implicated
in the regulation of feeding were significantly repressed in microarray experiments. However, transcripts relating to courtship behaviour were also abundantly down-regulated. Of particular interest is whether cSOD\textsuperscript{n108} mutants attempt mating. Observation of courtship behaviours, such as the generation of acoustic cues, need to be examined given that cSOD\textsuperscript{n108} mutants demonstrate a crinkled wing phenotype that possibly interferes with wing vibration. In addition, observation of courtship duration, copulation duration and mating frequency would also be of interest (O’Dell, 2003). Also, the examination of feeding behaviour in cSOD\textsuperscript{n108} mutant larvae may provide further insight into the how oxidative stress affects metabolism since larvae feed in a relatively hypoxic environment.

The characterization of neurophysiology remains critical to elucidate the basis of observed cSOD\textsuperscript{n108} mutant phenotypes. Electrophysiological assessment of sensory neurons may determine whether cSOD\textsuperscript{n108} mutants are capable of detecting sucrose and odorants stimulus. In addition, calcium imaging may determine sensory neuron activation.

In conclusion of this thesis, we are able to integrate both genomic and behavioural techniques to determine correlations between gene expression and behaviour. This approach has identified several candidate genes that possibly function to alter behaviour in cSOD\textsuperscript{n108} mutants. Future work will help decipher the complex interactions that occur between transcription, physiology and behavioural output. Understanding these interactions will potentially provide insight on aging and ROS-associate diseases, which may lead to the identification of novel means to modulate stress resistance.
References


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models of mutant SOD1-mediated familial ALS. Prog Neurobiol 85, 94-134.
production of reactive oxygen species by cytochrome P450. Toxicol Appl Pharmacol 199, 316-331.


Web Pages:

Appendix A

Supplemental Data for Chapter 2
Table S1: Summary of top diverging gene changers with a ratio between fold values >2. Gene changers were extracted from clusters generated using an optimized Pearson Uncentered algorithm to compare heat maps for 2-3d male and 5-6d male flies; T0 versus T5.

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<tr>
<th>Gene ID</th>
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<th>5-6d M</th>
<th>Gene Name</th>
<th>Gene Function</th>
</tr>
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<td></td>
</tr>
<tr>
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<td>6.02</td>
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Table S1: Summary of top diverging gene changers with a ratio between fold values >2. Table continued from previous page.

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Table S2: Summary of top diverging gene changers with a ratio between fold values >2. Gene changers were extracted from clusters generated using an optimized Pearson Uncentered algorithm to compare heat maps for 2-3d male and 2-3d female flies; T0 versus T5.

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### Table S2: Summary of top diverging gene changers with a ratio between fold values >2.
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<tr>
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<td>Dmel_CG34367</td>
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<tr>
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<tr>
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Table S2: Summary of top diverging gene changers with a ratio between fold values >2.
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Table S2: Summary of top diverging gene changers with a ratio between fold values >2.
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Table S3: Common up-regulated genes overlapping in cSOD\textsuperscript{n108} mutant flies as compared to transgenic rescue controls for 2-3-day old males, 5-6-day old males and 2-3-day old females. **VENNY** was used to generate list of genes in common between groups. Gene Name and Gene Function obtained from FlyBase.

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<tr>
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<td>3.01</td>
<td>7.74</td>
<td>Dmel_10265</td>
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</table>
Table S4: Common down-regulated genes overlapping in cSOD<sup>n108</sup> mutant flies as compared to transgenic rescue controls for 2-3-day old males, 5-6-day old males and 2-3-day old females. *VENNY* was used to generate list of genes in common between groups. Gene Name and Gene Function obtained from *FlyBase*.

<table>
<thead>
<tr>
<th>Gene ID</th>
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<th>5-6dM</th>
<th>2-3dF</th>
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<th>Fold Change</th>
</tr>
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<td>-82.95</td>
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<td>serine-type endopeptidase activity</td>
</tr>
<tr>
<td>CG32751</td>
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<td>-7.97</td>
<td>-23.03</td>
<td>Vanin-like protein 2</td>
<td>pantetheine hydrolase activity; hydrolase activity; nitrogen compound metabolic process</td>
</tr>
<tr>
<td>CG3513</td>
<td>-18.09</td>
<td>-46.05</td>
<td>-11.11</td>
<td>Dmel_CG3513</td>
<td>serine-type endopeptidase inhibitor activity</td>
</tr>
<tr>
<td>CG30360</td>
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<td>-16.88</td>
<td>-7.62</td>
<td>Dmel_30360</td>
<td>carbohydrate metabolic process; glycoside hydrolase</td>
</tr>
<tr>
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<td>-11.43</td>
<td>-18.53</td>
<td>Dmel_CG11784</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>CG2071</td>
<td>-13.58</td>
<td>-15.44</td>
<td>-6.95</td>
<td>Serine protease 6</td>
<td>proteolysis</td>
</tr>
<tr>
<td>CG17876</td>
<td>-11.95</td>
<td>-12.02</td>
<td>-6.88</td>
<td>Amylase distal</td>
<td>carbohydrate metabolic process</td>
</tr>
<tr>
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<td>-9.97</td>
<td>-16.53</td>
<td>thioredoxin-2</td>
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</tr>
<tr>
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<td>-11.45</td>
<td>-6.94</td>
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<td>carbohydrate metabolic process</td>
</tr>
<tr>
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<td>-14.70</td>
<td>-5.81</td>
<td>Dmel_CG9377</td>
<td>serine-type endopeptidase activity</td>
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<td>-13.58</td>
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<td>dipeptidyl-peptidase activity</td>
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<td>Dmel_CG30083</td>
<td>serine-type endopeptidase activity</td>
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<td>CG31039</td>
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<td>-3.88</td>
<td>Jonah 99Ci</td>
<td>serine-type endopeptidase activity</td>
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<td>-8.66</td>
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<td>-3.82</td>
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<tr>
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<tr>
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<td>-2.64</td>
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<td>haemolymph juvenile hormone binding</td>
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<td>-2.35</td>
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<tr>
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<td>-2.31</td>
<td>-2.15</td>
<td>Jonah 25Biii</td>
<td>endopeptidase activity</td>
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</table>
Table S5: Common up-regulated genes overlapping in cSOD
mutant flies as compared to transgenic rescue controls for 2-3-day old males and 5-6-day old males. VENNY was used to generate list of genes in common between groups. Gene Name and Gene Function obtained from FlyBase.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>2-3d M</th>
<th>5-6d M</th>
<th>Gene Name</th>
<th>Gene Function</th>
</tr>
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<td>lipase activity</td>
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<td>34.58</td>
<td>44.24</td>
<td>Probable cytochrome P450 28a5</td>
<td>electron carrier activity; P450</td>
</tr>
<tr>
<td>CG1483</td>
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<td>68.60</td>
<td>Microtubule-associated protein 205</td>
<td>mitotic cell cycle</td>
</tr>
<tr>
<td>CG4650</td>
<td>24.49</td>
<td>21.95</td>
<td>Dmel_CG4650</td>
<td>serine-type endopeptidase activity</td>
</tr>
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<tr>
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<td>9.64</td>
<td>18.76</td>
<td>Companion of reaper</td>
<td>DNA damage response; induction of apoptosis</td>
</tr>
<tr>
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<td>16.72</td>
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<td>transmembrane transport</td>
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<tr>
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<tr>
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<tr>
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<td>glucuronosyltransferase activity</td>
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<tr>
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<td>5.97</td>
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<td>polyamine biosynthetic process</td>
</tr>
<tr>
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<td>5.74</td>
<td>Dmel_CG13793</td>
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<td>Transcription factor, fork head</td>
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<tr>
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<td>serine-type endopeptidase inhibitor activity</td>
</tr>
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<td>2.97</td>
<td>Dmel_CG13941</td>
<td>Arc2; Retrotransposon gag protein</td>
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</tbody>
</table>
Table S5: Common up-regulated genes overlapping in cSOD\textsuperscript{n108} mutant flies as compared to transgenic rescue controls for 2-3-day old males and 5-6-day old males. Table continued from previous page.

<table>
<thead>
<tr>
<th>Gene ID</th>
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<th>5-6d M</th>
<th>Gene Name</th>
<th>Gene Function</th>
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<tbody>
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<td>Insect allergen-related</td>
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<td>5.11</td>
<td>Heat-shock-protein-70Ab</td>
<td>response to hypoxia; response to heat</td>
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<td></td>
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<td>Heat-shock-protein-70Aa</td>
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</tr>
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<td>oxidation reduction</td>
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<td>phospholipase activity; lipid metabolic process</td>
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</tbody>
</table>

*a pseudo gene was removed from the table
Table S6: Common down-regulated genes overlapping in cSOD\textsuperscript{n108} mutant flies as compared to transgenic rescue controls for 2-3-day old males and 5-6-day old males. \textit{VENNY} was used to generate list of genes in common between groups. Gene Name and Gene Function obtained from \textit{FlyBase}.

<table>
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<tr>
<th>Gene ID</th>
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<th>5-6d M</th>
<th>Gene Name</th>
<th>Gene Function</th>
</tr>
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<td>pantetheine hydrolase activity; nitrogen compound</td>
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<td>metabolic process</td>
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<tr>
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<td>-11.43</td>
<td>Dmel_CG11784</td>
<td>glutathione transferase activity</td>
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<td>proteolysis</td>
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<td>alpha-amylose activity; carbohydrate metabolic</td>
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<td></td>
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<td>process</td>
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<td>-9.97</td>
<td>thioredoxin-2</td>
<td>cell redox homeostasis; determination of adult lifespan; response to DNA damage stimulus; response to oxidative stress.</td>
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<td>alpha-amylose activity; carbohydrate metabolic</td>
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<td>serine-type endopeptidase activity</td>
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<td>perception of smell</td>
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<tr>
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<td>sensory perception of chemical stimulus</td>
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Table S6: Common down-regulated genes overlapping in cSOD<sup>n108</sup> mutant flies as compared to transgenic rescue controls for 2-3-day old males and 5-6-day old males. Table continued from previous page.

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<td>galactose binding; Gram-negative bacterial cell surface binding; induction of bacterial agglutination</td>
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Table S6: Common down-regulated genes overlapping in cSOD\textsuperscript{n108} mutant flies as compared to transgenic rescue controls for 2-3-day old males and 5-6-day old males. Table continued from previous page.

<table>
<thead>
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* a pseudo gene was removed from the table
Table S7: Common up-regulated genes overlapping in cSOD<sup>108</sup> mutant flies as compared to transgenic rescue controls for 2-3-day old males and 2-3-day old females. *VENNY* was used to generate list of genes in common between groups. Gene Name and Gene Function obtained from *FlyBase*.

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Table S7: Common up-regulated genes overlapping in cSOD<sup>n108</sup> mutant flies as compared to transgenic rescue controls for 2-3-day old males and 2-3-day old females. Table continued from previous page.

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Table S8: Common down-regulated genes overlapping in cSOD\textsuperscript{n108} mutant flies as compared to transgenic rescue controls for 2-3-day old males and 2-3-day old females. \textit{VENNY} was used to generate list of genes in common between groups. Gene Name and Gene Function obtained from \textit{FlyBase}.

<table>
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<td>endopeptidase activity</td>
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Table S9: Summary of all functional categories generated for cSOD\textsuperscript{n108} mutant flies as compared to transgenic rescue controls for the following groups: 2-3-day old males, 5-6-day old males and 2-3-day old females. DAVID Bioinformatics Resource was used to generate GO Ontology terms corresponding to GO Biological Process (BP, level 5), Molecular Function (MF, level 5), INTERPRO and KEGG (P<0.05). Symbols indicate the presence (+) or absence (-) of significantly up-regulated or down-regulated functional categories for each group. An abbreviated version of this table is represented in Chapter 2 (Table 2.3).

<table>
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<th>5-6d M</th>
<th>2-3d F</th>
<th>Down 2-3d M</th>
<th>5-6d M</th>
<th>2-3d F</th>
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<td>Amylase activity</td>
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Table S9: Summary of all functional categories generated for cSOD^n108 mutant flies as compared to transgenic rescue controls for the following groups: 2-3-day old males, 5-6-day old males and 2-3-day old females. Table continued from previous page.

<table>
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<tr>
<th>Category</th>
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Figure S10: GO graph showing up-regulated transcripts annotated with GO Biological Process in cSOD<sup>108</sup> mutant flies as compared to transgenic rescue controls for 2-3-day old males. GO graph generated using the Babelomics 4 analysis tool. Node scores indicate enrichment with higher node scores reflecting that more transcripts are annotated within that category.
Figure S11: GO graph showing down-regulated transcripts annotated with GO Molecular Function in cSOD$^{108}$ mutant flies as compared to transgenic rescue controls for 2-3-day old males. GO graph generated using the Babelomics 4 analysis tool. Node scores indicate enrichment with higher node scores reflecting that more transcripts are annotated within a category. This figure is also represented in Chapter 2 (Figure 2.5).
Figure S12: GO graph showing up-regulated transcripts annotated with GO Biological Process in cSOD\textsuperscript{108} mutant flies as compared to transgenic rescue controls for 5-6-day old males. GO graph generated using the Babelomics 4 analysis tool. Node scores indicate enrichment with higher node scores reflecting that more transcripts are annotated within that category.
Figure S13: GO graph showing down-regulated transcripts annotated with GO Molecular Function in cSOD\textsuperscript{n108} mutant flies as compared to transgenic rescue controls for 5-6-day old males. GO graph generated using the Babelomics 4 analysis tool. Node scores indicate enrichment with higher node scores reflecting that more transcripts are annotated within that category.
**Figure S14:** GO graph showing up-regulated transcripts annotated with GO Biological Process in cSOD<sup>108</sup> mutant flies as compared to transgenic rescue controls for 2-3-day old females. GO graph generated using the *Babelomics 4* analysis tool. Node scores indicate enrichment with higher node scores reflecting that more transcripts are annotated within that category.
Figure S15: GO graph showing down-regulated transcripts annotated with GO Molecular Function in cSOD<sup>n108</sup> mutant flies as compared to transgenic rescue controls for 2-3-day old females. GO graph generated using the Babelomics 4 analysis tool. Node scores indicate enrichment with higher node scores reflecting that more transcripts are annotated within that category.
Appendix B

Supplemental Data for Chapter 3

Several of the assays presented were performed in conjunction with Umar Farooq (Figures S16-17) and Karen Pacheco (Figures S18-19).
Figure S16: Blue dye-feeding assay. A) Average absorbance (630 nm) readings for males. B) Average absorbance (630 nm) readings for females.
Figure S16: Blue-dye feeding assay. N=15 with 5 flies per sample (total of 75 flies for each group). Blue-dye samples were paired with non-dye controls. Absorbance values from non-dye controls were averaged and subtracted from the equivalent blue-dye value in order to account for background noise. **A**) Average absorbance (630 nm) readings for males. **B**) Average absorbance (630 nm) readings for females.
Figure S17: Average weight of each genotype. A) Average male weight. B) Average female weight.
**Figure S17:** Average weight of each genotype. **A)** Average male weight. **T0** males weigh significantly less than T5 but have no difference in weight compared to Oregon-R and T0/Oregon-R (P<0.05, ANOVA on Ranks: Tukey Test). **B)** Average female weight. **T0** females show no significant difference in weight as compared to T5 and T0/Oregon-R (P<0.05, ANOVA on Ranks: Dunn’s Method). **T0** females weigh significantly less than Oregon-R (P<0.05, ANOVA on Ranks: Dunn’s Method).
Figure S18: Olfactory response to 3-Octanol (undiluted) as measured in a T-maze assay. Performance index (PI) indicates the proportion of flies making a choice between the odour and the control. A) Male response to undiluted 3-Octanol. B) Female response to undiluted 3-Octanol.
**Figure S18**: Olfactory response to 3-Octanol (undiluted) as measured in a T-maze assay. Performance index (PI) indicates the proportion of flies making a choice between the odour and the control. Male or female flies (2-3-days old) were tested in groups of 30 (total of 540 flies tested for each genotype). Flies were transferred to empty vials via aspiration for 30 minutes prior to testing. Flies were then placed at the choice point between the two arms of the T-maze and allowed 1 minute to decide between the odour and the control. The performance index was calculated as previously described (see Materials and Methods). Statistical tests were performed using one-way ANOVA and Tukey for all pairwise multiple comparisons. **A**) Male response to undiluted 3-Octanol. There is not a statistically significant difference between the genotypes ($P = 0.269$), One-way ANOVA. **B**) Female response to undiluted 3-Octanol. There is not a statistically significant difference between the genotypes ($P = 0.369$), One-way ANOVA.
Figure S19: Olfactory response to yeast (1%) for unstarved flies as measured in a T-maze assay. Performance index (PI) indicates the proportion of flies making a choice between the odour and the control. A) Male response to yeast (1%). B) Female response to yeast (1%).
**Figure S19**: Olfactory response to yeast (1%) for unstarved flies as measured in a T-maze assay. Performance index (PI) indicates the proportion of flies making a choice between the odour and the control. Assay performed as described in the legend of Figure S18. A) Male response to yeast (1%). There is a statistically significant difference between genotypes ($P = 0.008$), One-way ANOVA. B) Female response to yeast (1%). There is a statistically significant difference between genotypes ($P = 0.003$), One-way ANOVA.
**Figure S20:** Average male hourly activity as measured using *Drosophila* Activity Monitors. **A**) Average male activity hourly activity for T0 and T5. **B**) Average male hourly activity for Oregon-R and T0/Oregon-R.
Figure S20: Average male hourly activity as measured using Drosophila Activity Monitors. Locomotor activity was measured in constant darkness (Dark/Dark). Flies were previously entrained for 3 days (L/D). Activity counts were generated using the number of infrared beam breaks detected by monitors. Counts were averaged for each hour. A) Average male activity hourly activity for T0 and T5. B) Average male hourly activity for Oregon-R and T0/Oregon-R.
**Figure S21:** Average female hourly activity as measured using *Drosophila Activity Monitors.*  
**A**) Average female activity hourly activity for T0 and T5.  
**B**) Average female hourly activity for Oregon-R and T0/Oregon-R.
Figure S21: Average female hourly activity as measured using *Drosophila* Activity Monitors. Locomotor activity was measured in constant darkness (Dark/Dark). Flies were previously entrained for 3 days (L/D). Activity counts were generated using the number of infrared beam breaks detected by monitors. Counts were averaged for each hour. A) Average female activity hourly activity for T0 and T5. B) Average female hourly activity for Oregon-R and T0/Oregon-R.