Evaluating the effects of oxidative stress on microRNA accumulation in Drosophila melanogaster and their regulatory roles in cellular protection and degeneration

BY

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Department of Biological Sciences

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Winnipeg, Manitoba

© August 2017

Acknowledgements

I am extremely grateful to my advisor, Dr. Steve Whyard, for his endless support, guidance, and knowledge during the course of my degree. This thesis would not have been possible without your patience and encouragement. You have been instrumental in my growth as a student at the University of Manitoba and success in both my research and career endeavors in the field of medicine.

I thank my thesis committee, Dr. Mark Belmonte, Dr. Deborah Court and greatly appreciate all of the support, and advice they have given to me.

I thank all my lab mates, past and present, who have helped and supported me. I would especially like to thank Aditi Singh and Cassidy Erdelyan for teaching me a variety of lab techniques, as well as Alison Partridge, Dave Giesbrecht, Suresh Desai, and Dave Boguski for their friendship and advice over the years. An additional thank you goes to the summer students that assisted me in experiments throughout the years: Cassie DuGray, Gabriel Dmytryszyn, Richard Jung, and Ingrid Hougen.

Special thanks to Dr. Erwin Huebner, Dr. Bill Diehl-Jones, and the Cardona Lab for the use of their lab space and equipment.

Finally, I must thank my family and friends, especially my husband, parents, and sister, who have supported and encouraged me every step of the way.

This research was funded by the National Sciences and Engineering Research Council (NSERC), and I gratefully acknowledge additional funding through the University of Manitoba Faculty of Science Graduate Studentship Awards.

Abstract

Oxidative stress is considered to be an underlying mechanism in the pathogenesis of many cellular degenerative processes. In this study, microRNAs (miRNAs) are examined as potential epigenetic regulators of the oxidative stress response. In an effort to identify miRNAs up- or down-regulated during oxidative stress, we conducted a microarray analysis to evaluate miRNA accumulation changes in *Drosophila melanogaster* exposed to hyperoxic versus normoxic conditions. Several miRNAs were further evaluated using qRT-PCR to determine their accumulation in whole bodies and heads as well as changes over extended hyperoxia exposures. *Dme-miR-8, -11,* and *-970* were found to be up-regulated in both whole bodies and heads after 5 days hyperoxia exposure. *Jaguar* (*jag*), *castor* (*cas*), and *derailed* (*drl*) were identified as putative targets of these three miRNAs using miRNA target prediction algorithms. Reporter gene-based assays were used to examine the interaction of the miRNAs with the target mRNAs, and confirmed functional suppressive relationships between *miR-11:cas* and *miR-970:drl*, but not *miR-8:jag*. Cell-based assays were also used to assess the ability of candidate microRNAs to suppress expression of several different predicted target genes with known antioxidant activities: *superoxide dismutase* (*Sod*), *heat shock protein cognate70-4* (*Hsc70-4*), *sniffer* (*sni*), *thioredoxin-2* (*trx-2*), and *catalase* (*cat*). *Sod* was not significantly down-regulated by any miRNA, but *mir-927:Hsc70-4*, *mir-964:Hsc70-4*, *mir-277:sni*, *mir-1013:trx-2*, and *mir-1012:cat* interactions were all functionally verified in addition to some pairings having correlational accumulation/expression profiles under hyperoxic stress conditions.

Key words: Oxidative stress, *Drosophila melanogaster*, microRNA, antioxidant genes

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Chapter 1: General Introduction

1.1 Oxidative Stress

A challenge for all aerobic species is the homeostatic regulation of molecular oxygen's metabolites, namely, the reactive oxygen species (ROS). Whether ROS are generated exogenously or endogenously, an intracellular rise in ROS can damage cell organelles and activate specific signalling pathways, which is collectively termed oxidative stress (D'Autréaux & Toledano 2007). There are many human disease states linked to oxidative stress and mitochondrial dysfunction. At a cellular level, excessive ROS can alter cellular homeostasis by directly oxidizing and thereby inhibiting the functions of many cellular macromolecules, while at the tissue level, defective or incomplete repair of ROS-induced cellular damage can result in broader scale damage, such as neurodegeneration, bone marrow failure, and cancer (Kim *et al.* 2015). Alterations in mitochondrial DNA, as a known oxidative stress precursor, is associated with the pathogenesis and progression of myoclonic epilepsy, ragged red fibres syndrome, skeletal muscle diseases, and ischemic heart diseases (Greaves & Taylor 2006; Wu *et al.* 2010; Powers & Jackson 2008; Tsutsui *et al.* 2011; Pei *et al.* 2016; Lightowlers *et al.* 2015). Additionally, mitochondrial biogenesis plays a central role in cellular activity, especially in neurons, where it promotes development, activity, connectivity, plasticity, and survival (Uittenbogaard & Chiaramello 2014). The brain is a particularly vulnerable tissue as it contains large amounts of polyunsaturated fatty acids (PUFA), which are prone to free radical attack due to the double bonds within membranes, allowing easy removal of hydrogen atoms by ROS such as OH^- (Frederickson & Bush 2005). Clearly, the imbalance of oxygen homeostasis that triggers

oxidative stress is an important component of many disease etiologies and pathophysiology, particularly in neuronal tissue.

Oxidative stress, a state of lost balance between the oxidative and anti-oxidative systems of the cells and tissues, results in the over-production of oxidative free radicals and reactive oxygen species (ROS), which is a collective term for superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^-) (Zhao *et al.* 2010; Rani *et al.* 2016). ROS can be formed outside the cell by UV irradiation, ozone, pollutants, and cigarette smoke (Praticò 2008). Endogenously, their rate of formation can also be enhanced by genetic mutations and cell membrane sources including NAD(P)H oxidase (NOx) and cytochrome P450s (Reddy & Beal 2008). H_2O_2 can also undergo Fenton reaction chemistry in the presence of metals such as iron (Fe^{2+}) to generate OH^- (Reddy & Beal 2008). Mitochondrial sources include the electron transport chain, which produces cytosolic O_2^- that can be converted to H_2O_2 by superoxide dismutase (SOD) and back to O_2^- by xanthine oxidase (XO) (Praticò 2008). It is estimated that approximately 1-3% of all normal O_2 is converted into ROS in mammals due to inefficiencies of the electron transport chain and this ROS production can be enhanced if mitochondria are not functioning optimally due to hypoxic, hyperoxic, or other stress-related conditions (Figure 1.1) (Heis *et al.* 2003).

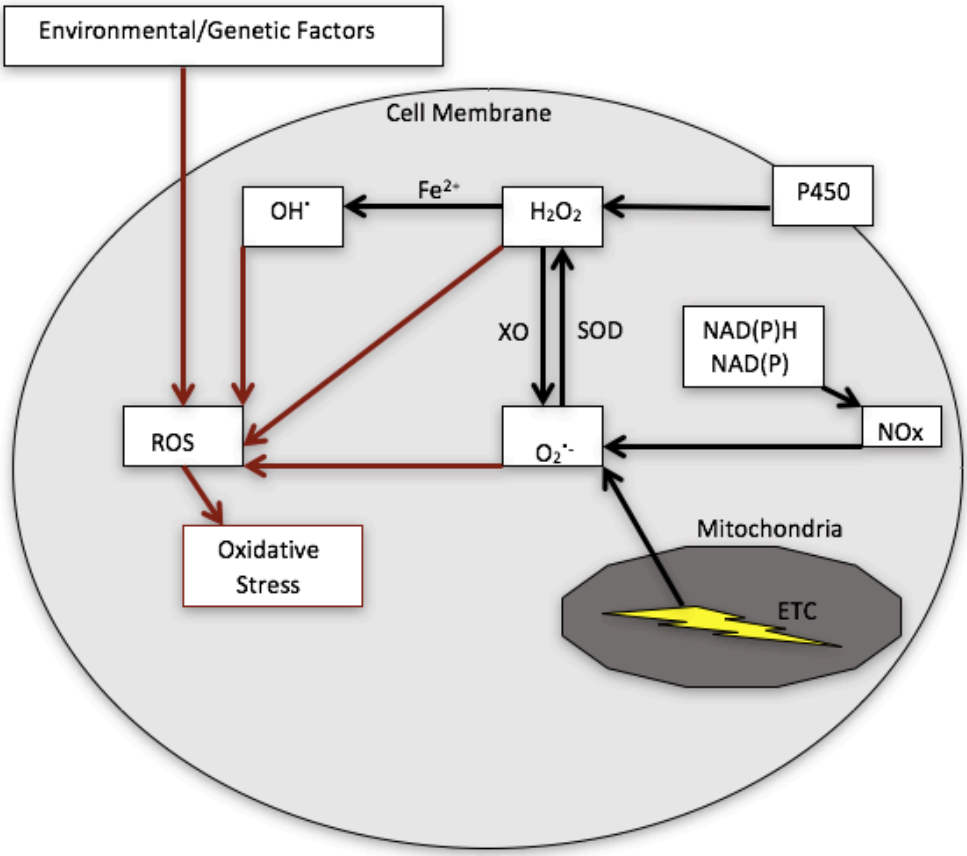

Figure 1.1. *In vivo* sources of oxidative stress. ($O_2^{\circ-}$=superoxide, H_2O_2=hydrogen peroxide, OH° =hydroxyl radical, ROS=reactive oxygen species, NOx=NAD(P)H oxidase, P450=cytochrome P450, ETC=electron-transport chain, SOD=superoxide dismutase, XO=xanthine oxidase, Fe^{2+}=Fenton reaction). Adapted from Praticò 2008.

It is also well established that mitochondrial function declines with age and is correlated with oxidative stress and accumulated gene defects that are particularly abundant in the brain, heart, and muscles (Beal 1995). Functional decline can be attributed to mitochondrial DNA mutations and deletions, which are associated with syndromes characterized by neurodegeneration indicating that mutations acquired with aging may disrupt the efficiency of electron transport and augment oxidative stress (Mecocci *et al.* 1993; Chinnery *et al.*2002). This is further supported by the fact that mitochondrial DNA has a mutation rate 10 times greater than nuclear DNA and less effective repair mechanisms generating a 15-fold increase in oxidized

nucleotides in brain mitochondrial DNA with age (Mecocci *et al.* 1993). In fact, cells may not be able to trigger an effective response until oxidative stress activates a signaling pathway after mitochondrial dysfunction is already well advanced causing delayed repair from slow communication between mitochondria and the nucleus (Wu *et al.* 2014).

These highly reactive and unstable ROS molecules can form during normal metabolic reactions, but generally, cell-generated antioxidant proteins such as superoxide dismutase (catalyzes the dismutation of superoxide radicals), catalase (converts H_2O_2 to H_2O and O_2), and glutathione peroxidase (metabolize H_2O_2 and lipid peroxides) mitigate the adverse effects of ROS (Heis *et al.* 2003; Svensson & Larsson 2007; Praticò 2008). A secondary defense system is ROS scavenging provided by vitamins E & C, beta-carotenes, glutathione, urates, bilirubin, as well as others, which help mitigate overproduction of ROS (Kirkwood 2005). Excessive ROS attack cellular proteins, lipid membranes, and nucleic acids leading to cellular dysfunction including loss of energy metabolism, altered cell signaling and cell cycle control, genetic mutations, altered cellular transport mechanisms, and overall decreased biological activity, as well as immune activation and inflammation (Figure 1.2) (Rani *et al.* 2016).

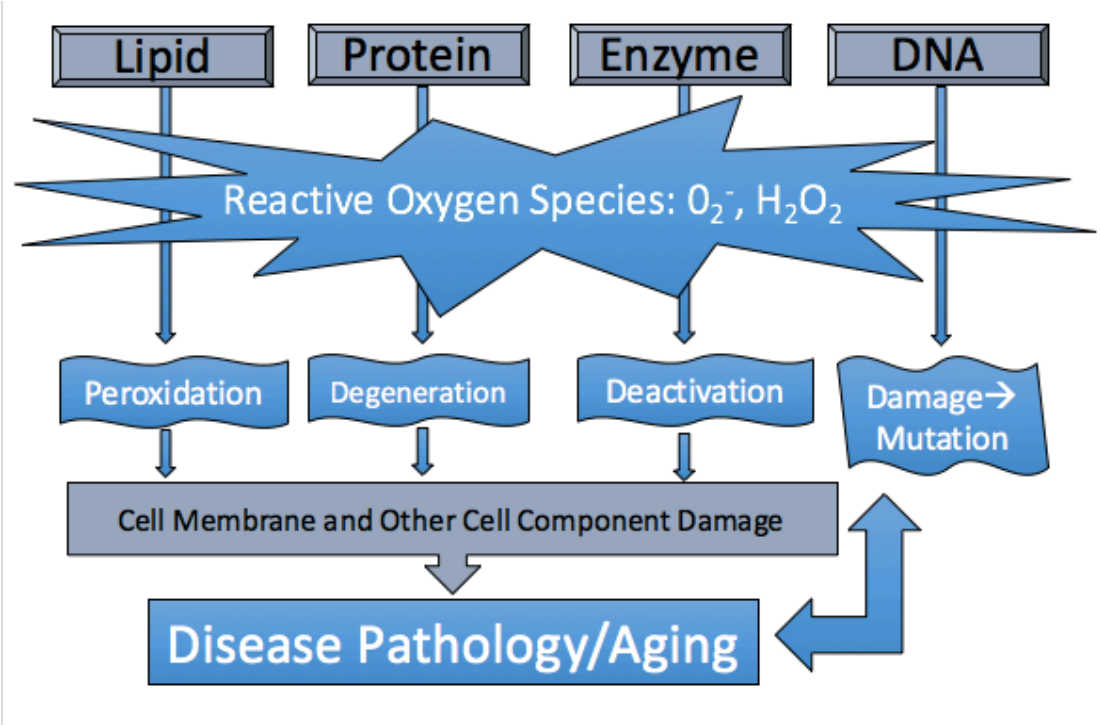

Figure 1.2. Schematic diagram illustrating the harmful effects of ROS on the cells' molecular components and subsequent outcomes.

Reactive nitrogen species (RNS) are also naturally produced and contribute to oxidative stress. They are primarily derived from the nitric oxide radical (NO^-), which is produced by nitric oxide synthase and serves signaling and immune defense roles when NO^- reacts with O_2^- to produce peroxynitrite ($ONOO^-$) (Fang 2004). Unfortunately, shifting this reaction to the right, for example, by reduced SOD proficiency, leads to elevated O_2^-, which results in $ONOO^-$ overproduction (Fang 2004). $ONOO^-$ usually reacts with the cellular abundant HCO_3^- to generate carbonate radicals, but will also react readily with heme-proteins, sulfur groups, and selenium groups of relevance to metal homeostasis and oxidative stress control (Squadrito & Pryor 1998). $ONOO^-$ also oxidizes cysteines to cysteine bridges or oxygenated side chains and "nitrosative stress" manifests itself as nitrosylations of protein side chains to impair protein function and stability and the deamination of DNA affecting both transcription and mitochondrial metabolism (Squadrito & Pryor 1998).

Metal ions play key roles in both ROS production and clearance. Metal ions readily bind ROS and RNS as ligands, and both copper and iron produce hydroxyl radical in solvent-exposed cellular environments, notably via the Fenton reaction (Jomova & Valka 2011). Fenton chemistry unites metal ion dis-homeostasis with oxidative stress pathogenesis, which is strongly aggravated by free copper and iron (Jomova & Valka 2011). Additionally, disturbed metal homeostasis resulting in increased concentrations of free intracellular metal ions will itself generate ROS leading to oxidative stress (Jomova & Valka 2011). Given the links between metal ions and ROS, it is not surprising that a vast number of copper, zinc, and iron containing proteins are involved in oxidative stress modulation (Rivera-Mancia *et al.* 2010).

ROS also play important roles as secondary messengers, mediating numerous cellular functions in stem cells such as self-renewal, differentiation, and proliferation, which can improve pathophysiological outcomes (Dröge 2002; Sarta *et al.* 2015). ROS are implicated in various important biochemical processes linked to healthy maintenance such as the mitochondriogenesis (Suliman & Piantadosi 2014). They also have functional roles in innate and adaptive immunity, by initiating secondary signal transduction processes (Nathan & Cunningham-Bussel 2013; Zuo *et al.* 2014). Hence, ROS production has opposing effects, depending on the level and duration of the stress induced. The physiological effects of short lived ROS in activating the redox-sensitive signaling pathways has been linked to longevity via studies of caloric restriction or exercise, while chronic excess activates aging processes and reduces longevity (Bianchi & Falcioni 2016). Muscle exercise is an important stimulator of ROS production as it activates peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α) stimulating mitochondriogenesis as well as gene transcription in skeletal muscle, liver, and heart (Handschin & Spiegelman 2006; Ji *et al.* 2016). Activation of PGC-1α can prevent mitochondrial

dysfunction for treating various pathologies, such as diabetes, muscular dystrophies, neurodegenerative diseases, or cancer (Wareski *et al.* 2009; Villena 2015). Clearly, ROS have important physiological roles to play, but uncontrolled overabundance proves eventually deleterious.

1.2 Cellular degeneration and oxidative stress theory of aging

Throughout an organism's life, the efficiency of various physiological processes decline with advancing age, making the process irreversible and progressive (Kirkwood 2005; Hayflick 2007). Oxidative stress is considered to be an underlying mechanism in the pathophysiology of cells in any aerobic organism, and is considered an important component in the cellular basis of aging due to its contribution to progressive physiological deterioration (Stadtman 1992; Beckman & Ames 1998; Finkel & Holbrook 2000). As advocated by the free radical theory of aging, slower metabolic rate induced by moderate hypoxia enhances life span by producing less radical oxidative damage from mitochondrial activity, whereas hyperoxic conditions shorten life span of cultured cells and organisms as a whole (Wallace 2005). The role of ROS in chronic diseases has also been shown to be influenced by sex steroids that decrease with age (Bianchi & Falcioni 2016). Comparative studies have shown that most variation in lifespan between species is driven by ROS production and susceptibility of proteins and lipid to damage, but not differences in antioxidant defenses (Magwere *et al.* 2006). In general, species with longevity tend to have lower levels of antioxidant defenses, but only because of a disproportionate reduction in susceptibility to oxidative stress or rates of ROS production (Barja 2002).

Reduced fatty acid unsaturation in tissue cellular membranes and lower rates of mitochondrial ROS production are considered to be the strongest correlated factors to longevity

in vertebrate animals and mammals (Ku *et al.* 1993; Pamplona *et al.* 1998; Pamplona *et al.* 1999; Pamplona *et al.* 2002; Barja 2004; Hutter *et al.* 2007; Lambert *et al.* 2007). Severe disease phenotypes and shortened lifespan are reversible upon partial restoration of ubiquinone levels and mitochondrial function, which strongly suggests that the irreversible degenerative phenotypes are not secondarily caused by the gradual mitochondrial dysfunction, but that the damage at the mitochondrial level could be a consequence of aging (Wang *et al*. 2015*)*. Additionally, it has also been shown that oxidative damage in mitochondrial DNA is low in long-lived animals (Barja & Herrero 2000). The mitochondrion is both the source and target of ROS, making it a very delicate and influential organelle in oxidative stress processes.

Again though, a little bit of oxidative stress has some protective properties. Studies on caloric restriction have shown that oxidative damage to lipid, DNA, and protein was reduced and in general it was found that caloric-restricted rodents compared to rodents fed ad lib were more resistant to oxidative stress (Sohal & Weindruch 1996; Yu 1996; Barja 2002; Bokov *et al.* 2004). Mutations in the insulin and insulin-like growth factor-1 (IGF-1) signaling pathways (*age-1*, *daf-2*, and *daf-16* mutants) have also been shown to increase the lifespan of *Caenorhabditis elegans* and increase resistance to oxidative stress by reducing oxidative damage via reduced cellular glucose uptake (Ishii *et al.* 2002). In general, the balance of ROS has proven to be a highly complex yet influential process in any and all cellular organism's longevity.

1.3 Drosophila melanogaster as a model species

Drosophila melanogaster, the vinegar fly, has long been used as a genetic tool to yield fundamental insights into mammalian biology due to the abundant sequence homology between *D. melanogaster* and mammalian genomes (Adams *et al.* 2000). With its fully sequenced, and

relatively compact, minimally redundant genome, *D. melanogaster* is a far easier organism than many vertebrates to assess gene functions and to perform genetic manipulations (Adams *et al.* 2000; Helfand & Rogina 2003; Khurana 2008). Availability of constantly updated and readily shared genetic tools that allow for detailed genetic analyses also makes *D. melanogaster* an ideal model species. In addition to the genetic rationale, working with *D. melanogaster* produces quicker and more cost efficient results due to their short life cycle, low maintenance cost, and ease of drug and treatment testing. Additionally, numerous research groups have shown the utility of examining the effects of oxidative stress in *D. melanogaster* and their ability to generate significant and multigenerational data on gene expression (Gruenwald *et al.* 2009; Zhao *et al.* 2010; Harrison & Haddad 2011; Zhao & Haddad 2011; Zhao *et al.* 2011; Weber *et al.* 2012; Bosco *et al.* 2015).

Flies exhibit a wide range of complex behaviors that are relevant to mammalian and other higher organism behaviors. These include circadian rhythms, sleep, learning and memory, courtship, feeding, aggression, grooming, and flight navigation (Greenspan & van Swinderen 2004). Flies also have neurotransmitter systems similar to those found in humans and include those that use serotonin, dopamine, glutamate, GABA, acetylcholine, histamine, adenosine, and neurokinins, utilizing both metabotropic G-protein coupled receptors and ionotropic receptor channels (Nichols 2006; Jeibmann & Paulus 2009; Lu & Vogel 2009). Within these neuronal signaling pathways, the catalytic domains of many neurotransmitter biosynthesis and receptor proteins share greater than 80% similarities with their counterparts found in higher organisms (Nichols 2006). For these reasons, the fly has and will continue to serve as an essential platform for the development of novel therapeutics for various neurological diseases (Bilen & Bonini 2005; Cowan *et al.* 2011).

1.4 Oxidative Stress in *Drosophila*

Given the high frequency of sequence identity between *Drosophila* and mammalian genes, these insects are a valuable model species to study genetic and molecular changes that occur in animals undergoing oxidative stress. Additionally, the free radical theory of aging has been extensively examined and strong evidence supporting the role of oxygen radicals in aging metazoans has come from *Drosophila* studies. The rates of mitochondrial O_2^- and H_2O_2 production tend to increase during the post-reproductive phase of life, especially in post-mitotic cells (Mockett *et al.* 1999; Mansfield *et al.* 2004; Morrow & Tanguay 2008). Many studies have shown increases in the rate of mitochondrial H_2O_2 production is a consistent feature of the aging process in various species, including *Drosophila*, and is therefore a shared phenomenon (Mockett *et al.* 1999; Begel *et al.* 1999; Fu *et al.* 1999). One conserved transcription factor involved in oxidative stress regulation is cap-n-collar (CncC/Nrf2) (Grimberg *et al.* 2011). *Drosophila* cells that are pretreated with H_2O_2 adapt to oxidative stress by up-regulation of CncC/Nrf2-dependent 20S proteasome expression, which is a phenomenon also seen in mammalian cell lines (Grimberg *et al.* 2011). It has also been shown that decreased proteasome expression is related to CncC/Nrf2 dysfunction in older flies (Tsakiri *et al.* 2013). Enhanced proteasomal activity by CncC/Nrf2-induced gene expression represents a fundamental strategy in the protection against oxidative stress not only in flies, but also in nematodes and mammals (Loboda *et al.* 2016).

Interestingly, resistance to induced oxidative stress in *Drosophila* and mice overexpressing antioxidant enzymes (e.g. SOD or catalase), has not led to prolongation of life span and these enzymes were not found to be up-regulated in *Drosophila* exposed to hyperoxic conditions (Gerschman *et al.* 1954; Walker *et al.* 2006; Gruenewald *et al.* 2009).

Selective breeding of *Drosophila* strains with enhanced longevity produced flies with a suite of other up-regulated antioxidant defense system enzymes (Arking *et al.* 2000), but some researchers have argued that enhancing just a single component of the antioxidant system is not sufficient to increase longevity (Hulbert *et al.* 2007). However, decreased lifespan in *Drosophila* has been observed with increased flight activity because of a change in membrane fatty acids making them more prone to lipid peroxidation (Magwere *et al.* 2006). There seems to be a clear correlation between oxidative stress and aging, but a definitive link is yet to be elucidated and *D. melanogaster* is an ideal model species to aide in this endeavor.

Recent transgenic studies provide more evidence for the oxidative stress theory of aging in *Drosophila*. Overexpression of Peptide-*S*-methionine sulfoxide reductase (MsrA), was found to increase average life span in up to 85% of independent breeding lines (Ruan *et al.* 2002). In a similar overexpression study, the most pronounced longevity was observed with MsrA overexpressed in motor-neurons, but this study also demonstrated age-related decrease in spontaneous activity and fertility when overexpressed in other tissues (Parkes *et al.* 1998). Global overexpression glutamate-cysteine ligase results in increase glutathione, a primary antioxidant in both *Drosophila* and mammals, and extended the mean life span of *Drosophila* up to 24% (Orr *et al.* 2005)**.** Neuronal overexpression of the glutamate-cysteine ligase extended mean and maximum life span up to 50%, without affecting the rate of oxygen consumption/metabolic rate and produced the longest living *Drosophila* strain to date (Orr *et al.* 2005). *In vitro* studies on isolated mitochondria demonstrate ROS production is dependent on proton motive force, and can be significantly decreased by chemical uncouplers (Skulachev 1996; Muller 2000; Esteves *et al.* 2005). Expressing human *uncoupling protein-1* (*UCP)* in mitochondria of adult fly neurons results in decreased ROS production, reduced oxidative

damage, resistance to the free radical generator paraquat, and an extension of *Drosophila* life span (Fridell *et al.* 2005). These experiments strongly support the free radical theory of aging in *Drosophila*.

It is still unclear whether decreased longevity in response to increased oxidative stress is due to "oxygen poisoning" or that oxidative stress just accelerates aging. Oxygen poisoning is less likely given that survival in early fly development (i.e. pre-eclosure) is unaffected by 40% O_2 environments (Frazier *et al.* 2001). Additionally, gene expression patterns in young flies treated with 100% oxygen is complementary to the gene expression changes seen in old flies (Landis *et al.* 2004). This suggests that oxidative injury can have a role in normal fly aging and that life-span shortening with hyperoxia may just be accelerated aging. Additionally, hyperoxia induces severe *Drosophila* flight muscle mitochondrial malformations, which are characterized as "swirls" and are present in old flies reared under normoxic conditions (Walker & Benzer 2004). "Hyperswirl" mutants exhibit accelerated formation of these swirls and reduced life span (Walker & Benzer 2004). This study demonstrates that the two phenomena are not independent and that screens under hyperoxia can be used as a successful strategy to identify aging-related gene and molecular processes (Mockett *et al.* 1999).

Many studies with antioxidant-supplementation have been conducted in *Drosophila*, but the conclusions drawn from these studies have been mixed, with some studies confirming that a given antioxidant can clearly extend life span, while in others, the antioxidant has no effect (Le Bourg 2001; Beckman & Ames 1998). One such supportive study showed that genetic inhibition of the antioxidant defense enzyme, *Sod*, in *D. melanogaster* enhanced tau-induced neurodegeneration in oxidative stress fly brains (Dias-Santagata *et al.* 2007). Though some studies have shown that manipulation of a single enzyme can effect ROS production, oxidative

stress, and longevity, there are still many other factors to consider in these regulatory processes. One such consideration is the epigenetic regulation of enzymes in cells undergoing oxidative stress, including microRNA post-translational regulation.

1.5. MicroRNAs as gene regulators

In recent years, several research groups have focused their attention on a new class of gene expression regulators, microRNAs (miRNAs), as potential epigenetic factors that regulate the cellular response to oxidative stress/aging (Satoh 2010; Zovoilis *et al.* 2011). MiRNAs are components of an endogenous RNA interference system with implications for regulation in virtually all eukaryotic biological functions (Ambros 2004; Selbach *et al.* 2008). MiRNAs are short non-coding RNAs of approximately 22 nucleotides that were first described in the nematode *C. elegans* in 1993 (*lin-4* and *let-7*), but have since been found in an ever growing list of eukaryotes, and are presumed to regulate the expression of genes associated with most biological functions (Lee *et al.*1993; Ambros 2004). MicroRNAs have also recently been identified the unicellular algae *Chlamydomonas reinhardtii* and viruses (Papaioannou *et al*. 2010). In animal cells, they act as post-transcriptional regulators that usually bind to complementary sequences on the 3` untranslated regions (UTRs) of target messenger RNAs, which can result in gene silencing via translational repression or target degradation, though some miRNAs have been found to target within the protein coding region (Reczko *et al.* 2012). MiRNAs are either expressed from independent transcriptional units or derived from introns of protein-coding genes or introns of long non-coding RNAs (Rodriguez *et al*., 2004; Griffiths-Jones 2007). MiRNAs are highly conserved across species and are involved in the regulation of different cellular processes such as developmental timing, cell differentiation, cell proliferation,

apoptosis, and metabolism (Brennecke *et al*. 2003; Xu *et al*. 2003; Chen 2004; Bushati & Cohen 2007; Ambros 2011). Although a plethora of miRNAs are found in many species, the function of the vast majority of them has not been identified yet (Ying *et al*. 2012). The deficit in functional miRNA knowledge provides a novel research platform in uncovering miRNAs' regulatory roles in oxidative stress processes.

1.6 Biogenesis and mechanism of microRNAs

The majority of miRNA genes are transcribed from inter- and intra-genic locations by RNA polymerase II into pri-miRNA transcripts, but a small group of miRNAs can be transcribed by polymerase III (Zhou *et al.* 2007; Faller *et al*. 2008). Polymerase II-derived pri-miRNAs are 5' capped, spliced, and poly-adenylated (Cai *et al.* 2004). In the nucleus, pri-miRNAs are processed by a multi-protein complex called Microprocessor, which cleaves the pri-miRNA into a shorter hairpin-structured precursor miRNA (pre-miRNA) (Siom *et al.* 2010). Microprocessor consists of DGCR (Pasha in invertebrates), a double-stranded RNA binding protein, and Drosha, an RNase III enzyme (Bartel 2004). DGCR8/Pasha binds to the junction between the single-stranded and double-stranded regions of the pri-miRNA stem and directs Drosha to cleave 11-bp away from the junction, resulting in a molecule of about 70 nucleotides long, with a two-nucleotide overhang at the 3' end (Bartel 2004).

The pre-miRNAs are exported from the nucleus by exportin-5, a nuclear membrane transport protein, which also exports the short hairpin RNAs (Yi *et al.* 2005). The two nucleotide overhang left by Drosha is recognized by exportin-5 and transports the pre-miRNAs into the cytoplasm via a Ran-GTP-dependent reaction (Okada *et al.* 2009). In the cytoplasm, pre-miRNAs are cleaved by Dicer, an RNaseIII enzyme, producing a 22-nt miRNA duplex that is

unwound by helicase (Kim *et al.* 2009). After Dicer cleavage, the duplex is separated by the RNA-induced silencing complex (RISC), which includes both TRBP, a double stranded RNA binding protein and argonaute-2 (Siom *et al.* 2010). One 22 nucleotide strand remains bound to the argonaute-2 protein on RISC as the mature miRNA (the guide strand), and the other strand (the passenger strand or miRNA*) is degraded (Siom *et al.* 2010). The thermodynamic stability at the two ends of the miRNA duplex determines which strand is the guide strand (Khvorova *et al.* 2003). The miRNA strand with the relatively unstable base pairs at the 5' end will be more frequently chosen as the guide, while the miRNA strand with relatively stable base pairs at the 5' end will be degraded (Khvorova *et al.* 2003). However, recent studies show that either the miRNA or miRNA* strands can be functional; in this case, the miRNA* strand is not degraded, but associates with argonaute-2 (Okamura *et al.* 2009). More recent evidence has shown that miRNAs in mammals can also bind to coding regions or even to 5`UTR sites of target mRNAs (Lytle *et al*. 2007; Schnall *et al*. 2010). Due to imperfect binding of miRNAs to mRNAs in animal cells, one miRNA can target many different sites on the same mRNA or many different mRNAs at the post-transcriptional level (Zhang *et al.* 2009). It is worth noting that miRNAs function slightly differently in plants (Jones-Rhoades *et al.* 2006). The main difference is that in plants, miRNAs bind with perfect or near-perfect complementarity, and therefore always induce cleavage of the mRNA target transcript rather than cleavage or repression depending on binding strength seen in animal cells (Figure 1.3) (Zhang *et al* 2009).

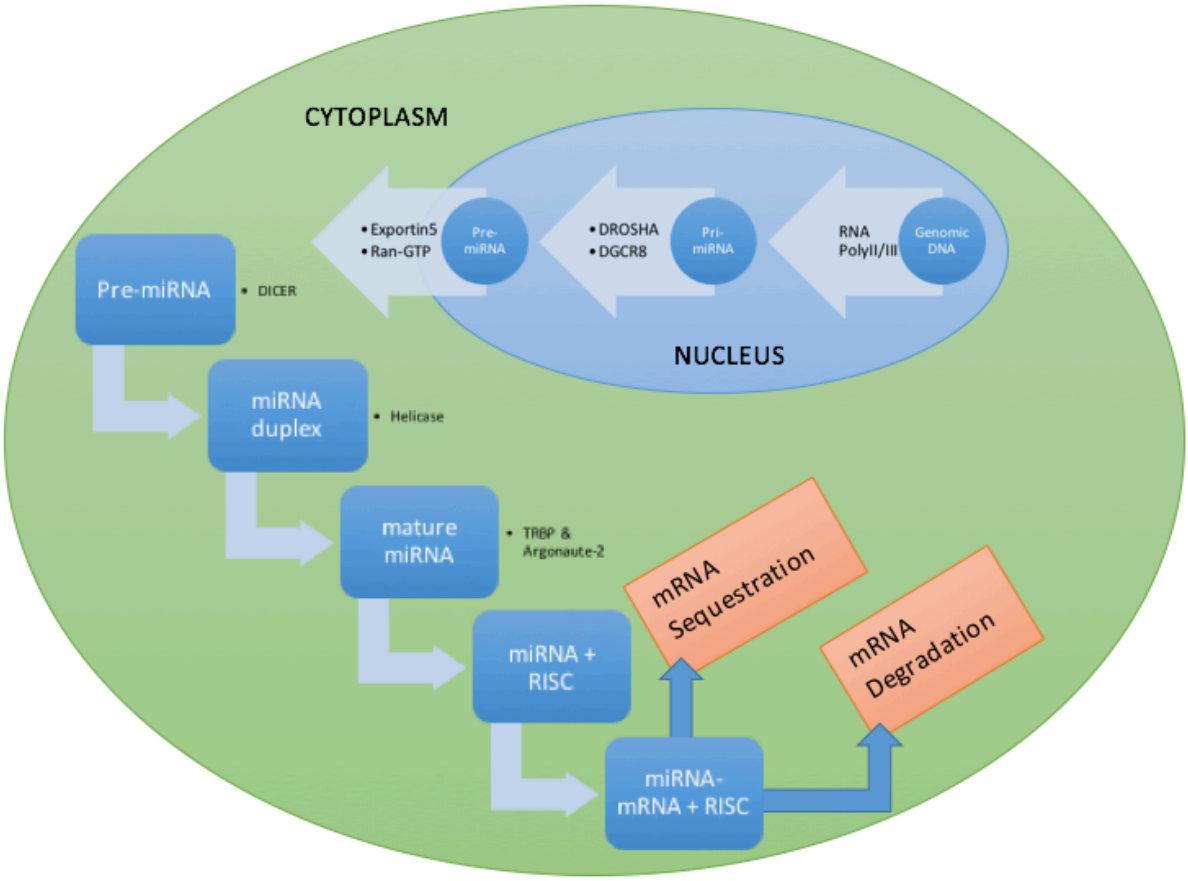

Figure 1.3. The microRNA biogenesis and mechanism pathway. (Adapted from Gan *et al.* 2015)

1.7 Computational tools for microRNA target prediction.

MiRNA target recognition is based on the complementarity of the eight nucleotide seed region, and this complementarity has been used to develop computer algorithms to predict miRNA:mRNA target binding (Watanabe *et al.* 2007). Several computational algorithms have been developed to predict miRNAs target mRNAs. These algorithms are mainly focused on sequence alignments to identify complementary elements between the seed region at the 5'-end of the miRNA and the 3'UTR of the mRNA, so more novel binding patterns are currently not well predicted (Yue *et al*. 2009). Most algorithms also use additional steps to refine the predictions and rank them according to statistical confidence (Ritchie *et al*. 2013).

MicroInspector, miRanda, and PITA algorithms for example, calculate the thermodynamic stability of miRNA:mRNA duplexes by searching for the strongest physical interactions between the seed region at the 5'-end of a miRNA and the 3'UTR of putative mRNAs (John *et al*. 2004; Rusinov *et al*. 2005; Kertesz *et al*. 2007; Grimson *et al.* 2007). This approach is limited by unidentified or inaccurate predictions of stable secondary structures (Ritchie *et al*. 2013). Another approach that is used for prediction of miRNA targets involves evaluating sequence conservation of predicted targets between different species. For example, TargetScan predicts biological targets of miRNAs by searching for the presence of conserved sites that match the seed region of a miRNA among different vertebrate species (Lewis *et al*. 2005). This approach reduces the number of false positive predictions and helps determine conserved miRNA:mRNA relationships among different species, but has little use in detecting species-specific binding sites (Ritchie *et al*. 2013).

The ability to catalog and predict targets is an essential tool in determining miRNA biological function once candidate miRNAs are uncovered, but the predicted target binding must be functionally tested. In addition, most prediction algorithms are restricted to examining the 3'UTR of the mRNA and do not incorporate evidence of functional binding between the miRNA and the 5'UTR or protein coding region of the mRNA (Thomson *et al*. 2011). Additionally, the stable pairing between miRNA and 3`UTR of the mRNA may not necessarily be functionally interactive, which may explain why the false positive predication rate by these algorithms is relatively high (Kuhn *et al*. 2008; Thomson *et al*. 2011). Therefore, functional miRNA:mRNA interaction is essential to identify molecular and physiologically relational miRNA targets.

1.8 MicroRNAs in oxidative stress

Environmental factors that cause cellular stress can influence miRNA expression (Maes *et al.* 2008; Maes *et al.* 2009). The miRNA biosynthesis apparatus is compromised during organismic aging and in cellular senescence, leading to a general decline of miRNA availability with age (Bu *et al.* 2017). Global decrease in miRNA accumulation was found in aging of different model organisms, suggesting an aging-associated alteration of miRNA biogenesis (Inukai & Slack 2013). Dicer down-regulation and subsequently reduced miRNA processing in adipose tissue is associated with accelerated aging, reduced life span, and reduced stress defense in different model organisms from *C. elegans* to mice as well as in humans (Mori *et al*. 2012). Longevity-promoting interventions (i.e. caloric restriction) prevent decline of Dicer and miRNA processing, while senescence-inducing stimuli, like oxidative stress or UV radiation, decrease Dicer expression (Martin-Montalvo *et al.* 2013; Mori *et al*. 2012; Noren Hooten 2016). Inhibiting DGCR8 expression in adult *C. elegans* resulting in loss of miRNA synthesis showed accelerated aging and reduced lifespan (Lehrbach *et al.* 2012). Clearly oxidative stress can reduce miRNA synthesis/accumulation by affecting proteins' biogenesis pathways. Additionally, through these experiments it could be extrapolated that oxidative stress can induce miRNA-mediated gene silencing during senescence induction, by either affecting the enzymes that process miRNAs or regulating (up-regulation or down-regulation) the expression of certain specific miRNAs.

Since miRNAs can generate rapid and reversible responses, they are ideal mediators for adaptive responses against oxidative stress through their capacity to fine-tune gene expression (Mendell & Olson 2012). MiR-34a has been found to induce oxidative stress-mediated cellular senescence by targeting *Situin* 1 (*SIRT1*) and other antioxidant pathway genes in different tissues

(Hermeking 2010; Ito *et al.* 2010; Li *et al.* 2011; Tabuchi *et al.* 2012). MiR-34a along with miR-335 were found to be up-regulated in aged rat kidney with respective mRNA targets being thioredoxin reductase 2 (Txn2) and superoxide dismutase 2 (SOD2) (Bai *et al.* 2011). Additionally, increased accumulation of miR-335 and miR-34a resulted in increased ROS and premature apoptosis of mesangial cells via suppression of both antioxidant enzymes (Bai *et al.* 2011). NRF2, a regulator of redox biology, is targeted by various miRNAs in order to fine-tune redox homeostasis (Cheng *et al.* 2013). One example of such regulation was when NRF2 expression was restored under caloric restriction by significantly decreasing miR-144 levels (Csiszar *et al.* 2014). MiRNA expression during hyperoxia may provide insight into epigenetic mechanisms involved in oxidative stress responses that are either mediating repair responses, malfunctioning, or at least not sufficiently functioning in cells undergoing oxidative stress.

Many studies have started to uncover the way in which miRNAs regulate and respond to states of oxidative stress or how miRNA dysregulation can adversely affect *Drosophila.* Dysregulation of 17 miRNAs in *D. melanogaster* was observed to modify expression of amyloid precursor protein (APP) (Kong *et al.* 2014). Several *D. melanogaster* aging induced miRNA candidates have recently emerged. *Dme-miR-34* expression increases with age in *D. melanogaster* and deletion of miR-34 has been shown to both shorten lifespan and accelerate brain degeneration (Liu *et al.* 2012). D*me-mir-8*, *7*, *9*, and *bantam* have been observed to regulate different aspects of neuronal function in *D. melanogaster* (Chawla & Sokol 2011). *Dme-miR-8* null mutants display progressive neurodegeneration and humans have 5 paralogues of this miRNA (*hsa-miR-200b*, *200a*, *429 200c* and *141*) (Karres *et al.* 2007). These candidate miRNAs have clear associations to the aging process, which could have applicative use in mammalian models if their mechanisms can be further elucidated.

1.9 Thesis Objectives

The primary aim of this project is to uncover novel miRNA mediators involved in the oxidative stress response. Additionally, this project sought to determine whether miRNAs could regulate key antioxidant genes involved in oxidative stress.

Therefore, the specific objectives for my M.Sc. were:

1. Elucidate novel miRNAs involved in regulating genes in the setting of oxidative stress

Oxidative stress has been well studied as the underlying cause of many disease processes and in aging overall. What is still unclear is the underlying mechanisms that regulate the balance between oxidative stress and innate antioxidant defenses. In this study, a global scan of all *Drosophila melanogaster* miRNAs was used to uncover novel miRNA regulators in oxidative stress. These candidate miRNAs were further evaluated for the functional roles they play through accumulation profiling using qRT-PCR. Predicted gene targets of select miRNAs were functionally tested in order to determine these miRNAs' ability to bind and suppress predicted gene targets. An attempt was also made to undercover the effect of eliminating key miRNAs *in vivo*.

2. Assess microRNAs' abilities to functionally regulate key antioxidant genes

MiRNA target prediction programs only suggest which miRNAs may bind and regulate specific genes, but do not provide confirmed functions of the miRNA:mRNA target relationship (Kuhn *et al*. 2008). In order to understand how key antioxidant genes may be regulated by miRNAs, a cell-based functional assay was used to determine the gene regulatory potential of miRNAs of their predicted antioxidant genes. Functional miRNA:antioxidant mRNA gene pairs underwent accumulation/expression profiling in order to correlate their functional relationship to oxidative stress conditions.

Chapter 2: Elucidating miRNAs and their regulatory functions in oxidative stress

2.1 Introduction

The oldest method of modulating *in situ* oxidative damage is through the manipulation of oxygen tension by creating an environment with elevated oxygen content. Harman's free radical theory of aging was supported with oxygen "poisoning" experiments where *in situ* oxidative damage was modulated through changing the oxygen tension (Gerschman *et al.* 1954). Because O_2 is the substrate for superoxide (O_2^-) production, an increased in O_2 tension can result in increased superoxide formation, in the cytoplasm as well as mitochondria (Boveris & Chance 1973; Li *et al.* 2004; Walker & Benzer 2004). This manipulation is most effective in organisms that lack ability to sufficiently regulate their oxygen tension, such as *Drosophila* (Frazier *et al.* 2001). Early experiments demonstrated an inverse linear relationship between life span and oxygen tension in *Drosophila* (Miquel *et al.* 1975; Baret *et al.* 1994). For example, if atmospheric oxygen is increased above 21% there is a resulting corresponding life span decrease (Miquel *et al.* 1975). Additionally, despite the potential to increase ROS production in hypoxic conditions, some studies have indicated that decreasing oxygen tension below 21% actually increases life span (Strehler 1977; Mansfield *et al.* 2004). For this reason, a hyperoxia chamber was developed to provide a ~95-100% hyperoxia environment for my experiments described herein.

Hyperoxia-exposed flies were used in a microarray analysis to identify candidate miRNAs, which were then subjected to further evaluation. This evaluation included observing how these miRNAs' accumulations vary between *D. melanogaster* whole body and head samples. Additionally, the response and corresponding accumulation of miRNAs has also been shown to change, depending on the length of time an organism is exposed to an environmental

stress (Kaur *et al.* 2016). I sought to determine when during the oxidative stress process these miRNAs accumulation patterns were most pronounced in order to better understand their physiological relevance. *Dme-miR-34* was added to this group of candidate miRNAs, even though it was not initially identified in the microarray screen, because its accumulation has been associated with adult-onset, brain-enriched, and age-modulated characteristics, and miR-34 loss triggers accelerated brain aging, late-onset brain degeneration, and a catastrophic decline in survival (Liu *et al.* 2012). Up-regulation of miR-34 has also been shown to extend median lifespan and counteract polyglutamine-mediated neurodegeneration (Liu *et al.* 2012). Additionally, I sought to identify some of the enzymes that these miRNAs are targeting. *Dme-miRNA-8*, *-11*, and *-970* were selected to investigate further, and *jaguar, castor,* and *derailed,* respectively, were the genes chosen to evaluate the impacts on gene expression.

To help understand the potential impacts of oxidative stress on neural or other physiological functions within the insects, it is worth highlighting the known roles of these proteins within *D. melanogaster*. *Jaguar* encodes myosin VI, an F-actin-based motor protein, in *D. melanogaster* (Kellerman & Miller 1992). Myosin V and VI have been shown to modulate axonal mitochondrial transport in *D. melanogaster* (Pathak *et al.* 2010). In addition, myosin VI may promote mitochondrial docking and anchoring along the actin-based cytoskeleton by moving mitochondria away from microtubule tracks and holding them there, as it has slow kinetic properties due to a small peptide insertion near the ATP binding pocket, which reduces accessibility of the modulating ATP (Ménétrey 2007). Inhibition of mitochondrial transport may result in the loss of mitochondria from synaptic terminals, which leads to dysfunctional synaptic transmission (Stowers *et al.* 2002; Yano *et al.* 2006). Hence, abnormal or insufficient myosin VI expression may contribute to mitochondrial dysfunction. The absence of mitochondria in

presynaptic terminals may reduce local ATP supply and thus affect ATP-dependent processes including myosin motors that transport synaptic vesicles, which indicates a complex, but dependent relationship between myosin and mitochondria (Stowers *et al.* 2002). Outside of the nervous system, *jaguar* has been shown to stabilize DE-cadherin at adherens junctions in the ovaries (Geisbrecht 2002). Myosin VI/Jar has also been implicated in the regulation of actin dynamics during sperm individualization (Rogat 2002). If *dme-miR-8* does bind and suppress *jaguar* it would implicate its role in neuronal mitochondrial as well as reproductive regulative processes.

Castor (*cas*) encodes a zinc finger protein and has multiple transcriptional activation domains, suggesting that it acts as a transcription factor necessary for the development of a subset of central nervous system neuronal precursors expressed in a subset of *Drosophila* glioblast cells where it controls neuronal differentiation (Mellerick *et al.* 1992). *Castor* interacts genetically with *linotte*, a transmembrane protein, and *no-bridge* (Hitier *et al.* 2001). *Cas* may also directly silence *nubbin*, a homeodomain transcription factor in the wing, expressed in early and late developing wing neuroblasts, given that *nubbin* contains a *cas*-binding site (Kambadur *et al.* 1998). In addition, increased production of Cas protein in all neuroblast lineages reduces *nubbin* expression (Kambadur *et al.* 1998). Embryos that lack *castor* expression have a diminished CNS axonal network and express *engrailed* aberrantly late during central nervous system development (Mellerick *et al.* 1992). Taken all together, it is clear that *castor* has a significant role in central nervous system growth, development, and regeneration.

Derailed (*drl*) encodes a known Wnt5 receptor of the protein-tyrosine kinase receptor family expressed in dendrites and the precise expression patterns of Wnt5 and Drl orient dendrites allowing them to target their final glomerular positions (Wu *et al.* 2014). Mutation of

drl results in derangement of the glomerular map, particularly the olfactory map; ectopic midline glomeruli; and the accumulation of Wnt5 at the midline (Yao *et al.* 2007). *Derailed* is expressed by a small subset of embryonic interneurons whose growth cones choose common pathways during development (Yao *et al.* 2007). In *derailed* mutant embryos these neurons fail to make the correct pathway choices and fail to establish the correct neuronal pathway recognition (Callahan *et al.* 1995). *Derailed* as a Wnt5 receptor, again plays a distinct role in central nervous system growth, development, and regeneration.

The interactions of the hyperoxia-induced miRNAs and these aforementioned target genes within *D. melanogaster* is discussed.

2.2 Methods

2.2.1 Insect rearing

A *Drosophila melanogaster* white-eyed strain (w1118) was used for all experiments. Stocks were maintained at room temperature under atmospheric oxygen on a potato flake medium (Ward's Instant Drosophila Medium). All experiments were performed using an approximately 50/50 random distribution of male and female flies.

2.2.2 Hyperoxia treatment

Hyperoxia treatments were performed by exposing groups of flies in a sealed glass container to a constant flux of ~99.5% oxygen under a low positive pressure. Two day old adult flies were exposed to hyperoxia for various treatment time points including: 6 hours, 12 hours, 1 day, 2 days, and 5 days. Control flies were handled under identical conditions of light and temperature, but kept in normoxia (normal atmospheric levels of oxygen).

2.2.3 Evaluating lipid peroxidation as an indirect measure of reactive oxygen species production using Thiobarbituric acid reactive substances (TBARS) Assay

Flies were treated as described in 2.2.2 Hyperoxia treatment. Five flies from each treatment (hyperoxia and normoxia at different time points) were frozen in liquid nitrogen then homogenized in PBS to a final volume of 250μL. Homogenates were centrifuged (5 min at 13,000 rpm) to pellet debris and the supernatant was divided into two 100 ul aliquots for experimental replicates (50 ul of homogenate debris was discarded). Lipid peroxidation of the 100 ul samples was determined using an OXItek TBARS Assay Kit (ENZO Life Sciences), according to the manufacturer's specifications.

2.2.4 Microarray analysis

Three replicates of 5 day hyperoxia and normoxia treatments were conducted. RNA was isolated from approximately 25 hyperoxia- or normoxia-treated flies using the miRNeasy® Mini Kit (QIAGEN). Samples were evaluated for purity and concentration by UV spectrophotometry, and RNA integrity was assessed by resolving 10% of the RNA sample on a 1% agarose gel in TAE buffer. The gel was stained using SYBR Gold and samples were visualized on a UV transilluminator. Approximately 5μg of RNA for each sample (two treatments in triplicate) were sent to LC Sciences for microarray analysis. The complete microarray protocol was carried out by LC Sciences (Houston, TX, USA) using μParaflo® Microfluidic Chip microRNA microarrays containing oligos for mature miRNAs cataloged in miRBase version 17 (425 unique mature miRNA probes). Validation of the microarray analysis was performed by quantitative RT-PCR (see section 2.2.5).

2.2.5 Accumulation profile of the miRNAs at various stages of hyperoxia exposure

Quantitative reverse-transcriptase PCR (qRT-PCR) was used to determine when microarray-identified miRNAs and predicted targets are transcribed under various stages of hyperoxia exposure. RNA was isolated from either approximately 25 whole bodies or 250 fly heads using the miRNeasy® Mini Kit (QIAGEN) for 1-day, 2-days, and 5-days hyperoxia and normoxia treatments. cDNA was synthesized using QuantiTect® Reverse Transcription Kit (Qiagen) with random hexamers. Transcript levels were assessed by quantitative RT-PCR (qRT-PCR) using a BioRad iQ5 Real-Time thermal cycler and SYBR Green dye. Primers were designed for microRNAs to amplify pre-miRNAs as described previously by Schmittgen *et al.* (2008) and primers for the predicted miRNA target genes were designed using Beacon

Designer^{TM} program (Premier Biosoft) (Table 2.1). In general, qRT-PCR primers have annealing temperatures within 1°C of each other and produce relatively small amplicons of fewer than 200 bp long. When designing qRT-PCR primers to the levels of a miRNA, the general rules of primer design were relaxed slightly (with slightly higher annealing temperature gaps and slightly longer amplicon lengths), as the primers needed to be designed to amplify the pre-miRNA sequence in order to have enough nucleotide sequence for amplification as well as specificity of the targeted miRNA and not the miRNA's target gene. For each cDNA sample, qRT-PCR was performed in triplicate using a BioRad iQ5 Real-Time PCR Detection System using 96-well plates with 20 µl reactions containing ~10 ng of cDNA, 10 µl of SYBR Green Supermix (BioRad), 1 µl each of forward and reverse primers (10 µM), and Nanopure water, using the following program: 95°C for 3 min, followed by 40 cycles of 95°C for 10 seconds, then 45°C for 30 secs, followed by a melt curve analysis to confirm that only a single PCR product was amplified. For this experiment, one set of primers was designed to target the Rpl32 as an internal reference gene. The relative amount of transcripts in the *Drosophila* samples was determined using the $2^{-\Delta\Delta}CT$ method (Livak and Schmittgen, 2001) where miRNA transcript levels are normalized to the internal standard (Rpl32) using the following equation:

Fold change in miRNA accumulation = $2^{-\Delta\Delta}CT$, where $\Delta\Delta CT = {}^{(CT,\ miRNA\ -\ CT,Rpl32)}$Hyperoxia - $^{(CT,\ miRNA\ -\ CT,Rpl32)}$Normoxia.

Table 2.1. Primers used for qRT-PCR analysis of microRNAs.

qRT-PCR Target	Sense Primer	Antisense Primer
dme-miR-8	5'-AAGGACATCTGTTCACATCT-3'	5'-ACACGGACGACATCTTTAC-3'
dme-miR-34	5'-AATTGGCTATGCGCTTTG-3'	5'-CGGCAGTGAAGATAGTGG-3'
dme-miR-11	5'- CACTTGTCAAGAACTTTCTC-3'	5'-CTCAGCAAGAACTCAGACT -3'
dme-miR-970	5'-TTTTATTTGGTAGCTGTAA -3'	5'-TTAGACAACGGTTATAGC -3'
dme-miR-2491	5'- TTGCAGTTGCTGTTTTCCAT-3'	5'- AAAGTGAATCACGAGTGCT-3'
dme-miR-313	5'- ATTTTCTGCTGCGGATGG-3'	5'-TTTCGGGCTGTGAAAAGTG-3'
dme-miR-10	5'- GTCGATCCGAATTTGTTTT-3'	5'- TCTCTAGAACCGAATTTGT-3'
RpL32 (Control)	5'-AAGGGACAGTATCTGATGC-3'	5'-CACCAGGAACTTCTTGAATC-3'

2.2.6 Identification of potential miRNA gene targets

MiRBase (http://www.mirbase.org/) was used to investigate the general features (sequences and predicted secondary structures) of candidate miRNAs uncovered from the microarray. Several databases were used to help determine predicted targets, including: DIANA lab (DNA intelligence analysis) (http://diana.cslab.ece.ntua.gr/DianaTools/index.php?r=site/home); miRNA.org – Targets and Expression (http://www.microrna.org/microrna/home.do); and TargetScanFly (http://www.targetscan.org/fly_12/). Each database uses its own algorithm to predict miRNA:mRNA 3'UTR binding based on various parameters, including the presence of conserved 8mer and 7mer sites that match the seed region of the miRNA, and the free energy of binding the miRNA to its predicted target (Lewis *et al.* 2005). Top predicted targets were compared among the three databases. Target genes that were identified by two or more of the aforementioned databases were then evaluated for physiological function using FlyBase (http://flybase.org/) gene ontology information.

2.2.7 Preparation of plasmids for miRNA functional analyses

2.2.7.1 Isolation of miRNAs and predicted miRNA target gene fragments (3'UTRs)

Genomic DNA was isolated from approximately 15 flies (0.02 g) using a Wizard® Genomic DNA Purification Kit (Promega). Predicted gene untranslated regions (UTRs) were PCR-amplified from genomic DNA using extended primers (Table 2.2). Extended primers of predicted miRNA regulators were designed to amplify *dme-miR* precursors plus 100 bp of flanking sequences on both ends of the stem loop (Table 2.2). PCR products were resolved by electrophoresis on a 2% agarose gel in TAE buffer. The gel was stained using SYBR Gold and the bands were visualized on a UV transilluminator. Bands were gel extracted using a QIAquick

Gel Extraction Kit and cloned into the pstBlue™ Novagene cloning vector according to the manufacturer's instruction.

Table 2.2. Extended primers used for PCR amplifying predicted gene untranslated regions and *dme-miR* precursors plus 100 bp of flanking sequences from genomic DNA.

Genomic PCR Target	Extended Sense Primer	Extended Antisense Primer
dme-miR-11	5'-TCGAGGATCCAAAAATTAAACAAATTAAACA -3'	5'- TCGAGCTAGCCATGATCATTTTGCATCCGCC-3'
Castor	5'- GGTACCAGGAATCGACCGAC-3'	5'- GGGCCCCGGAAAACATAA-3'
dme-miR-970	5'-TCGAGGATCCCAGGAGATTCAGGAGCAACTC -3'	5'- TCGAGCTAGCAAGAGAAGAAATTGGATCAAA-3'
Derailed	5'- GGTACCCCAGCGGTGCT-3'	5'- GGGCCCGTTTTTAAATATTATGCACG -3'
dme-miR-8	5'-TCGAGGATCCTGAGAACTTTGAGCTTCCTCT-3'	5'- TCGAGCTAGCAACTTGTTTTTCCTTCGACTT-3'
Jaguar	5'-GGTACCACCCCCAATACGAC -3'	5'- GGGCCCACGTTTAAGTTCTC-3'

2.2.7.2 miRNA and target gene cloning and ligation into expression vectors

3'UTR fragments and respective *dme-mirs* were PCR-amplified from pstBlue using T7 (5'-TAATACGACTCACTAGGG-3') and Sp6 (5'-GATTTAGGTGACACTATAG-3') primers. 3'UTR fragments and the miRSelect™ pMIR-GFP reporter vector were digested with *Bam*HI and *Apa*I restriction enzymes. Respective *Dme-mir* and the miRSelect™ pEP-miR expression vector were digested with *Bam*HI and *Nhe*I restriction enzymes. Fragments were resolved by electrophoresis on a 1% high-resolution agarose gel in TAE buffer. The gel was stained using SYBR Gold and the bands were visualized on a UV transilluminator. Bands were gel extracted using a QIAquick Gel Extraction Kit. 3'UTR and miRNA fragments were ligated into pMIR-GFP and pEP-miR vectors respectively using T4 DNA Ligase (Invitrogen). Ligated vectors were then transformed into either E.cloni® (Lucigen) or Subcloning Efficiency™ DH5α™ (Invitrogen) chemically competent cells according to the manufacturer's instruction. Vectors were isolated using a QIAprep® Spin Miniprep Kit (QIAGEN) and sent for DNA sequencing to the Robarts Research Institute (London, ON).

2.2.8 MiRNA functional assays in HEK293 cells

2.2.8.1 HEK293 cell culture and Lipofectamine™ transfection with miRNASelect™ vectors

HEK293 cells were maintained in DMEM 10% FBS media (+4.00mM L- Glutamine, 4500 mg/L Glucose, and Sodium Pyruvate) at 37°C in a 5% constant flow CO_2 incubator. Cells were evenly aliquoted into wells of a 96 well plate and grown until approximately 95-99% confluent. Cells were transfected with either pMIR-GFP-3'UTR, pEP-miR, or pMIR-GFP-3'UTR+pEP-miR treatments. Control cell transfections included pMIR-β-Gal, pEP-miR-Null, and pMIR-β-Gal+pEP-miR-Null vector transfections as well as non-transfection treated cells. Transfections were performed in triplicate with 0.2 μg vector with 0.5μl Lipofectamine™ in 100μL OptimMEM® reduced serum medium in each well. The transfection medium was changed after 4 hours. After 24 hours, transfected cells were treated simultaneously with puromycin (2μg/ml) to select for cells containing the pEP-miR plasmid and with neomycin (G418 Sulfate) (2μg/ml) to select for cells containing the pMIR-GFP vector. GFP fluorescence expression within the cells was recorded after 24 hours of vector selection (Figure 2.1) (Section 2.2.8.2).

2.2.8.2 Quantification of GFP expression

Cells were washed with PBS and covered with 100μL PBS to facilitate measurements of GFP fluorescence. The fluorescence was read using a BioTek® microplate reader using Gen5™ 1.09 software at 485nm excitation and 528nm emission, and the cell density was determined by reading the same plate at 600nm. The fluorescence of each well was normalized to blank wells containing only 100μL PBS.

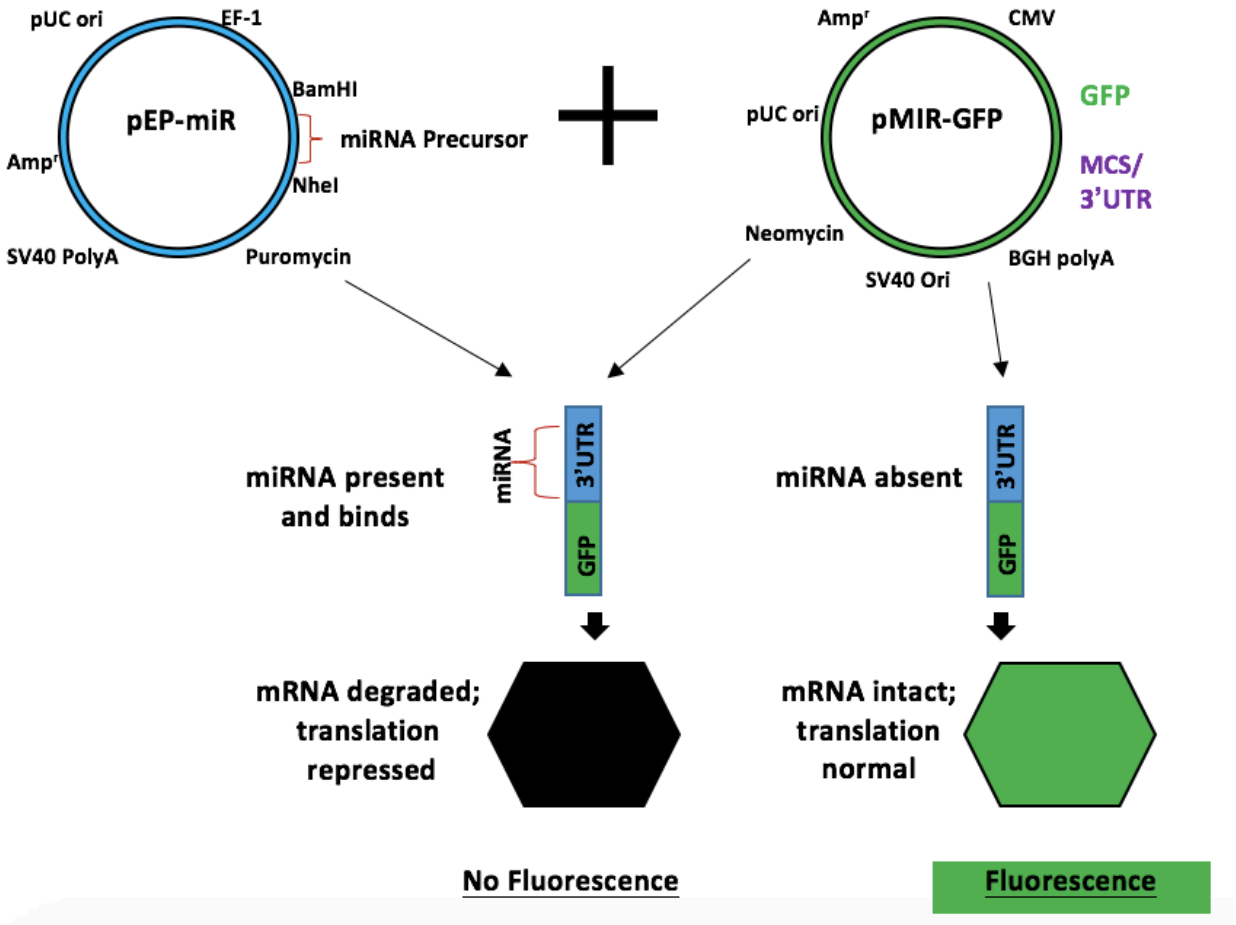

Figure 2.1. Assay principle for the miRSelect™ pEP-miR expression and miRSelect™ pMIR-GFP reporter vector system to functionally test predicted miRNA:3'UTR mRNA binding. Cells were transfected with both pEP-miR, containing the miRNA coding sequence, and pMIR-GFP, containing the testable 3'UTR fused to the GFP reporter gene. Loss of fluorescence is an indication that the miRNA can bind to the UTR and repress translation of the GFP transcripts.

2.2.9 *In vivo* suppression of *dme-miR-8* and *dme-miR-34* by antisense oligomer injections and bioassay

Antisense oligonucleotide constructs for dme-miR-8, dme-miR-34, and a GFP control were designed as chimeras of modified 2′-*O*-methyl RNA and phosphorothioate DNA nucleotides using GeneTools and ordered from IDT (Table 2.3). These oligomers were injected into adult flies using borosilicate glass needles. Needles were pulled on a P-97 Flaming/Brown Micropipette Puller (Sutter Instruments) using 50 µl glass capillary tubes and a program of: one

cycle of heat = ramp + 5, pressure = 500, pull = 30, velocity = 120, time = 125. Approximately 250-500 ηl of constructs were injected into one day-old *D. melanogaster* into the mesopleuron of the thorax. Four treatment samples of *D. melanogaster* were prepared for each miRNA (Hyperoxia + anti-miR, Hyperoxia + GFP Control, Normoxia + anti-miR, and Normoxia + GFP Control). The insects were then observed for 6 hours to ensure their viability before being placed in their respective treatment environments. These four treatments were compared by assessing changes in target gene expression (by qRT-PCR) at 1-day, 2-days, and 5-days post injection and treatment using qRT-PCR as previously described.

Table 2.3. Antisense oligonucleotide constructs for dme-miR-8, dme-miR-34, and a GFP control designed and ordered through IDT. (* = Phosphorothioate bonds; mN = 2′-*O*-Me RNA base)

Target	Antisense Oligomer Construct
dme-miR-34	mC*mG*mA*mC*A*T*C*T*T*T*A*C*C*T*G*A*C*A*G*T*mA*mU*mU*mA*mG*/3Phos/
dme-miR-8	mC*mG*mA*mC*A*T*C*T*T*T*A*C*C*T*G*A*C*A*G*T*mA*mU*mU*mA*mG*/3Phos/
GFP Control	mG*mC*mC*mA*C*A*A*C*G*T*C*T*A*T*mA*mU*mC*mA*mU*/3Phos/

2.2.10 *In vivo* detection of reactive oxygen species by dihydroethidium

Drosophila melanogaster brains were dissected under a dissecting microscope in Schneider's medium. Brains were then stained in a 30μM solution of dihydroethidium and washed 3 times prior to fixation in 7% formaldehyde in 1X PBS (Owusu-Ansah, 2008). Brains were visualized using a dissecting fluorescence microscope.

2.3 Results

2.3.1 Hyperoxia environment induces early death and oxidative stress in *D. melanogaster*

Adult *D. melanogaster* flies grown in hyperoxic conditions displayed progressive qualitative physical deterioration and death as treatment time progressed. Initially, flies were subjected to a 6-day hyperoxia exposure, as described by Gruenewald *et al.* (2009). However, by day 6, the majority of the flies were dead or dying and therefore considered unsuitable for miRNA extraction and accumulation analysis. Subsequently, all hyperoxia treatments were conducted for a maximum of 5 days, when the majority (>80%) of the flies were still active.

Two-days post-eclosure *D. melanogaster* were exposed to a ~99% O_2 chamber for 6 hours, 2 days, and 5 days. Enzo Life Sciences' OXY-TEK TBARS assay was then used to determine the extent of lipid peroxidation, to indirectly confirm that the hyperoxia environment was inducing some degree of cellular oxidative stress damage during the various hyperoxia exposure treatments (Oakes & Van Der Kraak 2003; Aksu *et al.* 2014). There was a significant increase in lipid peroxidation in all treatment time points (Figure 2.2). With these 3 treatment time points, there appears to be a peak in cellular lipid peroxidation at 2-days hyperoxia exposure, with a nearly 4-fold increase in lipid peroxidation relative to normoxia flies. At both 6 hours and 5-days hyperoxia there was an approximately 2.5-fold increase in lipid peroxidation relative to normoxia flies.

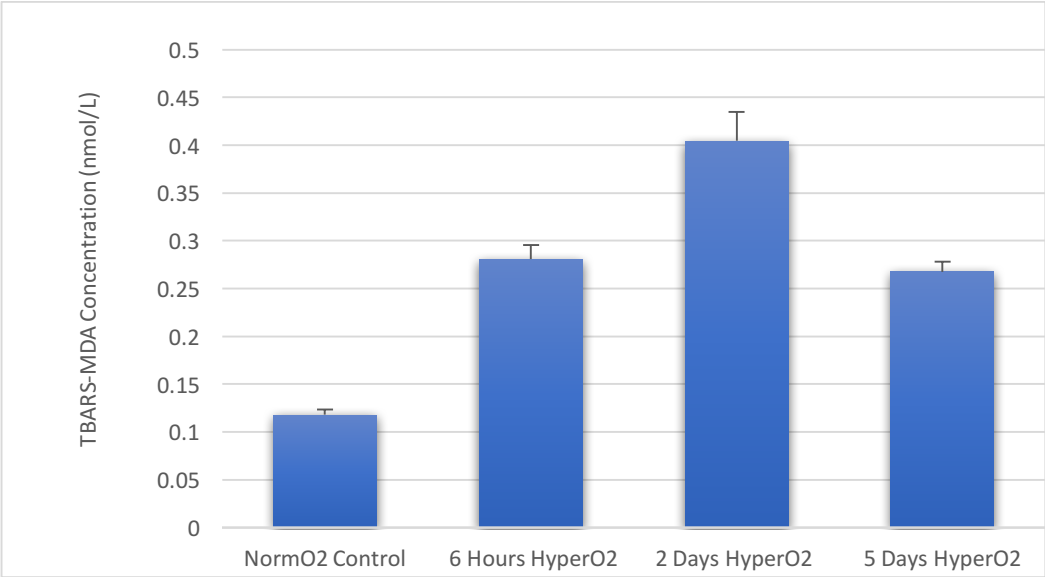

Figure 2.2. Extent of lipid peroxidation following different exposure lengths of hyperoxia in *D. melanogaster* adults. Thiobarbituric acid reactive substances (TBARS), in particular, malondialdehyde (MDA), a lipid peroxide byproduct, concentrations were measured, using a standardized curve that measured serial dilutions of MDA with a fluorometer with the excitation wavelength set at 530 nm and emission at 550 nm. All values were significantly different from the normoxia (NormO2) control (student t-test; $p<0.05$).

2.3.2 Hyperoxia whole body and head miRNA analysis

Gruenewald *et al.* (2009) showed that hyperoxia in *D. melanogaster* induces cellular degeneration and they identified a considerable number of genes with altered expression during oxidative stress in the flies. To extend on their findings, I aimed to determine whether miRNAs could be regulating the expression of genes during hyperoxia-induced oxidative stress by performing a genome-wide miRNA accumulation analysis using LC Sciences microarrays. This analysis identified seven miRNAs that exhibited statistically significant changes in the hyperoxia-treated flies, relative to those reared in normoxia (Table 2.4).

Table 2.4. Top seven miRNAs identified in a microarray screen (LC Sciences) that showed statistically significant changes in accumulation in whole bodies of flies subjected to normoxia relative to hyperoxia.

Mature dme-miR Identified	Accumulation Change	Amount of Change	p-value
miR-2491-3p	Down-regulated	.76	2.98E-02
miR-970-3p	Up-regulated	1.37	3.65E-02
miR-11-3p	Up-regulated	1.20	6.29E-02
miR-8-3p	Up-regulated	1.25	6.98E-02
miR-313-5p	Down-regulated	.22	7.40E-02
miR-10-5p	Up-regulated	1.28	8.21E-02
miR-4969-3p	Up-regulated	3.31	9.07E-02

Quantitative RT-PCR was used to confirm oxidative stress-mediated changes of accumulation in both *D. melanogaster* whole body and head samples. The qRT-PCR analyses confirmed the up- or down-regulation of six of the seven miRNAs identified by the microarray analyses; the only exception was *dme-miR-10*, which showed considerable variation in transcript levels in hyperoxia-treated flies, and hence, no significant difference from normoxia control levels was observed (Figure 2.3). Hyperoxia flies showed significant *dme-miR-8*, *-11*, *-34*, *-970*, and *-4969* up-regulation in both whole body and head samples. Interestingly, *dme-miR-2491* and *-313* had the opposite accumulation changes between whole body and head tissue, while *dme-miR-10* accumulation changes were not significant.

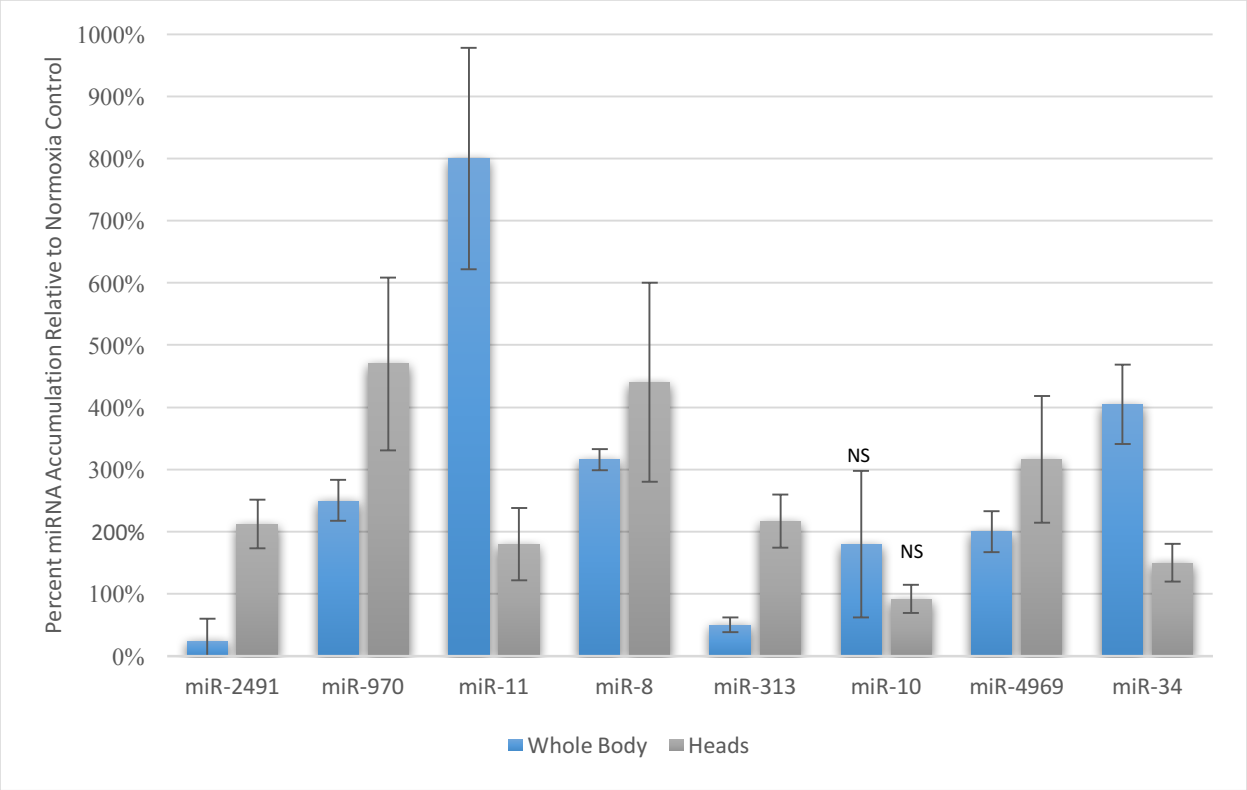

Figure 2.3. Accumulation levels of miRNAs elucidated by microarray global screen after 5 days hyperoxia treatment relative to the ribosomal protein gene RpL32 and adjusted to show dysregulation relative to normoxia treated flies, in both whole body and head samples of *D. melanogaster* subjected to hyperoxia growth conditions. The values represent means and standard errors for 3 replicate qRT-PCR experiments. All values were significantly different from Normoxia unless designation with NS (student t-test; $p<0.05$)

2.3.3 miRNA accumulations over continuous hyperoxia exposures

Following multi-day hyperoxia exposure, substantial oxidative stress and non-reversible cellular degeneration has already occurred (Zhao *et al.* 2010, Gruenewald *et al.* 2009). In order to determine if any of these identified miRNAs play a role in the earlier stages of oxidative stress responses, it was important to determine more precisely when they had altered levels of transcription. Preliminary experiments showed there are no significant changes in miRNA accumulation after the first 6 hours of hyperoxia exposure for any of the miRNAs previously identified (data not shown). However, qRT-PCR miRNA accumulation analysis confirmed that

miRNA levels had changed significantly after 1 day-post hyperoxia exposure. *Dme-miR-8, -34,* and *-11* all displayed consistent up-regulation at all treatment time points (Figure 2.4). *Dme-miR-2491* and *-313* showed up-regulation earlier in hyperoxia exposure, but were down-regulated by 5-days. *Dme-miR-970* and *-4969* were only significantly up-regulated at 5 days hyperoxia exposure. *Dme-miR-10* was never significantly different from normoxia treatments at any time point.

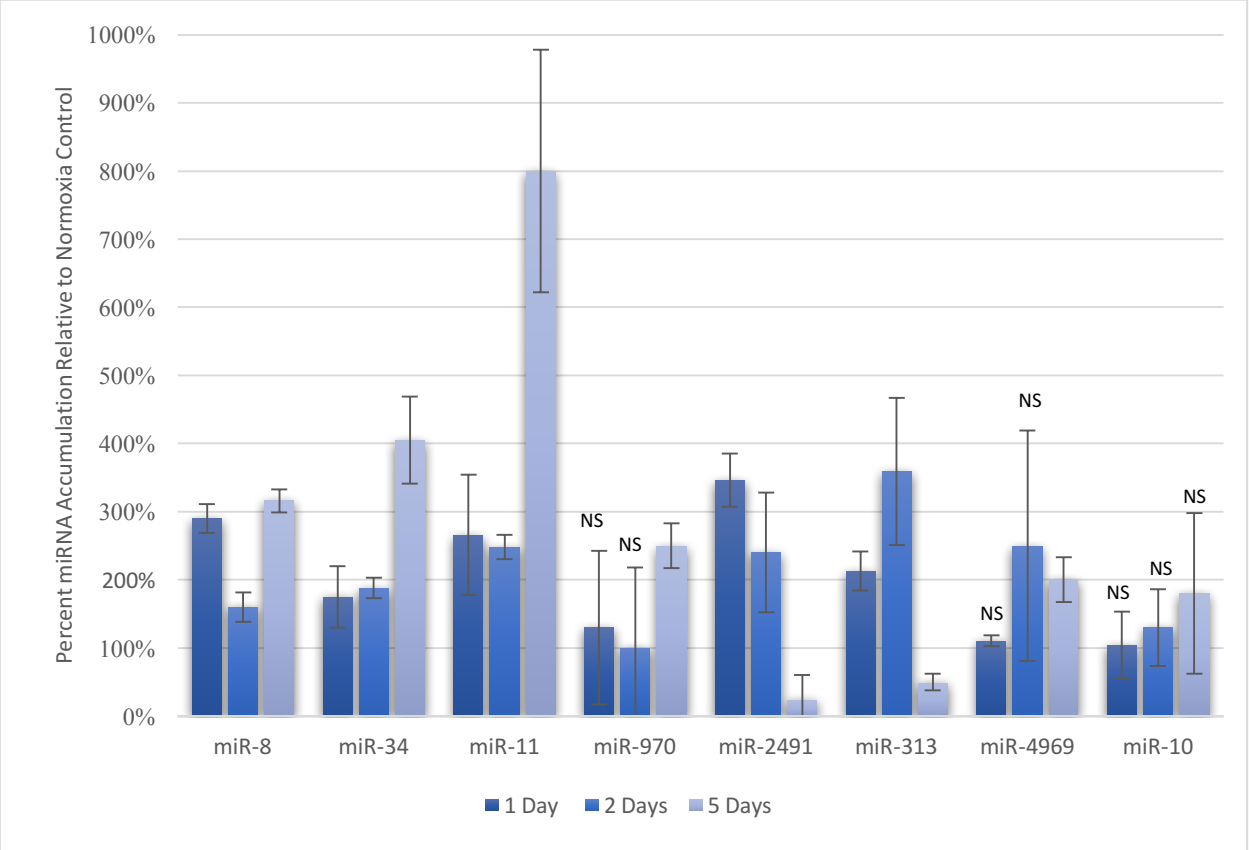

Figure 2.4. Relative accumulation levels of *dme-miR-8, -34, -11, -970, -2491, -313, -4969,* and *-10* in *D. melanogaster* treated in both normoxia and hyperoxia growth conditions for 1-day, 2-days, and 5-days and normalized to the normoxia treatments in whole body samples. The values represent means and standard errors for 3 replicate qRT-PCR experiments. All values were significant unless designation with NS (student t-test; $p<0.05$).

2.3.4 Selecting and evaluating *dme-miR-8, -11,* and *-970* predicted targets

The top 50 predicted gene targets for *dme-mir-8, -11,* and *-970* from DIANA lab, miRNA.org, and TargetScanFly computer algorithms were compiled and compared. For each of the three miRNAs, the highest ranking, functionally characterized, protein-encoding target gene was selected for further analysis. The *dme-mir-8* target was *jaguar,* the *dme-mir-11* target was *castor,* and the *dme-mir-970* target was *derailed,* which had two similarly-scoring *mir-970* binding sites. Table 2.5 shows the predicted consequential pairing of the target gene's region and respective miRNAs.

Table 2.5. Predicted 3'UTR targets of *Dme-miR-11, -970, and -8.* Seed match definitions - 8mer: an exact match to positions 2-8 of the mature miRNA (the seed + position 8) followed by an 'A'; 7mer-m8: an exact match to positions 2-8 of the mature miRNA (the seed + position 8); 7mer-1A: An exact match to positions 2-7 of the mature miRNA (the seed) followed by an 'A' (Lewis *et al.* 2005). Alignment data provided by TargetScanFly (http://www.targetscan.org/fly_12/).

3'UTR : miRNA	Predicted consequential pairing of target region (top) and miRNA (bottom)	Seed match	Branch-Length Score
Cas 3'UTR nt 326-332 *Dme-miR-11-3p*	5' ...UUAAGUUUGACUAAA-CUGUGAUG... 3' UCGUUCUUGAGUCUGACACUAC	7mer-m8	5.27
drl 3'UTR nt 812-818 *Dme-miR-970-3p*	5' ...UAGUUCUUAAGUUACUCUUAUGG... 3' UAUCGGCGCACACAGAAUACU	7mer-m8	3.39
drl 3'UTR nt 165-171 *Dme-miR-970-3p*	5' ...UAAAUGUACGAACGACUUAUGAG... 3' UAUCGGCGCACACAGAAUACU	7mer-1A	0.41
jar 3'UTR nt 84-90 *Dme-miR-8-3p*	5' ...UAUAUAUAUAUUUUACAGUAUUU... 3' CUGUAGAAAUGGACUGUCAUAAU	7mer-m8	5.27

To confirm whether or not *dme-miR-11, -970,* and *-8* bind to the respective 3'UTRs of *Cas*, *drl*, and *jar*, functional cell-based assays using pMIR-GFP-3'UTR reporter and pEP-miRNA expression vectors were performed. The 3'UTR sequences for three target genes and the three *dme-miR* genes were successfully PCR-amplified from genomic DNA and cloned into

their respective vectors. DNA sequencing confirmed that the entire 3'UTR sequences and the miRNA gene had each been successfully cloned. Cells were first transfected with the GFP reporter plasmid alone, and were observed to fluoresce strongly (results not shown), which confirmed that the 3'UTR sequences did not prevent GFP expression. Equal dosages of the pMIR-GFP-3'UTR reporter and the pEP-miR-927 expression vectors were then co-transfected into HEK293 cells, and 2-days post-transfection, the cells were monitored to assess whether the miRNAs were capable of suppressing expression of the GFP reporter gene. Cells that were co-transfected with the *dme-miR-11* and *dme-miR-970* expression plasmid and their respective 3'UTR-GFP reporter plasmid (*cas* 3'UTR and *drl* 3'UTR) showed a significant suppression of GFP fluorescence, relative to cells treated with the 3'UTR-GFP reporter plasmid alone (Figure 2.5). These results suggest that the *dme-miR-11* and *-970* can bind to the *cas* and *drl* 3'UTR sequences to reduce the reporter genes' expression. *Dme-miR-970* showed 88% translation reduction in pMIR-GFP-*drl*, while *Dme-miR-11* showed 36% reduction. Unfortunately, *Jaguar* does not appear to be regulated by *dme-miR-8* (Figure 2.5) as pMIR-GFP-*jar* showed no translation reduction when co-transfected with *dme-miR-8*.

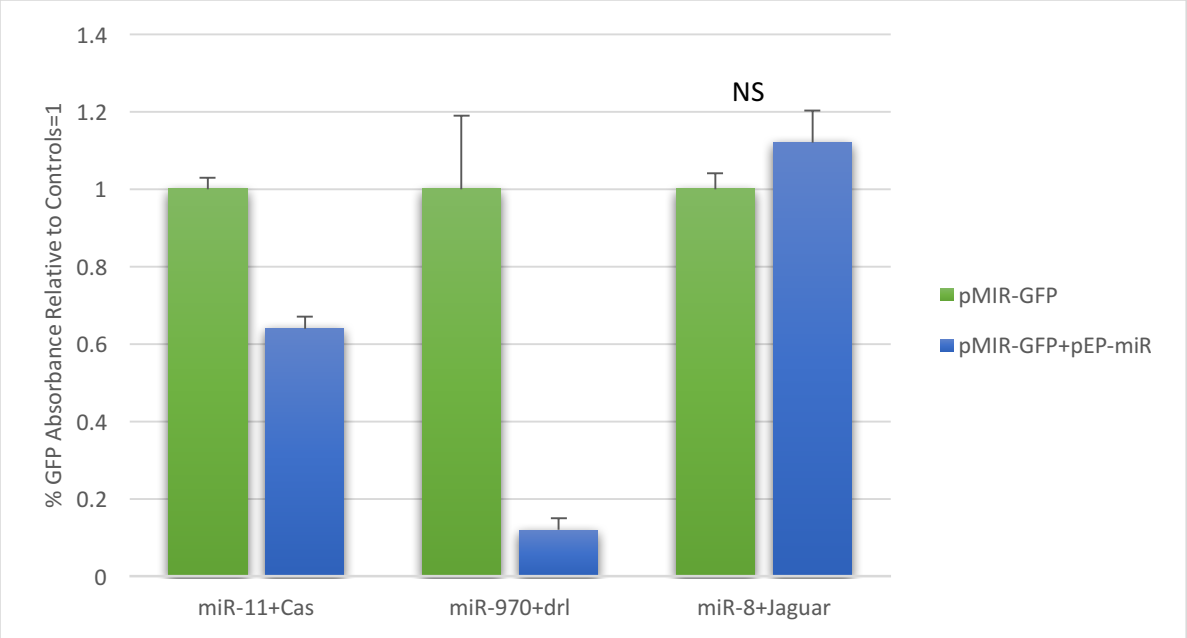

Figure 2.5. Cell based assays to assess whether the *dme-miR-11, -970, and -8* miRNA can bind to *Cas*, *drl*, and *Jar* 3'UTRs respectively. The pMIR-GFP-3'UTR plasmid alone and pMIR-GFP-3'UTR plasmid and the pEP-miR expression plasmid were transfected into HEK293 cells, and GFP fluorescence was measured 2-days post-transfection. GFP fluorescence was normalized to non-transfected cell (auto)fluorescence levels and values shown are % GFP fluorescence relative to pMIR-GFP-3'UTR + pEP-miR-Null. The values represent the means and standard errors for the experiment performed in triplicate. All values were significant unless designation with NS (student t-test; $p<0.05$).

2.3.5 *In vivo* suppression of *dme-miR-8 and dme-miR-34*

2.3.5.1 Evaluating the effect of antisense oligomer constructs to suppress miRNA accumulation

Dme-miR-8 and *-34* were two of the most significantly altered miRNAs following hypoxia stress in *Drosophila* and have been shown to have roles in aging, neuronal regulation, and neurodegeneration (Chawla & Sokol 2011; Liu *et al.* 2012). To functionally confirm the relevance of these two miRNAs in the opposing hyperoxia stress, *dme-miR-8* and *-34* antisense oligomers were designed with the aim to reduce the levels of these miRNAs before subjecting them to hyperoxia. The oligomers were injected into *D. melanogaster* in the mesopleuron of the thorax; control flies were injected with a nonsense oligomer with specificity to GFP (a sequence

not found within the *Drosophila* genome/transcriptome). Normoxia treated flies were first examined for evidence of miRNA knockdown (by qRT-PCR) at 1-day, 2-days, and 5-days post injection and treatment. Curiously, the oligomer injections had the opposite anticipated effect; anti-miRNA-8 induced increased accumulation of *dme-miR-8* transcripts after only 1-day post-injection and anti-miRNA-34 induced increased accumulation of its respective target after 2-days post-injection (Figure 2.6).

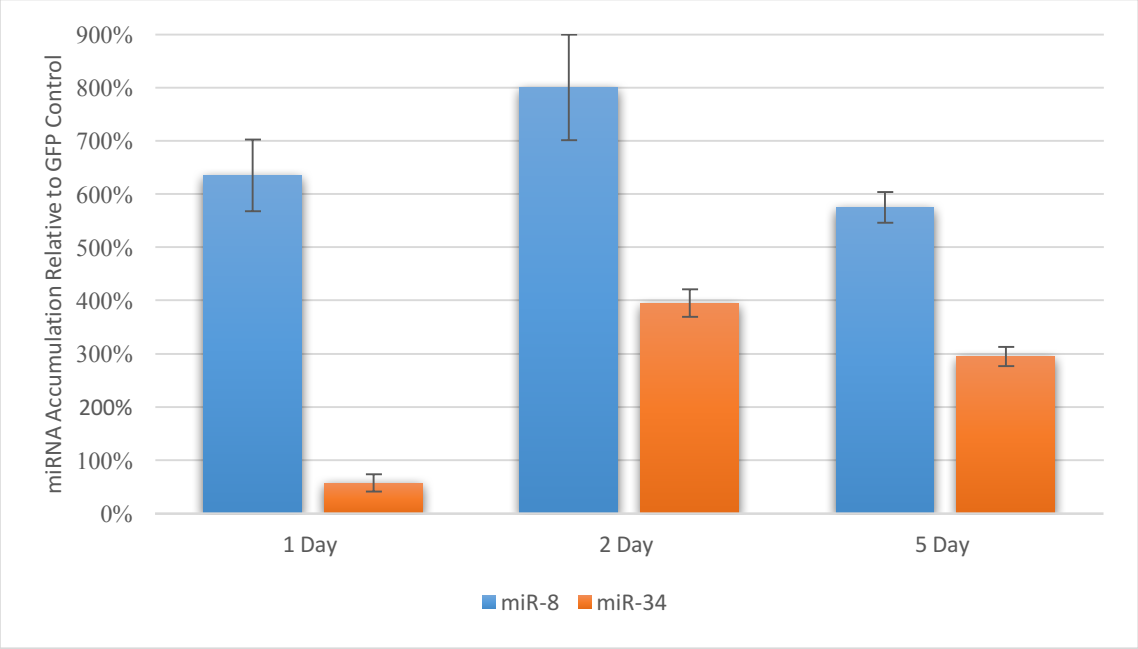

Figure 2.6. Relative accumulation levels of *dme-miR-8* and *34* in *D. melanogaster* injected with their respective antisense miRNA oligomers under normoxia growth conditions normalized to the GFP control injections. The values represent means and standard errors for 3 replicate qRT-PCR experiments. All values were significantly different from the normoxia treatment (student t-test; $p<0.05$).

2.3.5.2 Evaluating the effect of antisense oligomer constructs to alter miRNA in vivo functionality

Even though the miRNA accumulation change had the opposite of its intended effect with antisense oligomer construct injections, flies were monitored for survival with all 3

injection treatments in both normoxia and hyperoxia growth settings (Figure 2.7). Hyperoxia treated flies were placed in the hyperoxia environment 2-days post injection, to ensure mortality was not simply a result of the injection trauma. Overall, all treatments showed some degree of long term post-injection mortality. Anti-GFP control injections appear to have had better overall survival in both environment treatments, as they had the lowest mortality at day 6 post injection (4-days hyperoxia exposure) and had the lowest mortality in their respective treatment groups by day 9 post injection (7-days hyperoxia exposure). MiR-34 antisense injected flies showed the highest mortality in the normoxia treatment group. All three antisense oligomer-injected flies, including the negative control oligomer, showed similarly high mortalities by day 9 when the insects were subjected to hyperoxia, which only suggests that the injections themselves were too stressful for flies subjected to the hyperoxia treatments. The lack of any discernable difference in survival of flies injected with the different oligomers makes it difficult to conclude much more about the roles of these miRNAs.

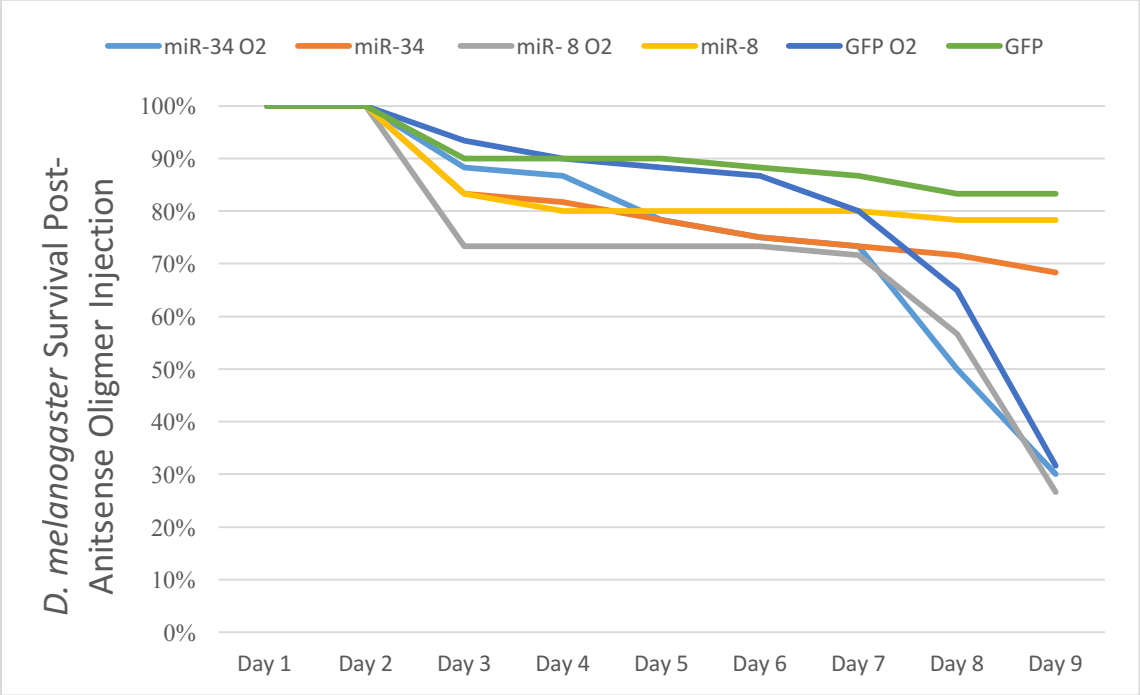

Figure 2.7. *D. melanogaster* survival post-antisense oligomer injection in Normoxia and Hyperoxia enviroments. Hyperoxia treated flies where place in their hyperoxia treatment setting 2-days post injection and are designated by O2 after their injection construct name. All experiments started with 60 flies 2-days post injections.

2.3.6 *In vivo* detection of reactive oxygen species by Dihydroethidium (DHE)

This experiment was originally intended to examine if decreased accumulation of key miRNAs would increase ROS accumulation, oxidative stress, and tissue specific damage. While the miRNA antisense oligomer injections seemingly had the opposite of the predicted effect, with the injection of the antisense oligomers seemingly failing to knock down the targeted miRNAs, the oligomer-injected flies were nevertheless examined for ROS-associated damage following hyperoxia treatment. DHE staining, which is indicative of superoxide accumulation, demonstrated ROS accumulation and damage was occurring within hyperoxia treated flies, specifically in the head (Figure 2.8).

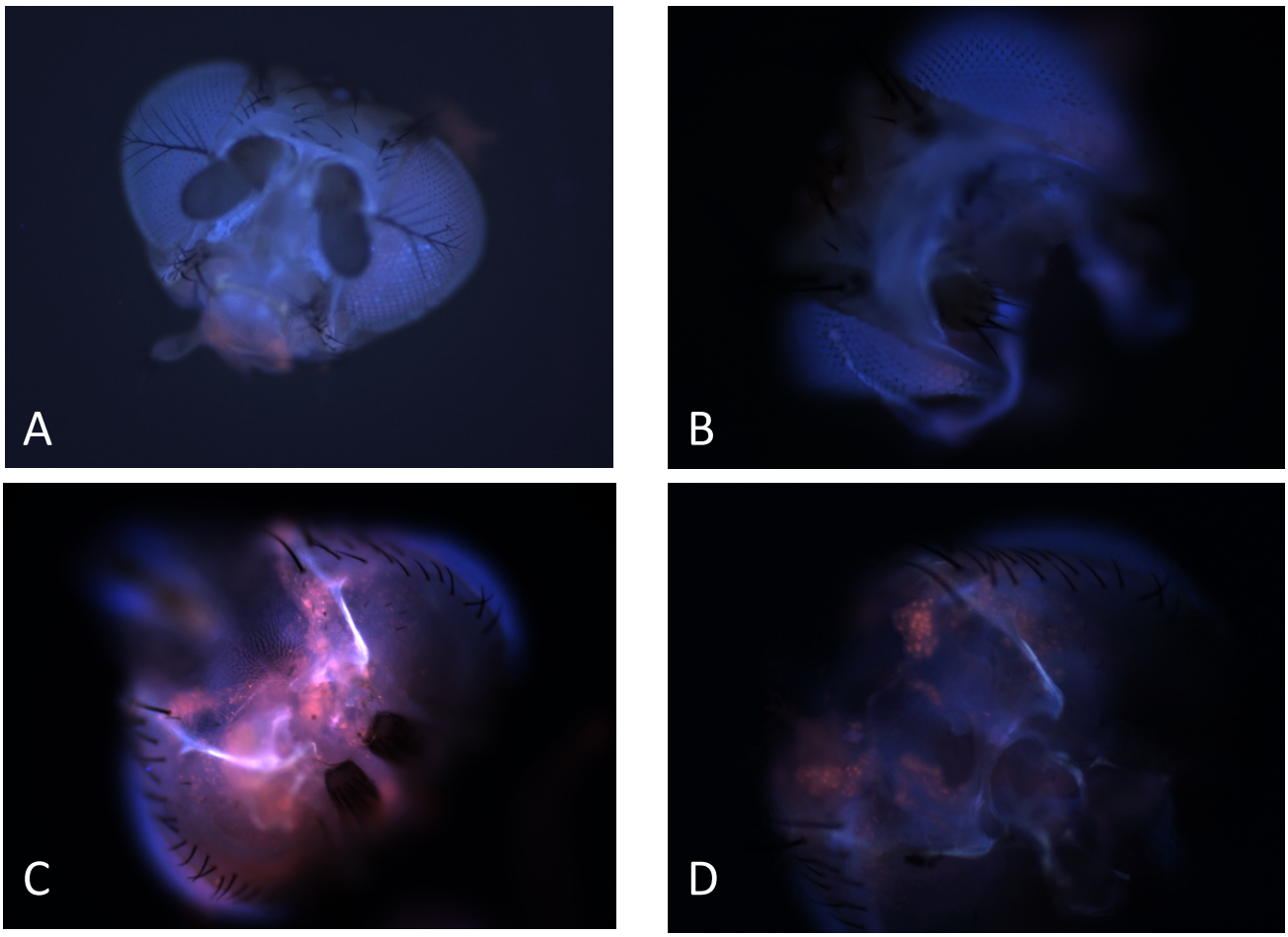

Figure 2.8. Normoxia and hyperoxia *Drosophila melanogaster* DHE head staining A&B) Normoxia fly heads dissected and incubated with Dihydroethidium (DHE) 5-days post-oligomer injection C-D) Hyperoxia fly heads dissected and incubated with DHE 5-days post-oligomer injection. Note the widespread red fluorescence in the hyperoxia-treated brain (C) and within clusters of neurons on the apical lobe of the brain (D).

In normoxia treated fly heads, red fluorescence was only faint and on the peripheral regions, and likely reflects a small degree of oxidative stress from cellular damage due to the dissection process. In contrast, hyperoxia-treated flies display distinct red fluorescence within neuronal cells in the head, indicating cellular damage prior to dissection. Three flies from each treatment were dissected and stained for a total of nine hyperoxia and nine normoxia examined flies, in which this effect was consistently seen.

2.4 Discussion

2.4.1 Hyperoxia and its effect on miRNA accumulation

Hyperoxia treatment has been shown to induce a range of cellular and tissue impacts, including: advanced aging processes; induced inflammation, neurodegeneration, and cardiomyopathies; and early mortality in *D. melanogaster,* presumably by constantly subjecting cells to oxidative stress through the creation of ROS (Gruenewald *et al.* 2009; Wu *et al.* 2010; Powers & Jackson 2008; Tsutsui *et al.* 2011; Pei *et al.* 2016; Lightowlers *et al.* 2015). Lipid peroxidation is the oxidative degradation of lipids in cell membranes and is used as a marker for oxidative stress-induced cellular damage. This process is quantified by indirectly measuring the malondialdehyde (MDA) end product reacting with thiobarbituric acid (TBARS test) to yield a fluorescent product (Marnettt 1999). There is a clear and significant increase in lipid peroxidation, and by association, oxidative stress in hyperoxia-exposed flies. A more interesting and novel finding is that maximal lipid peroxidation was seen at 2-days and not 5-days post-hyperoxia exposure. This may reflect the flies' capabilities to counter those particular oxidative stress effects, or simply reflect the maximal level of lipid peroxidation tolerated before apoptosis or cellular senescence produces the more toxic 4-hydroxynonenal secondary product, as the majority of flies died when exposed to 6-days of hyperoxic treatment (Ayala *et al.* 2014). The TBARS assay has been criticized for overestimating MDA levels because some aldehydic compounds, other non-lipid-related, and non-MDA substances are capable of reacting with TBA to produce absorbance spectra similar to those of the MDA-complex (Janero & Burghardt 1988). This could also be contributing to peak fluorescence 2-days post hyperoxia treatment.

Regardless, this experiment sufficiently demonstrates that lipid peroxidation and oxidative stress occurs in flies reared in hyperoxia environments.

Hyperoxia induces oxidant injury in almost every organ, with the brain being particularly susceptible (Webster *et al.* 1987). MiRNAs are emerging as critical factors in gene regulation during development and may hold the key to understanding the still undetermined modulation of neurodegenerative diseases. To initially identify miRNAs involved in neuronal protection against oxidative stress, flies were treated in a highly oxidative atmosphere and miRNA accumulation was analyzed using a microarray approach. The microarray analyses identified seven candidate miRNAs with altered accumulations following five days of hyperoxia treatment in whole body samples while head samples were assessed by qRT-PCR. Quantitative RT-PCR confirmed whole body changes in all identified miRNAs except for *dme-miR-10*. This is most likely due to *dme-miR-10* having the shortest pre-miRNA hairpin (only 71 nucleotides) and therefore insufficient nucleotide sequence to properly and consistently amplify using qRT-PCR as *dme-miR-10* accumulation was never found to be significantly different from any of the normoxia treatments and regularly only showed minimal or no amplification in qRT-PCR analyses.

Dme-miR-2491 and *-313* also showed down-regulation in whole body samples, but were up-regulated in head samples. These variances in miRNA accumulation profiles between whole bodies and heads is not surprising, as similar differences in whole body and head gene expression profiles have been observed in other *D. melanogaster* gene expression studies (Zhao *et al.* 2010). MiRNAs typically display tissue-specific accumulations, and potentially regulate different genes in different tissues (Mohammed *et al.* 2014), and hence, the differences in the responses of these two miRNAs in the different tissues likely reflect their different functions in

heads and other body tissues. These opposing responses in the two samples (heads vs bodies) were still associated with high levels of mortality and declines in lipid peroxidation, relative to the control normoxia flies, and hence necessitated evaluation of these miRNAs accumulation at earlier time points of hyperoxia and oxidative stress exposure.

Following prolonged hyperoxia exposure (5-days), substantial oxidative stress and non-reversible cellular degeneration had likely already occurred (Zhao *et al.* 2010, Gruenewald *et al.* 2009). MiRNAs are known to influence fundamental cellular activities in health and disease through their ability to post-transcriptionally regulate gene expression of most genes. MicroRNAs have been shown to change their accumulation as soon as 2-hours post-treatment; increased accumulation of miR-21, for example, was observed that soon after Interferon-alpha-induction (Yang *et al.* 2010). In my study, 6 hours of hyperoxia was selected as the earliest time point to examine miRNA accumulations, but no significant change in accumulation in any miRNA was observed. This lack of change in miRNA accumulation may simply reflect the duration of time required for ROS to accumulate to trigger an oxidative stress response and induce miRNA transcription (Lee & Choi 2003). Reinsbach *et al.* (2012) similarly observed no change in interferon-γ-induced miRNA accumulation until well after 12 hours, with the most significant changes in miRNA accumulations occurring at 24 hours. For this reason, the initial time point was increased to 1-day post-hyperoxia exposure.

As before, *dme-miR-10* showed no significant changes in accumulation over the course of the hyperoxia exposures. *Dme-miR-970*, and *-4969* showed no significant accumulation changes at earlier stages of oxidative stress, suggesting their roles are limited to terminal stages of oxidative stress assault. *Dme-miR-2491* and *-313* displayed initial up-regulation prior to a down-regulation at 5-days hyperoxia exposure. *Dme-miR-2491*'s accumulation displayed a gradual

decline, while *Dme-miR-313* initially had increasing accumulation until it was down-regulated. Presumably, these miRNAs have important regulatory roles in the early to mid-phase period of oxidative stress. The decline in their accumulation by day 5 may reflect widespread gene expression dysfunction, as oxidative stress-induced damage was irreparable after this long hyperoxia exposure. As there are currently no other studies that have examined the functions of these miRNAs in association with cell processes involved in oxidative stress, these findings are novel, but still demand future exploration to resolve their specific roles in response to oxidative stress.

Dme-miR-34 was added to the list of candidate miRNAs due to its strong link to regulating ageing processes (Liu *et al.* 2012). *Dme-miR-34,* as predicted, showed a gradual increase in accumulation as hyperoxic exposure increased. *Dme-miR-8* and *-11* also displayed consistent up-regulation at all three time points, with *Dme-miR-11* having a progressive increase in accumulation as well. *Dme-miR-11* is located in the last intron of *dE2f1*, a transcription factor, and its expression parallels that of *dE2f1* (Truscott *et al.* 2011). It has been shown that co-expression of miR-11 limits the pro-apoptotic function of *dE2f1* directly, as well as *reaper* and *head involution defective* indirectly after DNA damage by directly modulating dE2F1-dependent apoptotic transcription (Truscott *et al.* 2011). Its progressive up-regulation, indicates it may be trying to suppress cell induced apoptosis. *Dme-miR*-8's up-regulation, coupled with its potential to regulate over 250 conserved predicted target genes, is highly suggestive that it has a significant role in responding to oxidative stress (Grun *et al.* 2005). It has also previously been shown that *dme-miR-8* is essential in normal neuronal maintenance, and at least one of the genes that it regulates encodes atrophin, a protein associated with neuronal function in a wide range of species, from flies to humans (Karres *et al.* 2007). Given these three miRNAs' consistent up-

regulation and their affiliation with oxidative stress influenced processes, they seemed the ideal candidates to further explore functional validity.

2.4.2 Functional validation of *Dme-miR-8*, -11, and -*970* predicted neuronal targets

MiRNAs can potentially regulate many different genes, as they bind with imperfect complementarity to the target genes' 3'UTR (Bartel 2004). For this reason, computer programs are often used to identify putative miRNA target genes (Friedman *et al.* 2010; Hofer *et al.* 2011; Vlachos *et al.* 2012). This technology only continues to improve with software advancement and collaborative research input. For example, after the initial microarray results identified miRNAs associated with oxidative stress, two of the up-regulated miRNAs (*dme-miR-2491-3p* and *-4969-3p*) appeared to lack any predicted target mRNAs, but newly developed programs have now identified possible targets for these and other miRNAs. Given the growing number of theoretical target genes to pursue, it was prudent to narrow the focus of both miRNAs of interest as well as their predicted targets.

I opted to focus my attention on miRNAs that had clearly significant responses in both whole body as well as heads. Given *Dme-miR-970*'s consistent up-regulation in both heads and whole bodies during oxidative stress, it was included along with *Dme-miR-8* and *-11* for investigation into potential neuronal regulatory targets. 3'UTR seed-match pairings were initially used to identify potential miRNA interactions and branch-length scores were then calculated by combining computational evaluation of five general parameters in order to boost confidence in a target site's likelihood of interacting with a specific miRNA (Grimson *et al.* 2007). These parameters included: AU-rich nucleotide composition near the site, proximity to sites for co-expressed miRNAs (which leads to cooperative action), proximity to residues pairing to miRNA

nucleotides 13–16, positioning within the 3′UTR at least 15 nt from the stop codon, and positioning away from the center of long UTRs (Grimson *et al.* 2007; Kheradpour *et al.* 2007). *miR-11:Cas* and *miR-8:jar* both had the highest possible branch length score at 5.27, while *miR-970:drl* was found to have two regions for which *dme-miR-970* could bind to *drl* with branch-length scores of 3.39 and 0.41. Interestingly, even with the high scores for both *miR-11:cas* and *miR-8:jar* interactions, only *cas* was observed to be significantly suppressed by *dme-miR-11*, while *jag* was not significantly suppressed by *dme-miR-8*. This observation further supports the need for functionally testing miRNA:mRNA pairings, as there are obviously other mechanisms mediating their ability to bind and suppress even high-scoring predicted targets (Ritchie *et al*. 2013). Additionally, even though *miR-970:drl* had the lowest branch-length scores, it proved to have the highest level of mRNA suppression. This could be due to the ability of *dme-miR-970* to bind and suppress *drl* at two 3'UTR locations, providing an additive effect in suppression.

While *dme-miR-8* did not sufficiently bind and subsequently suppress *jaguar* in the functional assay, it does not mean that it has a reduced role in regulating oxidative stress cellular responses. On the contrary, its lack of functional suppression, which would potentially be deleterious to an already ROS-assaulted cell/neuron, may further attest to *dme-miR-8*'s helpful up-regulation in cells/neurons undergoing oxidative stress. Given that suppressing *jaguar* could cause insufficient myosin VI expression and contribute to mitochondrial dysfunction at synaptic terminals, *dme-miR-8*'s lack of *jag* suppression could be perceived as a benefit to the cell's survival (Pathak *et al.* 2010).

Dme-miR-11 binding to and suppressing *Castor* transcripts, which encode a zinc finger transcription factor mainly present in neuronal development, suggests that *dme-miR-11*'s up-regulation during oxidative stress could contribute to cellular energy conservation in oxidative

stress (Mellerick *et al.* 1992). This correlation can also be drawn for the functional relationship between *dme-miR-970* and *drl*, given that *drl* is expressed in embryonic interneurons and helps to facilitate pathway development as a Wnt5 receptor (Callaham *et al.* 1995). While this is only speculation, and there are of course numerous other potential *dme-miR-11* and *-970* regulatory targets, down regulating neuronal generation/regeneration during a time of stress could minimize subsequent aberrant neuronal regeneration.

2.4.3 Functional validation by *in vivo* suppression of *dme-miR-8* and *-34*

A relatively new approach to inducing miRNA loss-of-function is to use chemically modified antisense oligonucleotides, which bind to the mature miRNA, leading to functional inhibition of the miRNA (Torres *et al*. 2012; Stenvang *et al*. 2012). These antisense oligonucleotides are single-stranded RNA-based inhibitors that are chemically modified with phosphorothioate bonds and 2′-*O*-Me RNA bases to improve their activity and to increase their stability (Torres *et al*. 2012). This strategy has proven to be effective in examining phenotypic impacts of miRNA suppression in *Drosophila* embryos (Boutla *et al.* 2003). A similar strategy was applied in young adult *Drosophila* in this study. Unfortunately, upon evaluation of miRNA suppression following antisense miRNA injection using qRT-PCR, the opposite of the anticipated effect was seen. One day after the antisense oligomer injections, *miR-34* was suppressed, but *miR-8* was up-regulated when compared to the negative GFP injected control flies. Two-days after antisense oligomer injections, both miRNAs were up-regulated, and this up-regulation persisted for 5-days post-injections. It is possible that the oligomers did, in fact, initially inhibit or ‘mop up’ the miRNAs, but subsequently, triggered a responsive up-regulation. A study in HeLa cells and *Drosophila* embryo lysates suggested that antisense oligonucleotides

block the miRNA loaded miRISC complex in a stoichiometric and irreversible manner (Hutvágner *et al.* 2004). This suppression of both the miRNA-miRISC complex may trigger a compensating response, resulting in up-regulation of the targeted miRNA. Unfortunately, previous studies have not evaluated the change in miRNA accumulation following antisense oligonucleotide injection using qRT-PCR in adult *Drosophila*, so the exact mechanism for the results seen here remains unresolved.

Given this unintended result, it is difficult to interpret the experimental results that followed the miRNA antisense oligonucleotide injections. Increased morality was observed in both normoxia and hyperoxia flies injected with miR-34 and -8 antisense oligonucleotides as compared to GFP controls. Whether this effect is due to a true sequestration of these miRNAs or a general dysregulation of miR-34 and -8 remains unclear.

The antisense oligomer injections had been conducted with the aim to examine whether inhibition of miRNAs could enhance ROS-induced damage *in vivo*. Despite the apparent lack of miRNA inhibition using injected antisense oligomers, the injected fly heads and brains were nevertheless examined for cellular damage. The dihydroethidium staining confirmed that hyperoxia exposure results in distinct compartmentalized oxidative damage in the *Drosophila* head. This, along with qualitative observations of *Drosophila* behavior changes (slow mobility and reduced ability to climb or mate as previously described by Zhao *et al.* 2011) further supports the oxidative stress hypothesis that neurons are highly susceptible to ROS damage and that oxidative stress has a pronounced effect in the central nervous system (Webster *et al.* 1987; Greunewald *et al.* 2009).

Chapter 3: Antioxidant genes and their miRNA regulators

3.1 Introduction

Antioxidants are essential for cells in maintaining ROS homeostasis in order to prevent oxidative stress and its deleterious effects as previously described. I sought to uncover how miRNAs may be regulating antioxidant genes' expression by focusing on five specific antioxidants: Superoxide dismutase, Catalase, Heat shock cognate 70-4, Sniffer, and Thioredoxin-2.

3.1.1 Superoxide dismutase and Catalase – Essential antioxidant enzymes

Superoxide dismutase (SOD) catalyzes the conversion of superoxide to oxygen and hydrogen peroxide and occurs in both cytoplasmic and mitochondrial isoforms (encoded by *Sod1* and *Sod2*, respectively) in *Drosophila*. Catalase (cat) catalyzes the decomposition of hydrogen peroxide to water and oxygen and there is only one functional catalase gene in *Drosophila* (Mackay *et al.* 1989). Some of the first experiments to test the free radical theory of aging showed that artificial selection for increasing longevity results in increased SOD content and activity through elevated mRNA content of Copper/Zinc *SOD*, Iron/Magnesium *SOD*, and *catalase* (Arking 1987). In one study, concomitant overexpression of superoxide dismutase and catalase increased both average and maximum life span while in another it had no effect on life span, but had an increase in acute oxidative stress resistance (Sohal *et al.* 1995, Mockett *et al.* 2003). The overexpression of only Iron/Magnesium SOD increased life span, albeit to only a modest extent (Philips *et al.* 2000). Combined overexpression of *Sod1* and *Sod2* was shown to have an additive effect with the increase in life span proportional to the level of *SOD*

overexpression (Sun & Tower 1999, Sun *et al.* 2002, Sun *et al.* 2004). From these studies we see a clear link between oxidative stress, aging, and Sod/cat.

Sod1- and *Sod2*-null fly phenotypes are quite deleterious, resulting in 80% life-span shortening and accelerated aging via expression of the *wingless* gene (Reveillaud *et al.* 1994; Rogina & Helfand 2000). RNAi-mediated knockdown of the *Drosophila Sod2* resulted in larval lethality (about 10 days after hatching), which was only slightly worse than the *Sod1*-null mutant (Mockett *et al.* 2003). True knockout of *Sod2* was found to be larval-lethal (36-hours post-hatch), and established that mitochondrial superoxide dismutase is necessary for life (Duttaroy *et al.* 2003). Also, while complete loss of catalase activity results in fatality, partial levels are sufficient to ensure the viability of *Drosophila* to normal lifespans (Mackay *et al.* 1989). These results provide a strong rationale for manipulating individual antioxidant genes. However, there have been contradictory findings described in other studies: some have reported an increase in average life span; others reported no effect or a decrease in life span; and very high overexpression of superoxide dismutase was found to be lethal (Staveley *et al.* 1990; Seto *et al.* 1990; Reveillaud *et al.* 1991, Reveillaud *et al.* 1991). These conflicting results necessitate a better understanding of these antioxidant genes' expression as well as their miRNA epigenetic regulators.

3.1.2 Heat shock cognate 70-4

Heat shock cognate 70-4 belongs to the Heat Shock Protein 70 (Hsp70) superfamily of chaperones, which assist in numerous folding processes and are up-regulated in response to heat stress and toxic chemicals (Shaner & Morano 2007). Hsp70 chaperones share a highly conserved bipartite domain structure composed of an ATPase domain and a substrate-binding domain (Wang & Brock 2003; Shaner & Morano 2007). Hsc70-4's mutant allele is lethal (Chang *et al.*

2002). Extended longevity was also observed in transgenic flies overexpressing Hsc70-4 (Aigaki *et al.* 2002). Hsc70-4 controls rejuvenation of the synaptic protein pool by refolding proteins or by targeting them for degradation via facilitating endosomal microautophagy based on its membrane deforming activity (Uytterhoeven *et al.* 2015). When Hsc70-4 is able to oligomerize, it promotes endosomal microautophagy and the turnover of specific synaptic proteins, resulting in increased neurotransmission (Uytterhoeven *et al.* 2015). Hsc70-4 is also involved in clathrin-mediated endocytosis, at least in part by inhibiting the uncoating of clathrin-coated vesicles (Chang *et al.* 2002). Additionally, a screen to elucidate components of the RNAi pathway in *Drosophila melanogaster* uncovered Hsc70-4 (Dorner *et al.* 2006). Hsc70-4 has a clear role to play in managing of oxidative stress-induced protein damage through either repair or degradation, especially in the nervous system.

3.1.3 Sniffer

Sniffer (*Sni*) encodes a homodimeric NADPH-dependent carbonyl reductase that catalyzes the reduction of the lipid peroxidation and belongs to the short-chain dehydrogenase/reductase (SDR) superfamily of proteins. Sniffer catalyzes the reduction of the lipid-derived aldehyde 4-oxononenal (Martin *et al.* 2011). Its functions include protection against oxidative stress-induced neurodegeneration and prevention of apoptosis by removal of damaged cardiolipin (Martin *et al.* 2011). Mutant flies overexpressing sniffer have significantly extended life spans in a 99.5% oxygen atmosphere compared to wild-type flies (Martin *et al.* 2011). Sniffer's function has been found to be essential for preventing age-related neurodegeneration, as reduction of sniffer activity leads to neuronal cell death (Botella *et al.* 2004). Overexpression of *sniffer* confers neuronal protection against oxygen-induced apoptosis, increases resistance of

flies to experimental hyperoxia, and improves general locomotive fitness (Botella *et al.* 2004). *Sniffer* in another essential gene to try to better understand in terms of miRNA regulation during oxidative stress and neurodegeneration.

3.1.4 Thioredoxin-2

As *Drosophila melanogaster* does not contain glutathione reductase, the thioredoxin system has a key function for glutathione disulfide reduction in this and other insects (Candas *et al.* 1997). Thioredoxin-2 (Trx-2) is capable of reducing glutathione disulfide and represents up to 1% of the extractable protein extracted from either *D. melanogaster* Schneider cells or whole flies (Bauer *et al.* 2002). Thioredoxins are proteins that have thiol-reducing activity from the conserved WCGPC region, facilitating roles in defenses against oxidative stress and as electron donors for ribonucleotide-reductase (Svensson & Larsson 2007). In *Drosophila,* there are three classic thioredoxins with the conserved active site: deadhead, ThioredoxinT and Thioredoxin-2 (Svensson & Larsson 2007). Constitutive overexpression of thioredoxin reductase in long-lived *Drosophila* strains had no effect on life span, though an increase in acute oxidative stress resistance was present (Mockett *et al.* 1999). Trx-2 null mutants have decreased lifespans, and thioredoxin double mutant flies showed reduced oxidative stress tolerance, while flies carrying multiple copies of a Trx-2 rescue construct showed higher tolerance (Svensson & Larsson 2007). These findings suggest that Trx-2 has modest or redundant functions in *Drosophila* physiology under unstressed conditions, but could be important during times of oxidative stress.

In this chapter, I examined the changes in expression of these five genes following hyperoxia treatments and explored the potential for selected miRNAs to modulate their expression.

3.2 Methods

3.2.1 Preparation of plasmids for miRNA functional analyses

3.2.1.1 Isolation of miRNAs and predicted miRNA target gene fragments (3'UTRs)

Genomic DNA was isolated from approximately 15 flies (0.02 g) using Wizard® Genomic DNA Purification Kit (Promega). Fragments of antioxidant gene untranslated regions (UTRs) were PCR-amplified from genomic DNA using extended primers (Table 3.1). Extended primers of predicted miRNA regulators were designed to amplify *dme-miR* precursors plus 100 bp of flanking sequences on both ends of the stem loop (Table 3.1). PCR products were resolved by electrophoresis on a 2% agarose gel in TAE buffer. The gel was stained using SYBR Gold and the bands were visualized on a UV transilluminator. Bands were gel extracted using a QIAquick Gel Extraction Kit and cloned into the pstBlue™ Novagene cloning vector according to the manufacturer's instruction.

Table 3.1. Extended primers used for PCR amplifying predicted gene untranslated regions and *dme-miR* precursors plus 100 bp of flanking sequences from genomic DNA.

Genomic PCR Target	Extended Sense Primer	Extended Antisense Primer
SOD	5'-GGTACCGCGATAATCTATTCCGATGT-3'	5'-GGGCCCATGGGGCAATTTCAAAACAC-3'
Hsc 70-4	5'-GGTACCACCATTCACCCCCACACCTC-3'	5'-GGGCCCGCAATTCTCAAATTTATTTA-3'
dme-miR-927	5'-TCGAGGATCCATTTCATTTTATGCAGAATAT-3'	5'-GGGCCCTTATATCAAAAAATAAAGAA-3'
dme-miR-964	5'-TCGAGGATCCAAAACAAGGTAAATATCAGGT-3'	5'-TCGAGCTAGCTTTAATTCAACAGTAATTCAT-3'
Catalase	5'-GGTACCGCTGAGCGAGCGGATTCGAC-3'	5'-TCGCGCTAGCGCAAGCAATCAACTTGGTGAT-3'
dme-miR-971	5'-TCGAGGATCCCACACACACACTGACAGCTAT -3'	5'-TCGAGCTAGCCAAGAGTATAGAGGCGATGGC-3'
dme-miR-1012	5'-TCGAGGATCCTCAATGTCTGTAAGCCGGTGC-3'	5'-TCGAGCTAGCACTGGAGTTCTTGGCACATGG-3'
Sniffer	5'-GGTACCACGATGACAGCGGTTAGTTT-3'	5'-GGGCCCCTTGACTTTAGGAGTCCAGT-3'
dme-miR-978	5'-TCGAGGATCCAAACCAGTGGTGAGAGCTACC-3'	5'-TCGAGCTAGCTTGCCATCCAACAAAGCGCAC-3'
dme-miR-277	5'-TCGAGGATCCACTTACGCCGCGCCGTGCCGA-3'	5'-TCGAGCTAGCTTATTTATTGCTATTTCTTTT-3'
Trx-2	5'-GGTACCGTGGGCAGCGCATAGACGTC-3'	5'-GGGCCCCAAACGGCGAGTGTGTAATA-3'
dme-miR-1013	5'-TCGAGCTAGCATATCATTCCTACTCTGATAG-3'	5'-TCGAGGATCCCTGCGTGTACCCACTTCTCTC-3'

3.2.1.2 miRNA and target gene cloning and ligation into expression vectors

3'UTR fragments and respective *dme-mirs* were PCR-amplified from pstBlue using T7

(5'-TAATACGACTCACTAGGG-3') and Sp6 (5'-GATTTAGGTGACACTATAG-3') primers. 3'UTR fragments and the miRSelect™ pMIR-GFP reporter vector were digested with *Bam*HI and *Apa*I restriction enzymes. Respective *Dme-mir* and the miRSelect™ pEP-miR expression vector were digested with *Bam*HI and *Nhe*I restriction enzymes. Fragments were resolved by electrophoresis on a 1% high-resolution agarose gel in TAE buffer. The gel was stained using SYBR Gold and the bands were visualized on a UV transilluminator. Bands were gel extracted using a QIAquick Gel Extraction Kit. 3'UTR and miRNA fragments were ligated into pMIR-GFP and pEP-miR vectors respectively using T4 DNA Ligase (Invitrogen). Ligated vectors were then transformed into either E.cloni® (Lucigen) or Subcloning Efficiency™ DH5α™ (Invitrogen) chemically competent cells according to the manufacturer's instruction. Vectors were isolated using a QIAprep® Spin Miniprep Kit (QIAGEN) and sent for DNA sequencing to the Robarts Research Institute (London, ON).

3.2.2 MiRNA functional assays in HEK293 cells

3.2.2.1 HEK293 cell culture and Lipofectamine™ transfection with miRNASelect™ vectors

HEK293 cells were maintained in DMEM 10% FBS media (+4.00mM L- Glutamine, 4500 mg/L Glucose, and Sodium Pyruvate) at 37°C in a 5% constant flow CO_2 incubator. Cells were evenly aliquoted into wells of a 96 well plate and grown until approximately 95-99% confluent. Cells were transfected with either pMIR-GFP-3'UTR, pEP-miR, or pMIR-GFP-3'UTR+pEP-miR treatments. Control cell transfections included pMIR-β-Gal, pEP-miR-Null, and pMIR-β-Gal+pEP-miR-Null vector transfections as well as non-transfection treated cells. Transfections were performed in triplicate with 0.2 μg vector with 0.5μl Lipofectamine™ in 100μL OptimMEM® reduced serum medium in each well. The transfection medium was

changed after 4 hours. After 24 hours, transfected cells were treated simultaneously with puromycin (2μg/ml) to select for cells containing the pEP-miR plasmid and with neomycin (G418 Sulfate) (2μg/ml) to select for cells containing the pMIR-GFP vector. GFP fluorescence expression within the cells was recorded after 24 hours of vector selection (Section 3.2.3.2).

3.2.2.2 Quantification of GFP expression

Cells were washed with PBS and covered with 100μL PBS to facilitate measurements of GFP fluorescence. The fluorescence was read using a BioTek® microplate reader using Gen5™ 1.09 software at 485nm excitation and 528nm emission, and the cell density was determined by reading the same plate at 600nm. The fluorescence of each well was normalized to blank wells containing only 100μL PBS.

3.2.3 Expression profile of the antioxidant genes and their miRNA regulators at various stages of hyperoxia exposure

As described above (Section 2.2.5), quantitative reverse-transcriptase PCR (qRT-PCR) was used to determine when antioxidant genes and their miRNA regulators were being transcribed under various stages of hyperoxia exposure (Table 3.2).

Table 3.2. Primers used for qRT-PCR analysis of microRNAs and their antioxidant targets.

qRT-PCR Target	Sense Primer	Antisense Primer
Sod	5'-CCCACCAAGGTCAACATCAC-3'	5'-CCAAGATCATCGGCATCGG-3'
Hsc70-4	5'-GATCACCATTACCAACGACAAG-3'	5'-GTCTCCTTCTGCTTCTCATCC-3'
dme-miR-927	5'-GGCATACGAAATTCGGCAAAG-3'	5'-TAATGGATCGGTAGGGTTTCAG-3'
dme-miR-964	5'-ACTTGCCTTAGAATAGGGGAGC-3'	5'-TCAAATTGTCTTAGAACAGAGGCT-3'
Sniffer	5'-CGGAATGTACGCCTATCGCA-3'	5'-TTGCGGATACAGATCCACGC-3'
dme-miR-277	5'-TGAAGGTTTTGGGCTGCGTG-3'	5'-GATTGTACGTTCTGGAATGTCGT-3'
Trx-2	5'-GTCCTGAAGGTCGATGTGGA-3'	5'-TCTTGAGGAACACGAAGGTGG-3'
Catalase	5'-TGAATGTGACGGACAACCAG-3'	5'-ACAGCAGGAGGACAAGGC -3'

3.3 Results

3.3.1 Regulation of antioxidant genes by predicted microRNAs

A number of genes were previously identified in *D. melanogaster* that encode proteins that help cells protect themselves against oxidative stress (Gruenewald *et al.* 2009). The TargetScanFly algorithm was used to find miRNAs with the predicted capability of regulating these genes with known protective functions against oxidative stress: *superoxide dismutase* (*Sod*), *heat shock cognate 70-4* (*Hsc70-4*), *Catalase* (*Cat*), *Thioredoxin-2* (*Trx-2*), and *Sniffer* (*Sni*). TargetScanFly identified miRNAs with the potential to bind and suppress these key antioxidant genes' translations. The miRNAs with the strongest predicted binding strength were identified for further analysis (Table 3.3).

Table 3.3. Antioxidant genes (*Superoxide Dismutase*, *Heat Shock Cognate 70-4*, *Catalase*, *Sniffer*, and *Thioredoxin 2*) and their top candidate miRNA binders. Seed match definitions- 8mer: an exact match to positions 2-8 of the mature miRNA (the seed + position 8) followed by an 'A'; 7mer-m8: an exact match to positions 2-8 of the mature miRNA (the seed + position 8); 7mer-1A: An exact match to positions 2-7 of the mature miRNA (the seed) followed by an 'A' (Lewis *et al.* 2005). Alignment data provided by TargetScanFly (http://www.targetscan.org/fly_12/).

3'UTR : miRNA	Predicted consequential pairing of target region (top) and miRNA (bottom)	Seed match	Branch-Length Score
Sod 3'UTR nt 86-92 *Dme-miR-**927-5p***	5' ...AAACGAUAUACAUACUUCUAAAC... |||||| 3' CCAUUUCGCAUCCUUAAGAUUU	7mer-1A	0.0
Hsc70-4 3'UTR nt 98-105 *Dme-miR-**927-5p***	5' ...CUUAAACAAACUUGGAUUCUAAA... ||||||| 3' CCAUUUCGCAUCCUUAAGAUUU	8mer	4.48
Hsc70-4 3'UTR nt 98-104 *Dme-miR-964-5p*	5' ...CUUAAACAAACUUGGAUUCUAAA... |||||| 3' UUCAAUUCGAGGGGAUAAGAUU	7mer-1A	4.48
Catalase 3'UTR nt 84-90 *Dme-miR-**971-3p***	5' ...AAUUAUUCCAACACCAACACCAC... ||||||| 3' AGUGACAUUCUUCAUUGUGGUU	7mer-m8	0.32
Catalase 3'UTR nt 78-85 *Dme-miR-**971-3p***	5' ...GGAACUAAUUAUUCCAACACCAA... ||| ||||||| 3' AGUGACAUUCUUCA--UUGUGGUU	8mer	0.32

Catalase 3'UTR nt 118-125 *Dme-miR-1012-3p*	5' ...CCACCCAUUCCGAAA----UUGACUAA... |||| ||||||| 3' GAUACCCCUUUUAGAAACUGAUU	8mer	2.51
Sniffer 3'UTR nt 61-67 *Dme-miR-978-3p*	5' ...AUUGUUGUUGAAUAAACUGGACU... ||||||| 3' GACGUUAAAUGCCGUGACCUGU	7mer-m8	0.13
Sniffer 3'UTR nt 24-30 *Dme-miR-277-3p*	5' ...AGCGGUUAGUUUACCUGCAUUUU... ||||||| 3' ACAGCAUGGUCUAUCACGUAAAU	7mer-m8	1.43
Trx-2 3'UTR 217-223 *Dme-miR-1013-3p*	5' ...UGAGAAACUAAGUGGCUUUUAAA... |||||| 3' GCUCAAGCCGUAUGAAAAUA	7mer-1A	3.29

3.3.2 Functional validation of predicated miRNA:antioxidant gene regulation

To confirm whether or not predicted miRNAs bind to targeted 3'UTRs of antioxidant genes, functional cell-based assays using pMIR-GFP-3'UTR reporter and pEP-miR-*X* (where X represents a specific miRNA) expression vectors were performed. The 3'UTR sequences for the target genes and the miRNA genes were successfully PCR-amplified from genomic DNA and cloned into their respective vectors. DNA sequencing confirmed that the entire 3'UTR sequences and the miRNA genes had all been successfully cloned. Cells were first transfected with the GFP reporter plasmid alone, and were observed to fluoresce strongly (results not shown), which confirmed that the 3'UTR sequences did not prevent GFP expression. Equal dosages of the pMIR-GFP-3'UTR reporter and the pEP-miR expression vectors were then co-transfected into HEK293 cells, and 2-days post-transfection, the cells were monitored to assess whether the miRNAs were capable of suppressing expression of the GFP reporter gene. MiRNA:3'UTR mRNA pairings with stronger branch-length binding scores (>1) showed significant reduction in the translational levels of their predicted targets ranging from ~20%-35% reduction (Figure 3.1), which suggests that these miRNAs could indeed affect the expression of these antioxidant proteins within flies. Antioxidant genes with weaker branch length scores for miRNA binding (*miR-927:Sod, miR-971:Cat, miR-978:Sni*) displayed no significant reduction in

GFP expression, and therefore do not appear to have a strong functionally regulative relationship.

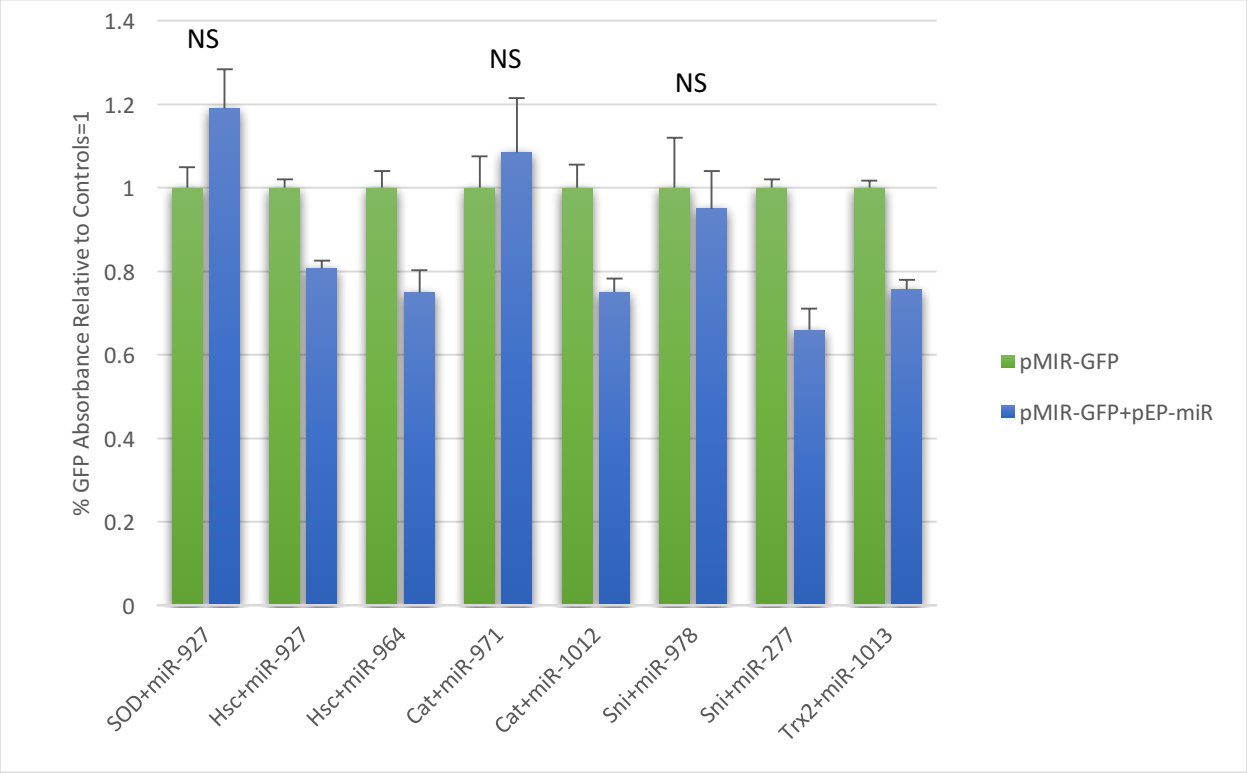

Figure 3.1. Cell based assays to assess whether antioxidant gene expression can be suppressed by miRNAs predicted by binding algorithms. The pMIR-GFP-3'UTR plasmid without predicted miRNA and pMIR-GFP-3'UTR plasmid and the pEP-miR expression plasmid were transfected into HEK293 cells, and GFP fluorescence was measured two days post-transfection. GFP fluorescence was normalized to non-transfected cell (auto)fluorescence levels and values shown are percent GFP fluorescence relative to pMIR-GFP-3'UTR + pEP-miR-Null. All miRNAs induced statistically significant reductions in GFP fluorescence except those designated with NS (student t-test; $p<0.05$).

3.3.3 Antioxidant gene and associated miRNA expression over continuous hyperoxia exposure

Quantitative RT-PCR was used to determine the expression of antioxidant genes and their predicted miRNA regulators over various durations of hyperoxia exposure (Figure 3.2). Transcript levels of *dme-miR-927* were initially insignificantly variable, but by day 5 of

hyperoxia, the level of this miRNA was significantly reduced, relative to normoxia flies. *Dme-miR-964* hyperoxia accumulation was found to be significantly down-regulated at 1 and 2 days hyperoxia exposure, but was up-regulated by 5 days. The down-regulation of these miRNAs predicted to bind and suppress Hsc70-4's translation corresponds with this protein's necessary expression in a hyperoxic environment. Similarly, *dme-miR-277* was down-regulated for 1 and 2 days of hyperoxia exposure, and then was up-regulated by 5-days, with its predicted target gene, Sniffer, showing consistent and increasing expression during the hyperoxia treatment. Unfortunately, *miR-1012* and *-1013* could not be successfully amplified in either normoxia or hyperoxia treated flies using qRT-PCR, presumably because they in are comparatively lower abundance than the other miRNAs examined here. Hence, it is not possible to show any regulatory relationship of these two miRNAs with their respective antioxidant gene targets (*Cat* and *Trx-2*), even though both of these genes showed up-regulation under oxidative stress. Catalase displayed consisted up-regulation, with peak translation at 1-day hyperoxia exposure, while *Trx-2* was initially down-regulated at day 1, but was up-regulated by days 2 and 5.

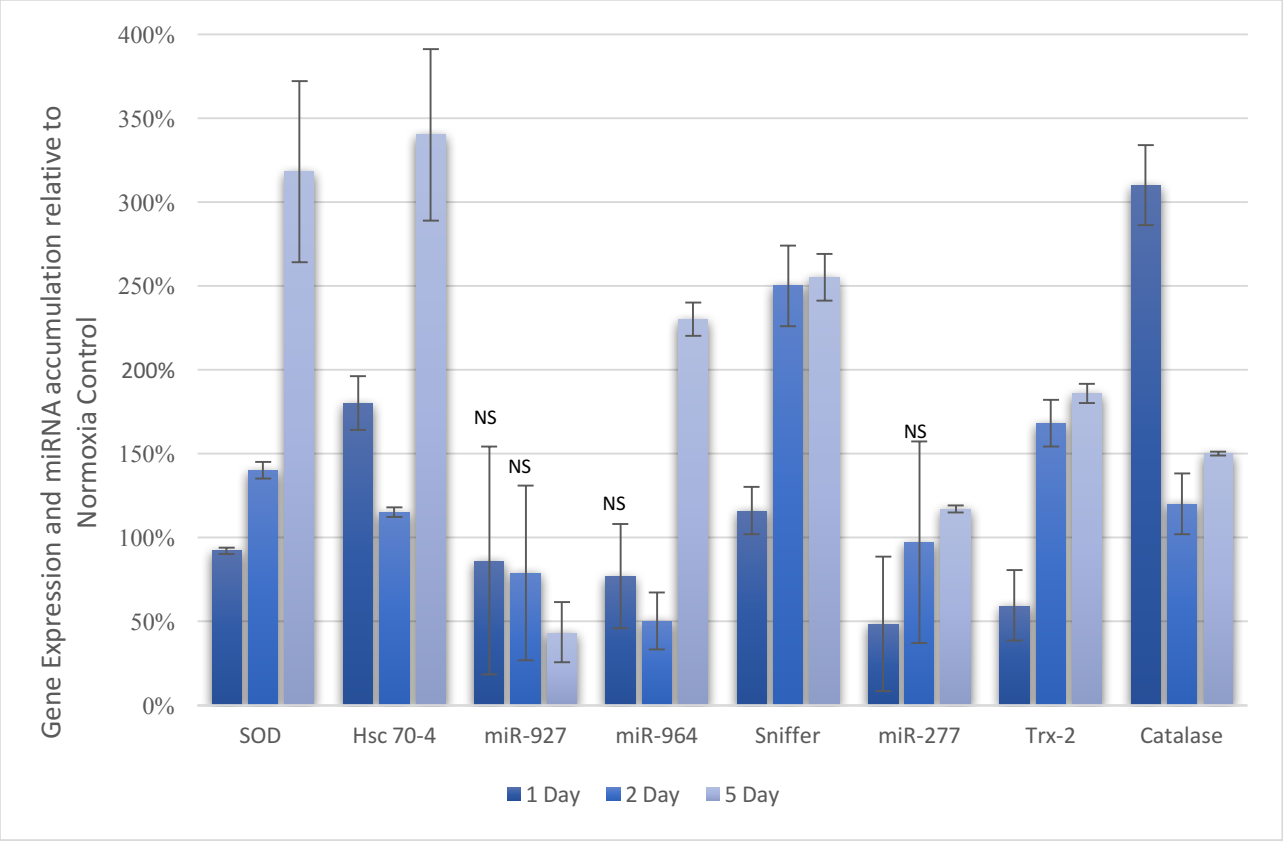

Figure 3.2. Relative expression and accumulation levels of *Sod, Hsc70-4, dme-miR-927, 964, Sniffer, dme-miR277, Trx-2 and Catalase* in *D. melanogaster* treated in both normoxia and hyperoxia growth conditions for 1-day, 2-days, and 5-days and normalized to the normoxia treatments in whole body samples. The values represent means and standard errors for 3 replicate qRT-PCR experiments. All miRNAs induced statistically significant reductions in GFP fluorescence except those designated with NS (student t-test; $p<0.05$).

3.3.4 Antioxidant gene and associated miRNA expression in head and whole body samples after 5 days hyperoxia exposure

Quantitative RT-PCR was used to determine the expression of antioxidant genes and their predicted miRNA regulators in head as compared to whole body samples after 5-days hyperoxia treatment. Down-regulation of *dme-miR-927* was observed in both head and whole body samples of hyperoxia-treated flies, relative to normoxia flies (Figure 3.3). Both *Sod* and *Hsc70-4* were up-regulated approximately 3.5-fold under hyperoxia stress in whole bodies, but only *Hsc70-4* had significant up-regulation in head samples.

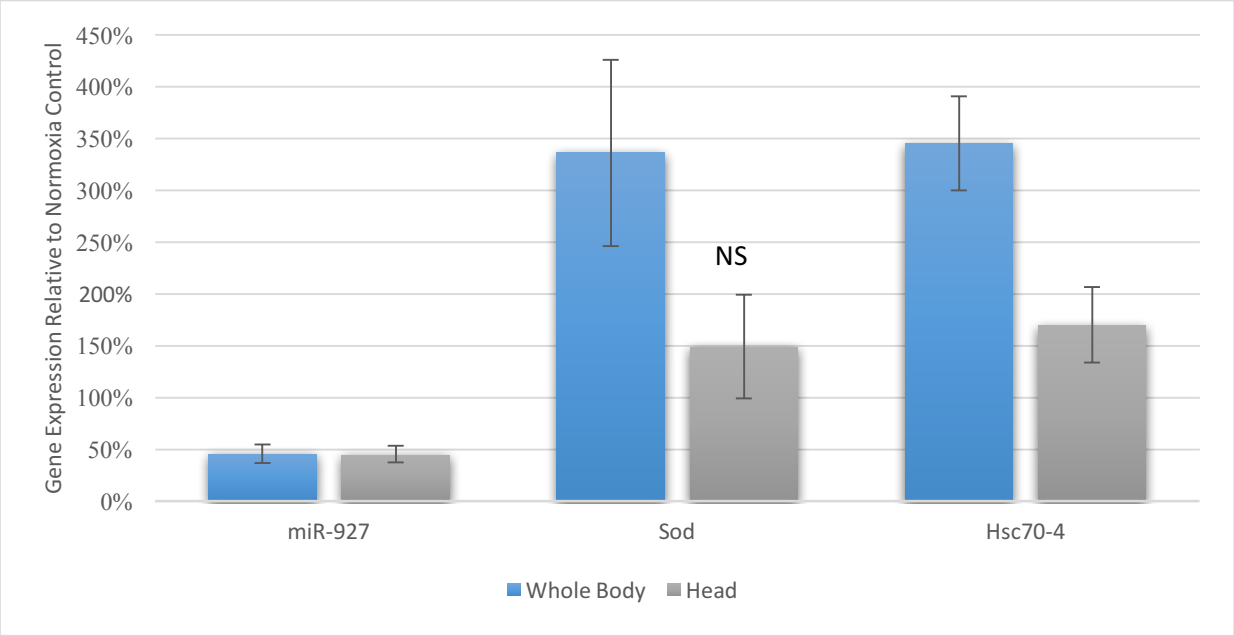

Figure 3.3. Relative expression and accumulation levels of *dme-miR-927, Sod,* and *Hsc70-4* in *D. melanogaster* treated in both normoxia and hyperoxia growth conditions for 5 days normalized to the normoxia treatments in head and whole body samples. The values represent means and standard errors for 3 replicate qRT-PCR experiments. All miRNAs induced statistically significant reductions in GFP fluorescence except those designated with NS (student t-test; $p<0.05$).

3.4 Discussion

3.4.1 Antioxidant genes and their miRNA regulators

The mechanisms underlying the control of antioxidant gene expression during the aging process and during extended periods of oxidative stress have not been fully elucidated. For this reason, I chose to examine the potential role of miRNAs as post-transcriptional regulators of antioxidant gene expression. The first step in this endeavor was to determine which miRNAs regulated 5 key genes involved in repairing and modulating oxidative stress. These genes were: *superoxide dismutase* (*Sod*)*, heat shock cognate 70-4* (*Hsc70-4*), *Catalase* (*Cat*), *Thioredoxin-2* (*Trx-2*), and *Sniffer* (*Sni*). MiRNAs with the highest likelihood of binding to and suppressing these antioxidant genes where chosen.

Interestingly *Sod1* only has one miRNA predicted to bind and target its 3'UTR, *dme-miR-927*, and this pairing had the lowest possible branch length pairing of 0.0 indicating that it contains a conserved pairing site, but with little to no potential of molecularly binding (Ruby *et al.* 2007). Additionally, *Sod2*, mitochondrial superoxide dismutase, currently has no predicted miRNAs. The lack of suppressive miRNAs for these two enzymes may reflect their physiological significance in combating oxidative stress, and an intolerance for down-modulation by miRNAs, especially considering null mutant produces fatal phenotypes (Duttaroy *et al.* 2003). Dme-*miR-927* is also predicted to bind and target *Hsc70-4* in addition to *dme-miR-964*, each with a much higher branch length score of 4.48. *Cat* had several potential miRNA regulators; *dme-miR-971* and *dme-miR-1012* were chosen as *miR-971* was predicted to bind to two different locations on *cat*'s 3'UTR and *miR-1012* had one of the higher predicted branch-length scores of 2.51. *Sni* also had a limited number of predicted miRNAs that might bind its 3'UTR, but *dme-miR-277* and *-978* were selected to study here as they had moderate branch

length scores of 1.43 and 0.13 respectively. *Trx-2* only had 1 conserved miRNA to choose from, which was *dme-miR-1013* and has a branch length score of 3.29.

Overall, microRNAs with higher branch-length scores were more successful in binding to and suppressing their predicted antioxidant 3'UTRs, and in general, any miRNA with a branch-length score >1.0 successfully bound to and suppressed GFP expression, though increased branch binding scores did not directly correlate to greater GFP suppression.

Sod was not suppressed by *dme-miR-927* while *Hsc70-4* was, in addition to being suppressed by *dme-miR-964*. *Dme-miR-927* has previously been shown to be mostly restricted to accumulation in the head while *dme-mir-964* was observed to be expressed in the head as well as other systems (Mohammed *et al.* 2014). Hsc70-4's concentration within the nervous system, combined with the fact that these two miRNAs are similarly expressed in the head of the insect, strongly suggests that they are indeed capable of modulating responses to oxidative stress. The additional finding that *dme-miR-927* was down-regulated in both head and whole body samples during prolonged (5-days) hyperoxia exposure suggests that Hsc70-4's expression could increase (due to the lack of *miR-927* modulation), and thereby provides a protective antioxidant role during oxidative stress induced neurodegeneration processes. The lack of significant *dme-miR-927* down-regulation at 1- and 2-days post-hyperoxia exposure in whole body samples could reflect its greater accumulation in the head or it could reflect its target *Hsc70-4's* more dominant antioxidant role in later stages of oxidative stress induced cellular- and neuro-degeneration. The variability of *dme-miR-964*'s accumulation between 2 and 5 days hyperoxia exposure in whole body samples is somewhat perplexing. Its alternation between decreased to increased accumulation from 2- to 5-days suggests that it may not have a large role in down-regulating its target mRNAs during early hyperoxia, but that is needed in later stages of oxidative stress. If

dme-miR-964 is indeed regulating *Hsc70-4*'s expression, then perhaps this is occurring in the peripheral nervous system during late hyperoxia, as this miRNA is not restricted to just the brain. Alternatively, *dme-miR-964* may be interacting in coordination with other miRNAs, such as *dme-miR-927,* to fine-tune *Hsc70-4*'s expression.

Sod and *Hsc70-4* were both up-regulated in whole bodies under oxidative stress, while only *Hsc70-4* expression was significantly increased in heads during oxidative stress. In another study, overexpression of *Hsc70-4* was able to extend the life span of hyperoxia-treated flies while *Sod* overexpression did not (Gruenewald *et al.* 2009). As *Hsc70-4* is a molecular chaperone involved in maintaining proper protein folding, these findings suggest that protein maintenance rather than ROS scavenging may be somewhat more important in organisms subjected to prolonged oxidative stress. However, Gruenewald *et al.* (2009) also showed that *Sod* was able to preserve the integrity of the dopaminergic system, highlighting the importance of this enzyme in neuronal integrity maintenance. Based on those findings, increased accumulation of *dme-miR-927* may have deleterious effects in the central nervous system of *D. melanogaster* through suppression of these key enzymatic antioxidant proteins involved in the oxidative stress response.

Catalase, even with *miR-971*'s two binding sites within its 3' UTR, was only suppressed by *miR-1012*. *Dme-miR-1012* has been shown to be globally translated in *D. melanogaster*, which further supports a *dme-miR-1012:cat* relationship (Mohammed *et al.* 2014). Unfortunately, *dme-miR-1012* was never successfully amplified using qRT-PCR as it is a small miRNA with only 59 pre-miRNA nucleotides, and hence difficult to amplify. Similarly, *dme-miR-1013* significantly suppressed *Trx-2* and has body-wide accumulation, but its small size rendered it impossible to amplify using qRT-PCR and thereby examine its changes during

oxidative stress. Both *cat* and *Trx-2* were up-regulated under hyperoxic conditions. *Catalase* had the highest expression in earlier oxidative stress conditions, as its up-regulation was likely triggered by early rising and peaking levels of H_2O_2, as evidenced by the peaking lipid peroxidation levels at 2-days post-hyperoxia exposure. *Trx-2,* on the other hand, was initially down-regulated then became up-regulated 2- and 5-days post-hyperoxia exposure indicating it has a greater antioxidant role once cellular degeneration ensues.

Sniffer was suppressed only by *miR-277* in the plasmid-based functional assays. *In vivo*, this miRNA was down-regulated on day 1 of the hyperoxia treatment, and this correlated with an increase in *sniffer* transcripts, but by day 2 to 5 of the hyperoxia treatments, this miRNA was accumulating along with its associated target. This could suggest that *dme-miR-277*'s accumulation is increasing in an effort to suppress or at least fine tune the translation of sniffer transcripts. Both *dme-miR-277* and *sniffer* are expressed almost exclusively in the head of the insects, which also supports a possible interaction between this miRNA and target mRNA (Mohammed *et al.* 2014). Interestingly, another study supported *dme-miR-277*'s role in modulating metabolism and lifespan, a result that is further supported here (Esslinger *et al.* 2013).

Unfortunately, due to time limitations, full head accumulation/expression profiling of all candidate miRNAs and antioxidant genes by qRT-PCR was not undertaken, limiting the interpretation of these genes' and miRNA's interactions in oxidative stress-induced neurodegenerative processes. The real proof of the regulatory roles of these miRNAs however, would involve measurements of the target proteins, to demonstrate that an increase in a particular miRNA leads to a predicted reduction in the predicted protein's levels. Such analyses are beyond

the scope of this study, but nevertheless, my findings provide insights that will help direct future studies in the roles of these miRNAs.

Chapter 4: Conclusions and Future Directions

From the studies described in Chapters 2 and 3, it appears highly likely that miRNAs play a role in the response to oxidative stress, given that their abundances change over the course of the stress period and that they are capable of interacting with and presumably destabilizing transcripts of genes associated with the oxidative stress responses. New miRNAs involved in advanced stages of the oxidative stress response were initially identified with the microarray analysis and then confirmed with qRT-PCR. Unfortunately, the microarray analysis only examined these miRNAs' accumulation variation during the final stages of the hyperoxia-reared flies' life spans. Hence, these analyses failed to identify miRNAs involved in the early phases of oxidative stress, at time points that might be critical to the organism's response to the oxidative stress. The cost of the microarray analyses was surprisingly high (>$5000), and precluded performing any additional microarray analyses of samples derived from earlier time points during the oxidative stress. As high-throughput RNA sequencing continues to improve and its cost continues to drop, it would be worthwhile using this method to examine changes in miRNA accumulation patterns during both early and late oxidative stress response periods. As the initial goal of the project was to examine miRNAs associated with neurodegenerative conditions arising from oxidative stress, it would be especially interesting to compare changes in miRNA abundance in both heads and the rest of the body, to identify those miRNAs exclusively associated with brain adaptation to oxidative stress.

Regardless of these initial limitations, it was still possible to examine whether these candidate miRNAs played an earlier role in oxidative stress response regulation using qRT-PCR. These experiments proved fruitful, and also helped narrow down key regulatory miRNAs, in order to start investigating potential miRNA targets. These functional assay experiments proved

that not all predicted target mRNAs, identified using a variety of publicly-available miRNA:mRNA binding algorithms, may be destabilized by the miRNA, at least using this somewhat artificial cellular assay system. Given the current results, it is worth pursuing alternative predicted miRNA targets for *dme-miR-8*, *-34*, and *-11*, as these three miRNAs had consistent up-regulation at all hyperoxia exposure points, and presumably must be regulating the suppression of many genes. Of particular interest would be to functionally verify the interaction between *dme-miR-11:dE2f1* given their transcriptional as well as correlative relation (Truscott *et al.* 2011). As of now, *castor* and *derailed* are the only functionally proven targets for these miRNAs (*dme-miR-11* and *-970* respectively), but the significance of these miRNA:mRNA target gene interactions are still unclear. Both castor and derailed are important genes in neural development and function, but they are not known to have any roles in dealing with oxidative stress. Perhaps these miRNAs may suppress these genes' expression during stress periods, to minimize any further physiological or developmental defects during periods of cellular repair.

We have much to learn about the number of genes that a single miRNA can regulate, and how that regulation actually impacts the cell's and/or organism's phenotype in both development and during times of cellular stress. While the cell-based assays are informative about the mRNA binding capabilities of miRNA, they do not inform us of the miRNA' *in vivo* functions. The use of a heterologous cell system (HEK) offers some security in that it may lack the mRNAs (3'UTRs) of interest, but it might still have similar miRNAs. In this way, the system is not perfectly clean as endogenous miRNAs may also bind to 3'UTRs of interest and disrupt the assay. Fortunately, the latter can be somewhat mitigated or at least accounted for by running parallel peP-miR-Null as well as pEP-has-miR-941 control vector assays. Additionally, since

more than one miRNA can bind to a single 3'UTR, some assay results may not reflect the actual biology as multiple miRNAs may need to work together to effectively reduce transcript stability.

With the unexpected result of antisense oligomer constructs having the opposite of predicted effect in suppressing *dme-miR-8* and *-34*, it seems an alternative method to knock down miRNAs would be worth pursuing in order to understand the *in vivo* consequences of these miRNAs' absences. An alternative method could be to create transgenic fly stocks with either *dme-miR-8* and/or *-34* sponges rather than just injecting antisense oligomers (Ebert and Sharp 2012). This method has proven to be more advantageous in biological experiments as it eliminates the need to continually inject antisense oligomers in order to reach desired miRNA suppression. Sponges also have the potential to be expressed using a tissue-specific promoter in order to keep the loss of a miRNA to just one tissue (brain), and not cause body-wide deficits. *Dme-miR-11, -927*, *-964*, and *-277* would also be miRNAs worth pursuing to identify potential *in vivo* knock-down targets in future studies.

The use of microRNA-inhibiting oligonucleotides can help define a microRNA's function, but as proven by this study, this method can be unreliable. Additionally, simply injecting or constitutively expressing miRNAs is complicated by the multiplicity of impacts that each microRNA has on any single cell, especially given the fact that most miRNAs display tissue specific accumulation (Mohammed *et al.* 2014). For this reason, an additional experiment worth pursuing to understand the roles of these candidate miRNAs would be *in situ* hybridization. This technique would help to determine in which tissue they are most predominantly accumulating in during oxidative stress (Song and Yan 2010). Once their primary tissue was uncovered, direct tissue suppression of the miRNA would yield the most indicative results of the miRNAs' regulatory function in oxidative stress.

While there are many leads to follow in the future, this study provides some thought-provoking findings that indicate a role for miRNAs in regulating a very important cellular protective mechanism, and demonstrates the utility of a simple insect model to explore complex cellular and genetic aspects of both disease processes and ageing. In conclusion, this study provides new insights into the role of some key miRNAs relevant to oxidative stress regulatory response. Although there is much more to understand about the complexity of miRNAs and their roles in regulating cellular functions, this study also provides some possible new directions in understanding the complex processes involved in oxidative stress.

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