PHENOTYPIC AND BEHAVIORAL EFFECTS OF METHIONINE SULFOXIDE REDUCTASE DEFICIENCY AND OXIDATIVE STRESS IN *DROSOPHILA MELANOGASTER*

by

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. David Binninger, Department of Biological Sciences, and has been approved by the members of her supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Science and was accepted in partial fulfillment of the requirements for the degree of Master of Science.

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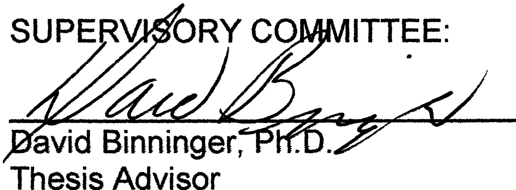

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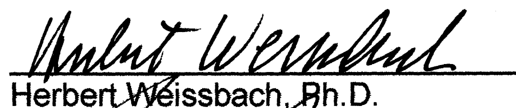

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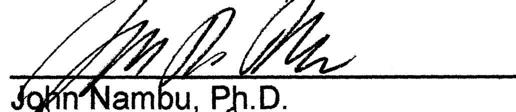

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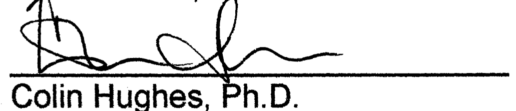

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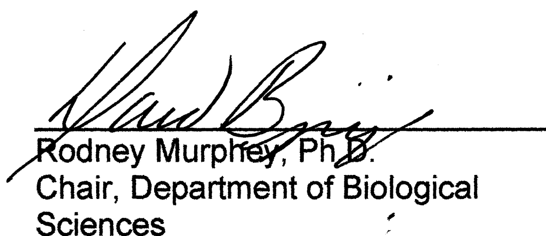

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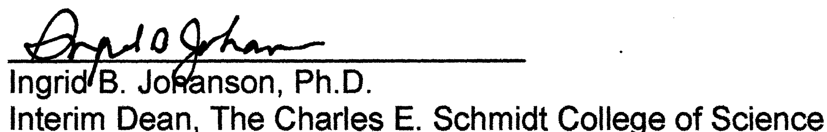

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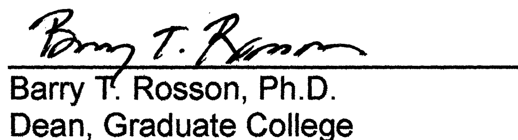

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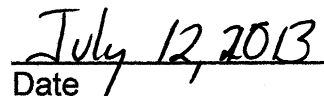

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ABSTRACT

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Harman's theory of aging proposes that a buildup of damaging reactive oxygen species (ROS) is one of the primary causes of the deleterious symptoms attributed to aging. Cellular defenses in the form of antioxidants have evolved to combat ROS and reverse damage; one such group is the methionine sulfoxide reductases (Msr), which function to reduce oxidized methionine. MsrA reduces the S enantiomer of methionine sulfoxide, Met-S-(o), while MsrB reduces the R enantiomer, Met-R-(o). The focus of this study was to investigate how the absence of one or both forms of Msr affects locomotion in *Drosophila* using both traditional genetic mutants and more recently developed RNA interference (RNAi) strains. Results indicate that lack of MsrA does not affect locomotion. However, lack of MsrB drastically reduces rates of locomotion in all age classes.

Furthermore, creation of an RNAi line capable of knocking down both MsrA and MsrB in progeny was completed.

DEDICATION

This work is dedicated to my late grandfather, Kornel Bleser, who, at his core, believed in the endless quest for knowledge and always encouraged me to pursuit my love of biology. I also dedicate this work to my parents, Annette M. Bleser and David E. Mulholland, Jr., for supporting me throughout this entire experience and lending words of encouragement when needed.

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CHAPTER 1: INTRODUCTION

Theories of Aging

Aging is generally described as a cascade of deleterious symptoms that increase in severity as organisms enter senescence. While there are several postulates explaining why individuals age, one of the most prominent theories is the free radical theory of aging, first proposed by Harman in 1956 [1]. The theory proposes that the cause of senescence is imputed to the increase of free radical attacks on cell components, resulting in a decline in metabolism and cellular function as well as an overall decrease in lifespan [1]. The mitochondrial theory of aging is similar, stating that a decline in overall health is attributed to mitochondria experiencing oxidative stress and subsequent damage. Compromised mitochondrial function can lead to a decrease in ATP production and overall diminution of energy and mobility [2].

An unavoidable consequence of aerobic respiration is generation of reactive oxygen species (ROS) by mitochondria. These ROS cause damage, called oxidative stress, and can lead to a loss of function of DNA, RNA, proteins, and lipids [2]. For example, nearly half of all proteins in elderly humans are oxidized [2]. This damage can be attributed, at least in part, to a decrease in efficacy of antioxidant and repair enzymes and an increase in oxidative stress as

humans age [3].

Since August Weismann first proposed that natural selection favors eradicating elderly individuals from populations, evolutionary biologists have developed theories of aging. The two most prominent are the mutation accumulation theory and the antagonistic pleiotrophy theory [4, 5]. The mutation accumulation theory explains how mutations that lead to deleterious effects only late in life, are effectively neutral with respect to natural selection, because so few individuals ever live long enough to suffer the phenotypic consequences. Genetic drift causes some of these mutations to become common in the human population [6]. For example, symptoms of the neurodegenerative disease known as Huntington's may not present until long after the affected individual has reproduced [7]. The eventual consequence of this pattern of inheritance over consecutive generations results in high mortality later on in life [5]. The antagonistic pleiotrophy theory explains how alleles conferring a benefit early in life and a cost late in life are readily favored by natural selection, because most individuals live long enough to receive the benefit, but few live long enough to pay the cost [5]. Alterations in population density can modify survival and fecundity, possibly leading to changes in the strength that natural selection exerts on different life periods [6]. Evolutionary biologists also distinguish between aging, the accumulation of number of days alive, and senescence, the late-life decline in condition and performance. However, here I use the words aging and

senescence interchangeably. Overall, it appears that multiple intrinsic and extrinsic properties bring about the cumulative changes leading to senescence.

Oxidative Stress and Disease

Links between age-related neurodegenerative diseases and oxidative stress have been well documented. Increased levels of both reactive oxygen species (ROS) and reactive nitrogen species (RNS) can contribute to diseases such as Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), and Huntington's disease (HD) [8]. ROS result from normal aerobic respiration occurring in the mitochondria; approximately 1-2% of oxygen utilized in the electron transport chain is converted to ROS. High levels of ROS damage mitochondrial membranes, compromising the efficiency of these organelles. Conditions involving activated microglia lead to increased levels of RNS such as peroxynitrate ($ONOO^-$) [9], as shown in Figure 1. Oxidative stress leads to damage of DNA and cellular organelles, oxidized proteins, decreased mitochondrial function, neuronal cell death, and an increase in apoptosis [10].

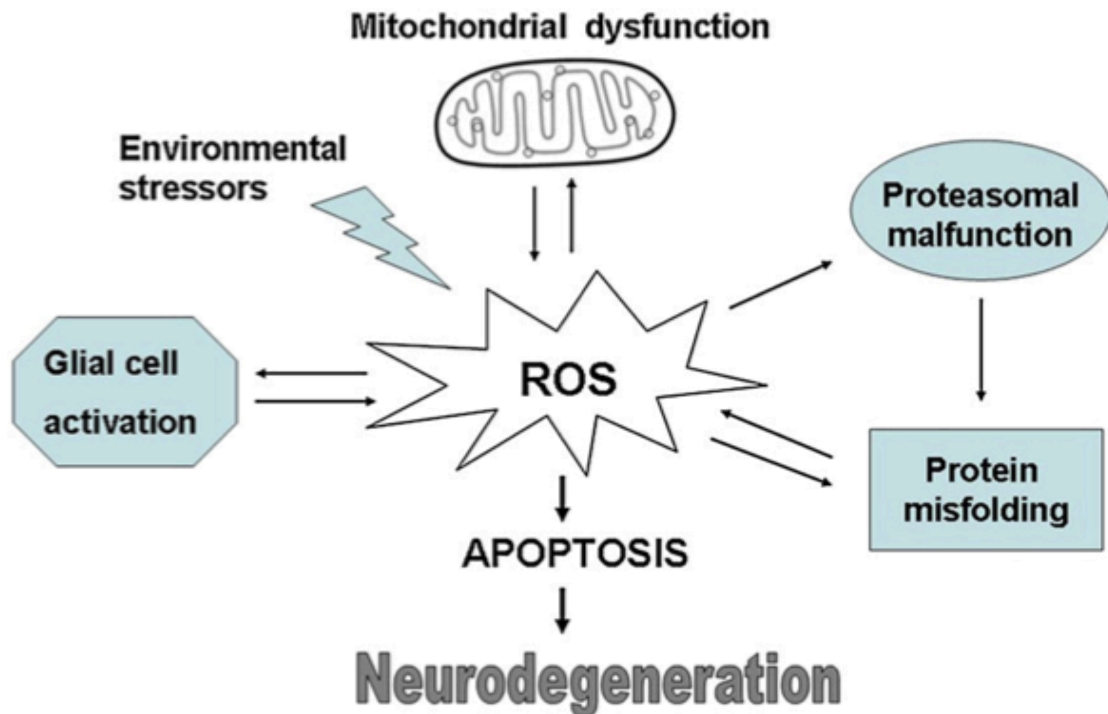

Figure 1. Cellular interactions leading to neurodegeneration. Interactions between cellular processes, such as mitochondrial dysfunction and ROS, lead to neurodegeneration and disease. Photo courtesy Antonio Federico, et. al [9].

Excitotoxicity refers to the process of activating excitatory amino acid receptors and leads to neuronal cell death. Slow excitotoxicity may occur as a result of low levels of ATP and has been implicated as a possible root of neurodegenerative diseases. Additionally, lack of sufficient ATP levels obstructs calcium buffering, giving rise to increased amounts of intracellular calcium. In vitro studies have shown that abnormally high levels of calcium induces free radical formation and nitric oxide synthase initiation [10]. Nitric oxide can be

detrimental to mitochondria for multiple reasons. Mitochondrial enzymes in complex IV and the iron-sulfur cluster containing enzymes found in complexes I and II can be inactivated in the presence of nitric oxide. Furthermore, poly (ADP-ribose) synthetase activity triggered by nitric oxide, leads to decreased intracellular levels of NAD and ATP, thereby blocking energy metabolism [11]. It has been suggested that many of these diseases may result from a deficiency in cellular repair and defense mechanisms [11].

Antioxidant Enzymes

Numerous endogenous antioxidant enzymes exist in virtually all organisms to counteract damage caused by oxidative stress. Enzymes such as superoxide dismutase (SOD), which catalyzes the conversion of superoxide radical to hydrogen peroxide, and catalase (CAT), which catalyzes the conversion of hydrogen peroxide into water, significantly decrease the amount of damage caused by ROS [1]. A study using *Drosophila* indicated that an overexpression of SOD could significantly extend lifespan, reduce relative amounts of oxidized protein, and prevent age related loss of mobility [12]. Furthermore, research has found that transgenic mice expressing human CAT within their mitochondria experienced a significant increase in lifespan of approximately eight months [13].

Methionine Sulfoxide Reductase

Methionine is one of only two amino acids containing sulfur atoms, making it particularly susceptible to oxidation by ROS. Previous studies indicate that oxidized methionine causes a decrease or loss of protein function [14]. However, methionine oxidation is one of the few types of oxidative protein damage that can be reversed. Oxidized methionine – called methionine sulfoxide – can be restored to functional methionine by a class of enzymes known as methionine sulfoxide reductases [15]. Methionine sulfoxide reductases (Msr) are a group of antioxidant enzymes found in a wide range of organisms including *E. coli, Saccharomyces, Drosophila,* and mammals. The two known classes of Msr, MsrA and MsrB, are monomeric enzymes capable of reversing damage of the two stereoisomers of oxidized methionine, Met-S-(o) and Met-R-(o), respectively. Weissbach and colleagues discovered MsrA in 1979 [16]. Later, simultaneous independent research by Weissbach and Grimaud led to the discovery of MsrB in 2001 [17,18]. MsrA specifically reduces the S enantiomer, Met-S-(o), while MsrB reduces the R enantiomer, Met-R-(o) [19]. Msr has three known functions. First, it repairs oxidized methionine residues through a thioredoxin (Trx)-dependent reaction [19, 20]. Second, the activity of some proteins can be modulated by the biochemical state of methionine, namely, whether or not it is oxidized. Third, Msr can function as an antioxidant by serving as an ROS sink. Methionine can be oxidized by ROS, thus avoiding damage to

other key cellular molecules. The resulting methionine sulfoxide is then reduced to reform methionine, which can then interact with other ROS [21].

The effects of mutations in MsrA and overexpression of the enzyme have been well studied. Researchers have discovered a potential relationship between lack of MsrA and Alzheimer's disease as well as smoker's emphysema in humans [17]. Overexpression of MsrA in human T cells increased their resistance to hydrogen peroxide induced oxidative stress. [20]. An initial study in mice found that MsrA knockouts experienced significantly reduced lifespan, reduced resistance to oxidative stress, and a phenotype specific to mice, an abnormal tiptoe walking pattern [22]. However, a subsequent study found only an increased sensitivity to oxidative stress in another MsrA knockout in a mouse [23]. Overexpression of bovine MsrA in the central nervous system of *Drosophila* resulted in increased resistance to free radicals and a longer lifespan [23].

Recent research by Zhang and Weissbach indicates that both MsrA and MsrB are present in nearly all eukaryotes [24]. However, a larger body of research is present for MsrA, which was discovered first. Studies in mammals on MsrB are more complicated than those of MsrA since there are three *MsrB* genes [25]. MsrB1 is targeted to the cytosol and nucleus, MsrB2 is found in the mitochondria, and MsrB3A is located in the mitochondria while MsrB3B is targeted to the mitochondria [26]. Fomenko and coworkers demonstrated that MsrB1 null mice display an overall decline in mobility and a slight decrease in oxidative stress resistance [25]. In another study, researchers found that

overexpression of human MsrB3A in *Drosophila* led to decreased susceptibility to oxidative stress induced by the chemical paraquat [26].

The cycle of methionine oxidation and reduction has been shown to modulate both calmodulin and voltage-gated potassium channels. *Drosophila* Shaker channels are voltage-gated potassium channels that are regulated by a ball and chain protein mechanism. The ball portion of the protein contains a distinct methionine residue that, when oxidized, causes inactivation of the channel. In the presence of MsrA, the methionine is reduced and the channel is reactivated [27, 28]. A similar effect is seen in the manner through which MsrA modulates the calcium binding protein calmodulin (CaM). This protein plays an important role in the cell by regulating enzymes, ion channels, and pumps. Oxidation of the methionine residues in CaM causes inactivation of the protein, resulting in instability of its structure. However, Msr is capable of reducing the oxidized methionine and reactivating CaM [21].

Evidence of a regulatory mechanism between MsrA and MsrB on each other has been seen in the mouse model though a biochemical mechanism for this interaction has not yet been described. Moskovitz and Stadtman have described a potential regulatory mechanism dependent upon specific methionine residues in an MsrA-mediated redox state, thereby transmuting its modification of MsrB expression [29].

Drosophila melanogaster

During the last hundred years, *Drosophila melanogaster* has become a widely used model organism in the study of genetics and molecular biology. Some of the advantages of using fruit flies include the following: reproduction and gestation are fairly short, its genome is fully sequenced, and there is a large amount of peer-reviewed literature widely available [30]. Also, use of the UAS-GAL-4 system and the availability of RNAi lines make fruit flies particularly convenient for gene over-expression studies [31].

There is one *Drosophila* MsrA gene and one MsrB gene that are both located on separate arms of the third chromosome (www.flybase.org). In fruit flies, MsrA was originally identified as *Ecdysone-induced protein 28/29kD (Dmel/Eip71CD)* (www.flybase.org). It is expressed ubiquitously with higher expression levels in the larval fat body and adult head, central nervous system, and midgut. Four known transcripts exist; three are putatively targeted to the cytoplasm while one is destined for the mitochondria, depending upon alternative splicing of exon one. Classified as a selenoprotein, MsrB is known as *SelR (Dmel/SelR)* and has eight known transcripts and corresponding polypeptides. The destination of each transcript also depends on the splicing pattern of exon one; three transcripts are intended for the mitochondria and five are cytoplasmic (www.flybase.org).

RNA interference

RNA interference, or RNAi, is a popular tool in molecular biology that can be utilized for many purposes, particularly for gene expression studies. A recently discovered phenomenon, the basic mechanism of RNAi is as follows. Dicer, a ribonuclease III enzyme, binds to long double stranded RNA and cuts it into shorter fragments known as small interfering RNAs (siRNAs) [32]. The siRNA fragments are complementary to the target mRNA sequence and, upon binding, an RNA-induced silencing complex (RISC) digests the target sequence, thereby preventing translation. The Vienna Drosophila Stock Center (VDRC) is creating RNAi transgenic lines that contain a transgene corresponding to a hairpin dsRNA consisting of 300-400 bp inverted repeats complementary to a target sequence, such as MsrA or MsrB. Flanking the RNAi sequence is an upstream activating sequence, or UAS. When a fly containing a GAL-4 driver is crossed to a fly containing the UAS RNAi sequence, the resulting progeny are not expected to express the target gene product [33]. RNAi can be combined with the use of tissue specific drivers to knock down gene expression in specific tissues while allowing the target gene to be expressed under normal conditions elsewhere in the organism.

Locomotion

In *Drosophila,* the ventral thoracic muscles are the main muscles involved in walking and jumping [34]. Central pattern generators (CPGs) are neural networks found within the central nervous system (CNS) that are capable of

producing rhythmic outputs. Singular CPGs regulate the steady movement of motor neurons involved in flexing and extending the individual leg joints of the fly. To further control the coordinated movements of the six legs, proprioceptive sensory inputs monitor the joints' stance and loads. Additionally, locomotor CPGs are regulated by visual, olfactory, and gravitational sensory modalities, enabling the fly to adjust motor behavior based on environmental cues [35].

Based on Harman's theory of aging, I postulated that mobility of *Drosophila* would decrease with age and that this effect would be more prominent in both the Msr single mutant and double mutant flies, due to a lack of Msr activity. I tested both climbing and walking behaviors using genetic mutants lacking one or both of the Msr genes in the presence or absence of chronic oxidative stress. To further address my hypothesis, I used two RNAi lines, each targeted to either MsrA or MsrB, and crossed them with an actin driver to achieve ubiquitous knockdown of MsrA or MsrB, respectively. Lastly, through a series of genetic crosses, an RNAi line targeting both MsrA and MsrB was created.

CHAPTER 2: MATERIALS & METHODS

Protein Isolation

Protein extracts from the indicated strains were prepared by homogenizing approximately thirty frozen flies with 150 μL of Buffer B, comprised of 50mM HEPES, potassium salt/pH 7.6, 50 mM KCl, 1mM EGTA, 1mM MgCl2, and 10% glycerol. Afterwards, the samples were centrifuged at 16,300 x g for 20 minutes. The Bradford protein quantification protocol was used to determine protein concentrations. All samples were stored at -80°C until further use.

Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Expression levels of MsrA and MsrB proteins in protein extracts obtained via homogenization were determined by SDS-PAGE and Western blotting. After separating the proteins using a 15% acrylamide separating solution, the proteins were transferred onto PDVF membranes. The membranes were incubated overnight in blocking solution consisting of 5% skim milk in 1X TTBS (10X TBS and 0.05% Tween 20). Next, the membranes were rinsed and incubated with the primary antibody for MsrA and MsrB for 1 hour at room temperature. A 1:400 dilution was used for dMsrA while a 1:700 dilution was used for dMsrB. After four subsequent washing steps, membranes were incubated with a secondary

antibody for one hour at room temperature. Lastly, Bio-Rad's Immun-Star chemilumiscent system was used to visualize the signals (www.bio-rad.com).

Lifespan Assay

A lifespan assay was performed on the MsrA and MsrB RNAi parental lines. One hundred male flies from each line were collected and placed on 4ml of standard *Drosophila* cornmeal agar medium, ten flies per vial. Flies were checked every other day and all deaths were recorded. All flies were transferred onto new aging food twice per week.

Climbing Assay

Fruit flies exhibit a natural inclination towards negative geotaxis and tend to climb up the sides of the vials they are contained within. Our lab evaluated mobility by utilizing a basic climbing assay consisting of four 30 cm tall transparent tubes (Figure 2), a modified version of a previously published method [36].

Figure 2. Climbing chamber. This climbing chamber was used to measure climbing ability in *Drosophila*. Ten male flies were placed into each tube without anesthetization and given ten seconds to climb 15 cm. Only flies that reached or exceeded the 15 cm mark were recorded. This procedure was repeated three times before flies were returned to food vials.

Flies from each of the four genotypes were transferred from stock vials to bottles and allowed to reproduce for approximately two weeks. Bottles were thoroughly cleared before the flies were collected at day four. Ten male flies were placed into each vial containing 4 ml of cornmeal agar and allowed 24 hours to recover from CO_2 exposure. Ten male flies were transferred without anesthetization into each tube, given five minutes to recover, and allowed to

climb for ten seconds; the number of flies that reached a height of 15 cm was recorded. The procedure was repeated three times for each set of flies before the data were averaged. Climbing assays were performed on four of the Msr lines described in Table 1, specifically lines WT60, A63, B54, and AB46.

Name of Line	Line Designation	Genotype
WT	WT77, WT60	$MsrA^{+}$ $MsrB^{+}$
MsrA null	A3, A63, A90	$MsrA^{LOF}$ $MsrB^{+}$
MsrB null	B22, B54	$MsrA^{+}$ $MsrB^{LOF}$
MsrAB double-null	AB113, AB46	$MsrA^{LOF}$ $MsrB^{LOF}$

Table 1. Genotypes of the Four Msr Lines. Three independently derived Msr strains, shown in the first column, were crossed to establish the lines shown in the second column. The third column designates the homozygous genotype of each line.

The Msr mutant lines used in this study were created by graduate students Katie Foss and Lindsay Bruce in our lab via imprecise excision of a P element transposon. The MsrA deletion is approximately 1.5Kb while the MsrB deletion is 2.5Kb. A significant segment of the amino acid coding portion of the mRNA is removed as a result of the deletion, thereby rendering these lines complete loss of function (LOF) mutants (Figure 3). RT-PCR and Western Blotting were unable to detect the presence of mRNA or protein in these lines

(data not shown). A complete description of the creation of the lines will be presented elsewhere.

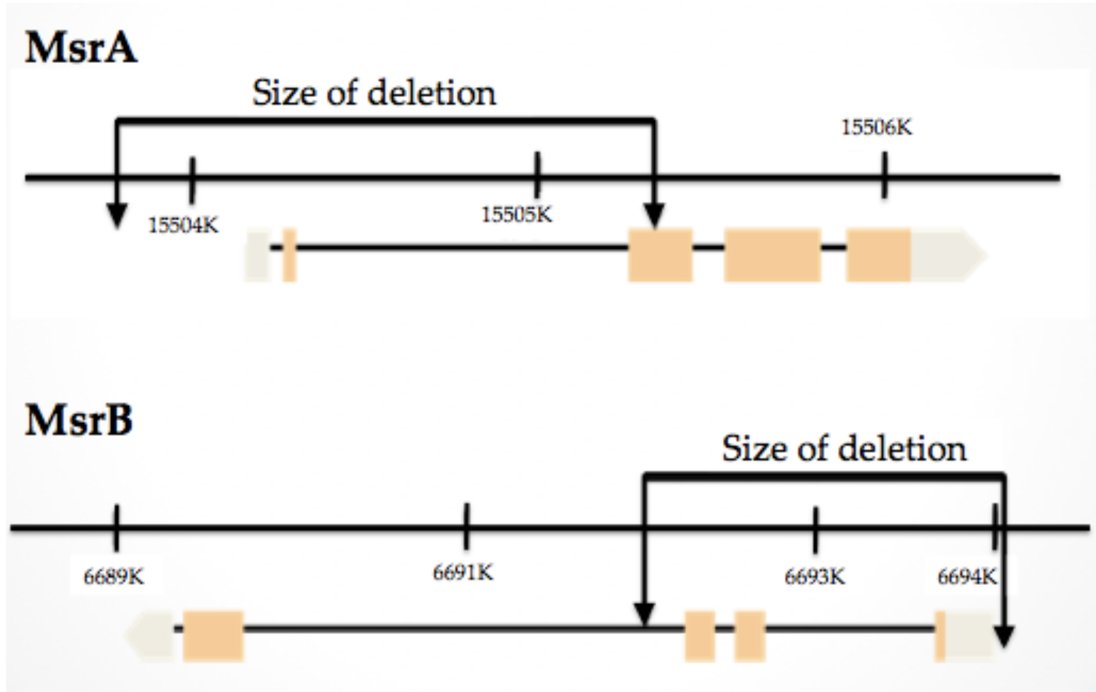

Figure 3. MsrA and MsrB gene deletion lines. The MsrA genomic deletion is between -300 and 1172 bp of the *Eip71C* gene on the 3L arm while the MsrB deletion lies between -364 and 2163 bp of the *SelR* gene on the 3R arm. A substantial portion of the amino acid coding sequence has been lost for all isoforms of both *MsrA* and *MsrB*. When RT-PCR and Western Blotting were used to confirm the deletion lines, no gene products were observed (data not shown). Figure courtesy Lindsay Bruce.

The RNAi lines including parental MsrA RNAi, parental MsrB RNAi, Actin-GAL4 driver, MsrA RNAi/Actin-GAL4 progeny, and MsrB RNAi/Actin-GAL4 progeny were also tested in the climbing assay.

Walking Assay – Four Msr Genotypes

The Drosophila Activity Monitor [Trikinetics – 37], also known as the DAM system, is used to test the mobility of fruit flies (Figure 4).

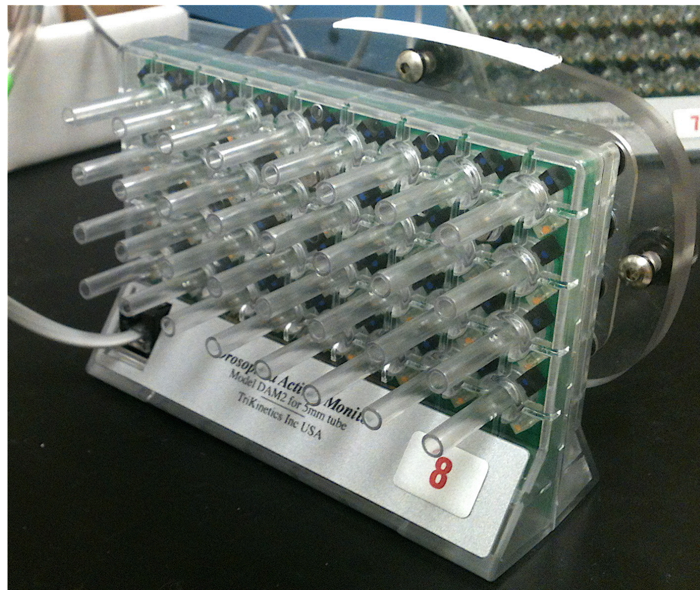

Figure 4. Drosophila Activity Monitor. This monitor was used in the walking assay. One fly is placed inside each tube and an infrared (IR) beam inside the monitor records fly movement for a given amount of time.

The DAM system can test up to 32 flies per experiment. Each fly is placed in an individual tube with a cotton plug at each end. The 32 tubes attach to a holding device, which is placed against the monitor, allowing each tube to fit through each individual hole in the monitor. An infrared beam measures activity in each tube; as each fly breaks the beam, the system records the activity. Flies were collected in the same manner as previously described for the climbing assay. Using a straw-like device, flies were transferred from their vials to the

tubes attached to the DAM system holder, with one fly in each tube. Eight flies of each genotype were loaded into each row of tubes so that one genotype was present in each row, allowing all four genotypes to be tested at once. This pattern of loading was rotated after each trial to ensure that each genotype was placed in a different row during the course of testing. The holders were placed into monitors and covered with a Styrofoam cooler to protect the flies from any light stimulus without affecting the temperature. After one hour, the flies were removed and returned to their respective vials. This was repeated four times over a period of five hours for each age class. All trials occurred during the hours of 12:00 PM and 5:00 PM to avoid changes in behavior due to circadian rhythms. The same individuals were tested during each age class. Twice per week, all flies were transferred onto new food. Flies were stored in the 25°C incubator when not being tested.

Analysis of the data was conducted using Microsoft Excel for Mac and GraphPad Prism [38]. The first ten minutes of each trial were omitted from the analysis to allow for a stabilization period. The average number of times each fly crossed the beam per minute was calculated using Excel. After calculating the standard deviation for each genotype, any individual fly average above or below three standard deviations was omitted from further calculations. The average number of times each fly crossed the beam per minute was designated as the activity level.

Walking Assay – Oxidative Stress

Standard *Drosophila* cornmeal agar was prepared with paraquat at a concentration of 2 mM. WT, MsrA null, MsrB null, and MsrAB double-null flies, specifically lines WT60, A90, B54, and AB46, were collected at day 4 and immediately placed on paraquat food. Flies were tested on the DAM system after allowing 24 hours to recover from exposure to CO_2. Testing occurred in the same manner as the flies that were not under stress with one exception. In order to prevent possible ingestion of paraquat aerosols, flies were tapped into a small funnel connected to the hose used to load the flies into the DAM system tubes. Upon completion of trials, flies were removed from the holder and returned to their respective vials. All flies were stored in the 25°C incubator when not under testing. Twice per week, all flies were flipped onto fresh food containing 2mM paraquat. Flies were tested until day 30 as all double mutant flies and the majority of single mutants flies had died by day 32. Following completion of the assay, flies were frozen for future protein extractions.

Walking Assay – RNAi Knockdowns

As previously stated, RNAi lines were obtained from the VRDC and tested for efficiency (see Figures 5 and 6). The RNAi and driver lines were backcrossed to a lab wild-type strain (YW) to isogenize the lines to help insure that any phenotypes are solely due to a lack of Msr. Upon completion of the backcrosses, the final line for the MsrB RNAi line was not reproductively viable and did not survive. The original MsrB RNAi line from the VRDC was tested on the DAM

system and utilized in the MsrB RNAi/Actin-GAL4 cross to create genetic knockdown progeny. The backcrossed MsrA RNAi line was crossed to the backcrossed Actin-GAL4 driver.

MsrA and MsrB loss-of-function (LOF) deletion mutants and parental lines were tested using the DAM system at the same ages as the four Msr genotypes. As before, the genotypes were rotated so that each genotype was present during each trial and placed in different rows of the holder during each trial. Data analysis was conducted using Excel and Prism, as described for the walking assay using the four Msr lines.

Statistical Analysis

As previously described, statistical analysis was conducted using GraphPad Prism Software [38]. Two Way ANOVAs were performed to determine the impact of each independent variable, namely age and genotype, on the dependent variable, mobility. Tukey's post hoc Honestly Significant Difference (HSD) test was performed to distinguish significant differences between the mean amounts of activity of each genotype [38].

RNAi Crosses

In an attempt to achieve a single fly line capable of using RNAi to knock down both MsrA and MsrB when paired with a tissue specific driver, crosses were performed to combine the MsrA RNAi line and the MsrB RNAi line (see Table 2).

	Parent 1	Parent 2	Progeny
Cross 1	RNAi – MsrA / cyo ♂ or ♀	RNAi MsrB ♂ or ♀	RNAi – MsrA / RNAi – MsrB
Cross 2	RNAi – MsrA / RNAi – MsrB ♀	Cyo / Sp ♂	RNAi – MsrA/RNAi – MsrB / Cyo
Cross 3	RNAi – MsrA/RNAi – MsrB / Cyo Single fly ♂ or ♀	Cyo / Sp ♂ or ♀	RNAi – MsrA/RNAi – MsrB / Cyo ♂ or ♀
Cross 4	RNAi – MsrA/RNAi – MsrB / Cyo Brother ♂	RNAi – MsrA/RNAi – MsrB / Cyo Sister ♀	RNAi – MsrA/RNAi – MsrB / Cyo (Final stock)

Table 2. RNAi Genetic Crosses. The purpose of these crosses was to achieve a fly line containing genes for both MsrA RNAi and MsrB RNAi. The final line was capable of knocking down expression of both MsrA and MsrB when paired with a tissue specific driver (see Figures 15 and 16).

CHAPTER 3: RESULTS

Western Blots – RNAi Lines

RNAi lines targeting both MsrA and MsrB were obtained from the VRDC [33]. UAS RNAi MsrA #26008, and UAS RNAi MsrB lines were each crossed with the Actin driver #4414 containing the GAL-4 promoter. These combinations are expected to result in the ubiquitous knockdown of all isoforms of MsrA or MsrB in progeny flies. Since these are the first experiments in our lab using the RNAi lines, it was necessary to confirm that these lines would indeed prevent translation of their respective target transcripts. Both of the RNAi lines were crossed with an Actin driver to induce synthesis of the dsRNA directed at either MsrA or MsrB mRNA. Protein extracts were prepared from progeny of these crosses along with the parental lines. Extracts of MsrA and MsrB loss-of-function (LOF) deletion mutants were included as negative controls. Protein concentrations were determined using the Bradford protein quantification protocol. Expression levels of MsrA and MsrB proteins were determined by Western blotting. Expression of MsrA was not detected in the RNAi knockdown line (see Figure 5).

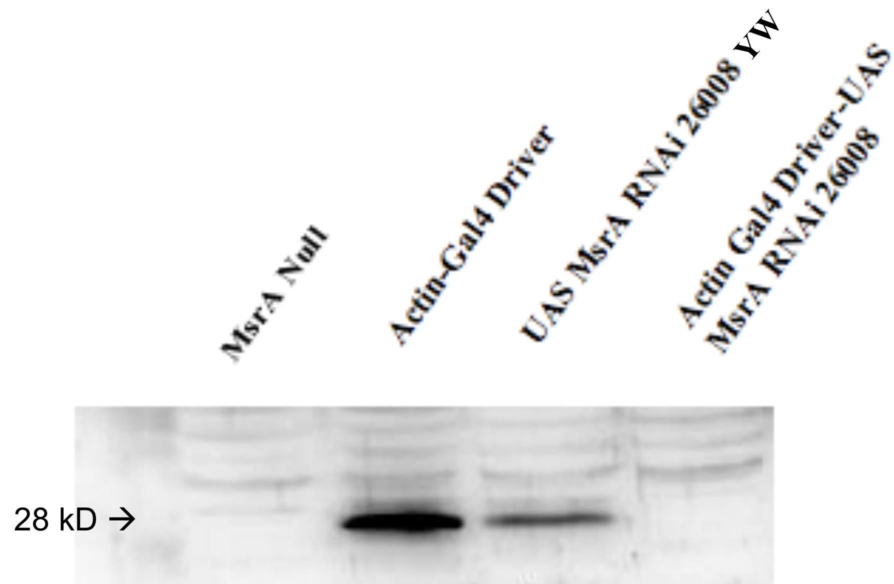

Figure 5. MsrA RNAi Western blot. Western Blotting was performed to determine expression levels of MsrA in MsrA RNAi knockdown flies. The membrane shows the presence of MsrA protein (28 kD in size), probed with MsrA antibody (1:400). Alpha tubulin was used to provide a loading control and indicated the presence of identical levels of protein in each sample (data not shown).

Expression of MsrB was not detected in the MsrB RNAi knockdown line (see Figure 6).

Figure 6**.** MsrB RNAi Western blot. Western Blotting was performed to determine expression levels of MsrB in MsrB RNAi knockdown flies. The membrane shows the presence of MsrB protein (17 kD in size), probed with MsrB antibody (1:700). Alpha tubulin was used to provide a loading control and indicated the presence of identical levels of protein in each sample (data not shown).

Climbing Assay – Four Msr Lines

The first climbing assay was performed using the following four Msr lines as described in Table 1, specifically the WT77, A3, B22, and AB113 lines. At the time of testing, these lines had not yet been backcrossed to the YW background. Thirty flies per genotype were tested at each age class. Results are shown in Figure 7.

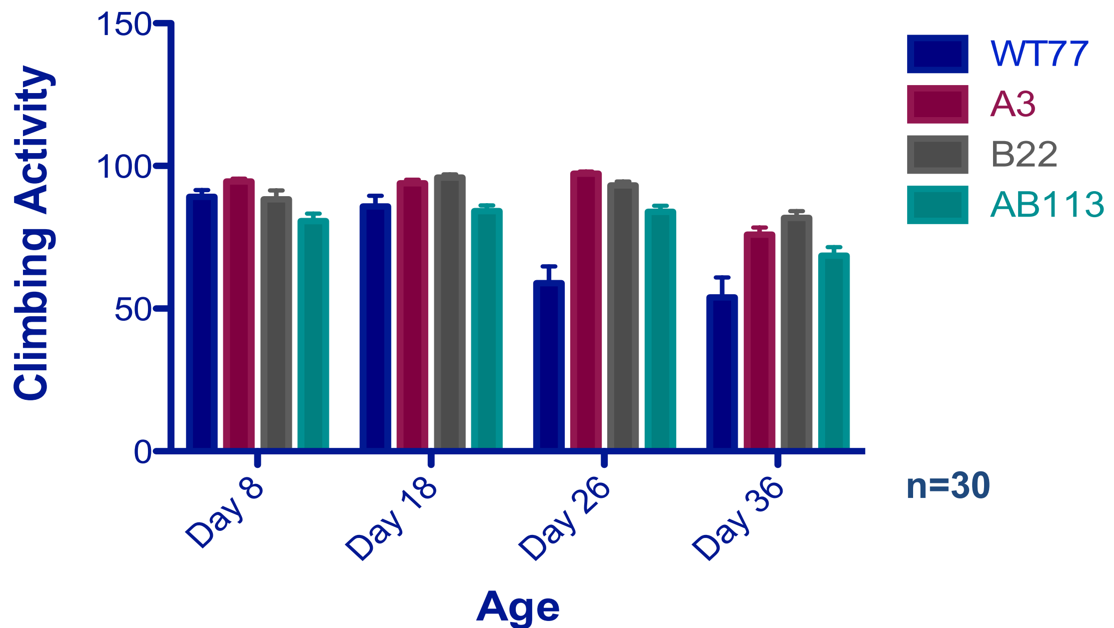

Figure 7. Climbing Assay Results using Four Msr Lines. The climbing assay measures the number of male flies capable of climbing 15 cm up the side of a chamber in ten seconds. The procedure was repeated three times for each set of flies before the data were averaged. Statistical testing was not performed on this data as replication of the results proved difficult.

Using this assay, there was no clear pattern to indicate any differences between genotypes or age. Statistical testing was not performed on this data as no clear pattern could be discerned. Visual inspection of the graphical results of the assay gives the impression that wild type flies possess a slower rate of climbing than the other three genotypes; however, differing results were observed upon replication of this assay. Several other students in our lab repeated this assay but results remained inconsistent.

RNAi Lifespan

A lifespan assay was performed on the RNAi parental lines and Actin driver line to assess overall health of these three lines; the survival curve can be seen in Figure A1 in the Appendix. The Actin driver line exhibited a median life span of ~33 days and a maximum life span of 63 days. The MsrA RNAi line displayed the longest maximum life span of the three lines, living to ~72 days with a median lifespan of ~46 days. The median lifespan of the MsrB RNAi line was ~40 days while the maximum lifespan was ~71 days. While the Actin driver and MsrB RNAi lines exhibited a gradual decrease in the number of living flies over time, the MsrA RNAi flies displayed a steep decline after day 35. These three lines were backcrossed to the YW background to isogenize the lines and improve their overall health.

Climbing Assay – RNAi Lines

The RNAi lines were each paired with the Actin driver to generate single mutant knockdown progeny. The climbing ability of the RNAi lines, the Actin driver line, and the single mutant RNAi knockdowns were tested in the climbing chamber, results are depicted in Figure 9.

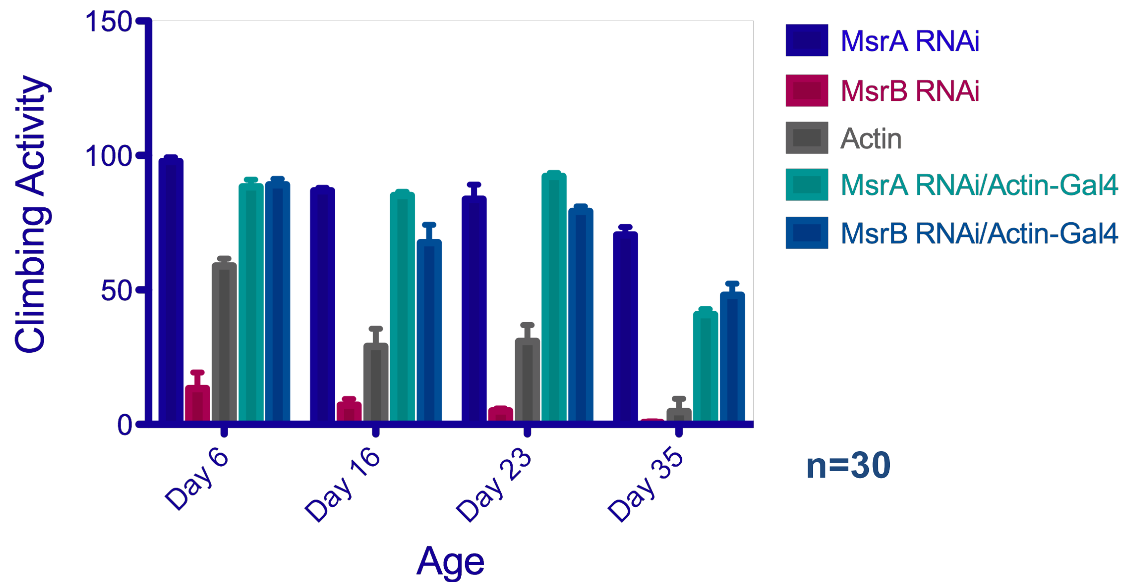

Figure 8. Climbing Assay Results using RNAi Lines. The climbing assay was repeated using the RNAi lines in the manner previously described in Materials and Methods. Each line was crossed with an Actin driver to create an MsrA knockdown flies and an MsrB knockdown flies. Climbing ability of both the parental and progeny lines was assessed.

The MsrA RNAi line climbed notably better than the MsrB RNAi line and the Actin Driver line through all age classes. Based on visual observation, the MsrB RNAi flies remained on the bottom of the climbing chamber and walked around. Very few of the flies actually climbed up the side of the tube and crossed the line; what little climbing ability the flies possessed decreased over time. The Actin driver flies climbed at a lower rate than the two knockdown lines and the

MsrA RNAi line but were at least double the rate of the MsrB RNAi line. The single mutant knockdown lines climbed equally well at day 6 and day 35 but the MsrA RNAi/Actin-GAL4 flies appeared to exhibit an increased rate of climbing during middle age as compared to the MsrB RNAi/Actin-GAL4 flies. Knocking down either MsrA or MsrB did not appear to have an observable effect on climbing ability.

Statistical testing was performed on the available data. Data between 0.01 and 0.05 was considered significant. Values between 0.001 and 0.01 were considered highly significant while any values of < 0.001 were extremely significant. Tukey’s post hoc HSD test was performed on the RNAi climbing data to compare each group to one another and determine if significant differences exist (results are shown in Table 3 in the Appendix). There was no significant difference between the two RNAi single knockdown lines at any age. *The MsrA knockdown progeny were significantly different from both parental lines only at day 35 while the MsrB knockdown progeny were highly significantly different from both parental lines at every age class.* A two-way ANOVA was performed using the RNAi climbing data (see Table 5 in the Appendix). All resulting P values were < 0.0001, indicating highly significant effects on mobility due to both age and genotype.

Climbing assay results indicate that climbing ability may decrease with age. Furthermore, with the exception of day 35, knocking down MsrA did not

impact climbing ability while it appears that MsrB knockdown progeny climb better than their respective parental lines.

Walking Assay – Four Msr Lines

When the results of the climbing assay were inconsistent, I decided to perform a walking assay utilizing the DAM system, a new piece of equipment our lab had recently obtained. The walking assay was performed using the four Msr lines (see Table 1). It was decided that the A90 line would replace the A63 after a lifespan assay performed by Lindsay Bruce in our lab indicated that the A63 might exhibit a reduced lifespan, thus impacting its overall health and potential mobility. Flies under testing were kept in the dark and the pattern of genotype loading in the DAM system holder was rotated at each trial, ensuring that each row of tubes contained a different genotype. Flies were tested twice during each age class to determine age specific effects on mobility. Mobility of the four Msr lines (Table 1) was tested, specifically WT60, A63, B54, and AB46, once for one hour during each of the following age classes: 4-9 days, 10-20 days, 21-29 days, 30-35 days, and 36-40 days. Overall results are depicted in Figure 9 while detailed depictions of age specific effects from the same experiment are represented in Figures 10 and 11. Again, the average number of times each fly crossed the IR beam per minute was designated as the activity level.

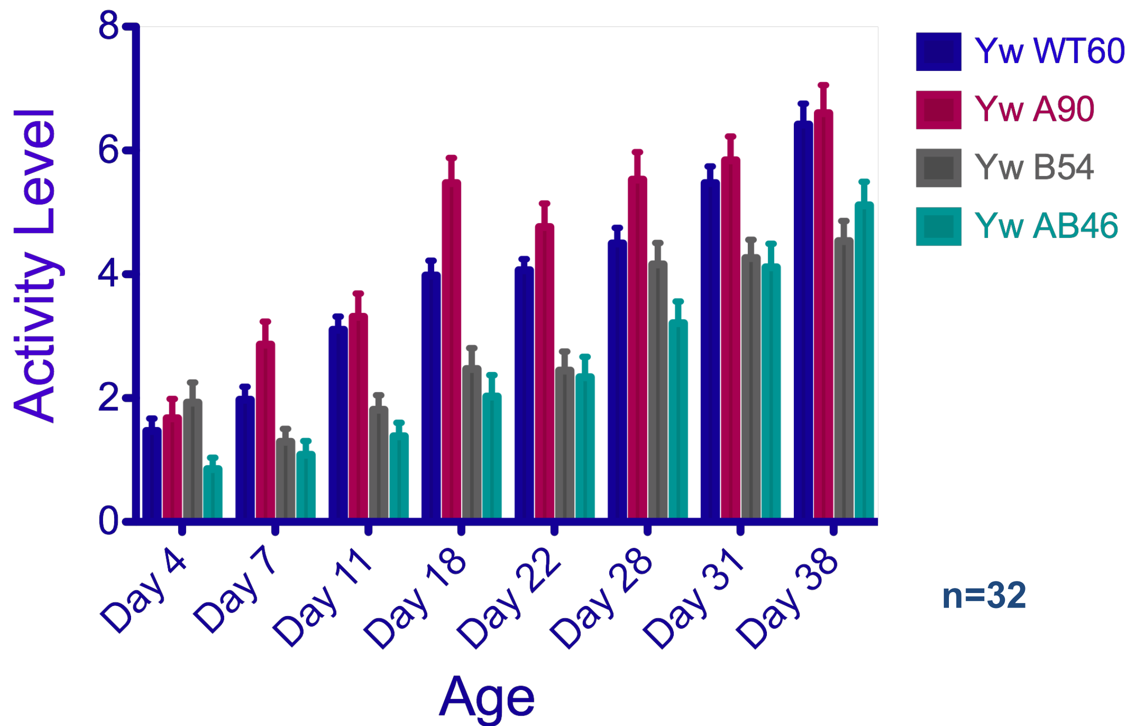

Figure 9. Walking Assay Results using Four Msr Lines. The DAM system walking assay was performed using the four Msr genotypes. One fly was loaded into each of the thirty-two tubes of the holder. Each row of tubes contained a different genotype; the pattern of genotype placement was rotated following each trial. All flies were tested in the dark to reduce potential impact of light changes on mobility. Mobility was tested for one hour. The first ten minutes of each trial were omitted from all data analysis. Activity levels, defined as the average number of times each fly crossed the IR beam within the DAM system monitor, were calculated, omitting any values above or below three standard deviations from the mean.

There was a clear overall trend of increasing mobility as the flies aged; every genotype experienced their highest activity level at day 38. The wild type and MsrA null lines were very similar throughout most age classes but the MsrA null flies exhibited the highest rate of activity overall. The MsrB null and MsrAB null lines displayed very similar activity throughout their lives and moved significantly less than the wild type and MsrA null flies. Figure 10 depicts activity levels of the four lines from days 4 through 18.

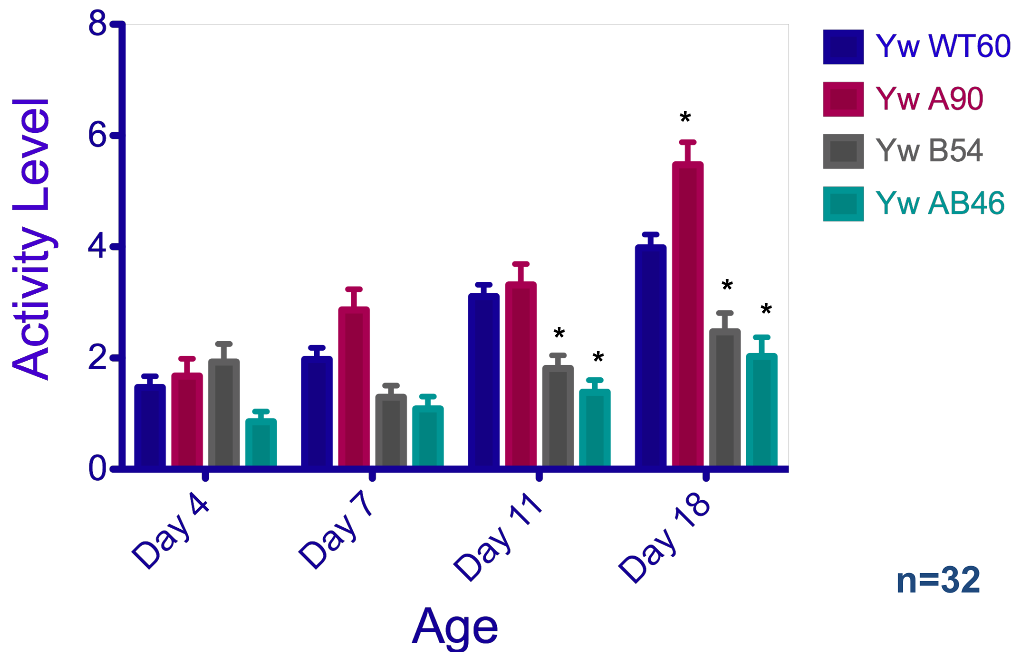

Figure 10. Walking Assay Results using Four Msr Lines –Young Flies. The DAM system walking assay was performed using the four Msr lines as previously described in Materials and Methods. A two-Way ANOVA with Tukey's post hoc HSD test was performed to assess the significance of each independent variable

as well as any significant differences between data sets (see Tables 5 and 6 in the Appendix. An asterisk indicates that activity level of the data set is significantly different than wild type.

When mobility of very young flies was tested, all four lines were very similar until day 7 when the activity of the wild type and MsrA null flies increased. At days 7 and 11, activity levels of MsrB null and MsrAB null flies were approximately fifty per cent lower than the activity of the wild type and MsrA null flies. Movement continued to increase with age. At day 18, activity of wild type and MsrA null flies increased significantly from day 11 while activity of the MsrB null and MsrAB null flies only increased slightly. The MsrA null flies displayed the highest activity level followed by the wild type flies. Again, activity levels of the MsrB null and MsrAB null flies were very similar and the lowest of the four lines. Figure 11 depicts mobility of middle to old aged flies.

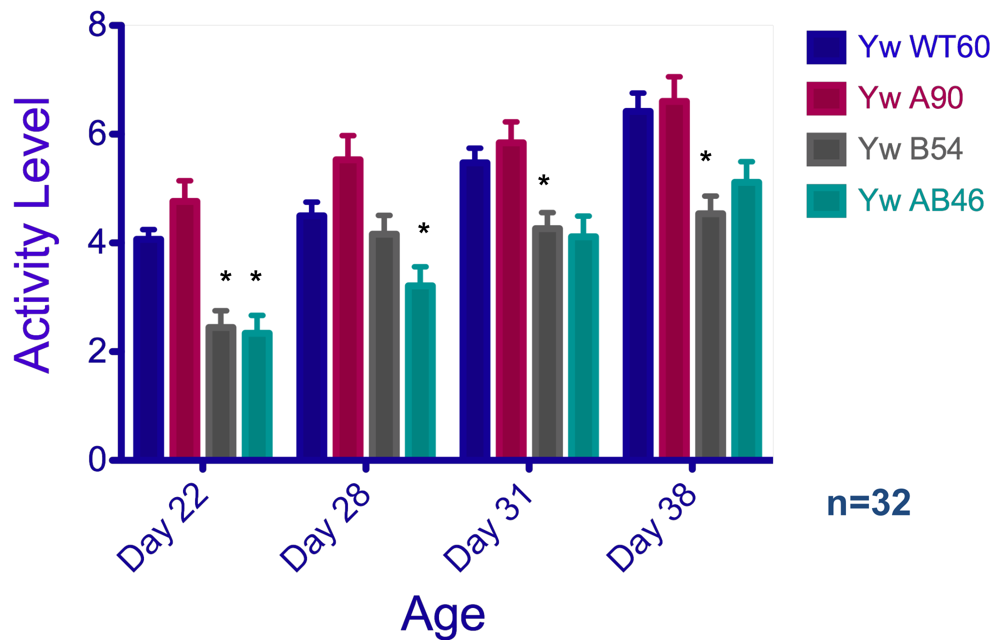

Figure 11. Walking Assay Results using Four Msr Lines – Old Flies. The DAM system walking assay was performed using the four Msr lines as previously described in Materials and Methods. A two-way ANOVA with Tukey's post hoc HSD test was performed to assess the significance of each independent variable as well as any significant differences between data sets (see Tables 5 and 6 in the Appendix). An asterisk indicates that a data set is significantly different than wild type.

Again, activity of MsrA null flies met or exceeded activity levels of wild type flies. Mobility of wild type flies was slightly below the MsrA null flies from middle age to senescence. The MsrB null flies moved significantly less than the wild type and MsrA null flies with the exception of day 28 when activity of the wild type and MsrB null flies was similar. In middle to old aged flies, activity of the MsrAB

double null flies was just slightly lower than the MsrB null flies with the exception of day 38 when the activity of the MsrAB double null flies was slightly higher than the MsrB single mutants.

Tukey's post hoc HSD test was performed to determine the relationships between each genotype at every age class; results are shown in Table 5 of the Appendix. Results indicate that the wild type and MsrA null flies were very similar at all age classes with the exception of day 18. Mobility of wild type and MsrB null flies was found to be statistically different in middle age and at day 38. The relationship between wild type and MsrAB null flies was statistically different only during middle age. With the exception of day 4, the two Msr single mutant lines were statistically different from each other in all age classes. A two-way ANOVA was performed on the data obtained from the second walking assay using the four Msr genotypes; results can be seen in Table 6 of the Appendix. P values for both the column and row factors were extremely significant at < 0.0001. The P values indicate that genotype and age exert an effect on the mobility of *Drosophila* when tested using the DAM system.

Overall, it appears that there is no statistical difference in mobility between wild type flies and those lacking MsrA regardless of age. On the contrary, flies lacking MsrB – whether MsrA was present or not – experienced a phenotype of severely lowered activity at all ages. All genotypes experienced an increase in locomotion from youth through day 38.

Walking Assay – Effects of Oxidative Stress

To determine the effects of chronic oxidative stress on mobility, the chemical paraquat was used. Redox cycling of paraquat results in formation of oxygen free radicals [39]. The four Msr lines were tested on the DAM system after being placed on food containing 2mM paraquat from day 4 until death. Thirty-two flies were tested at days 7, 15, 26, and 30. Results can be seen in Figure 12.

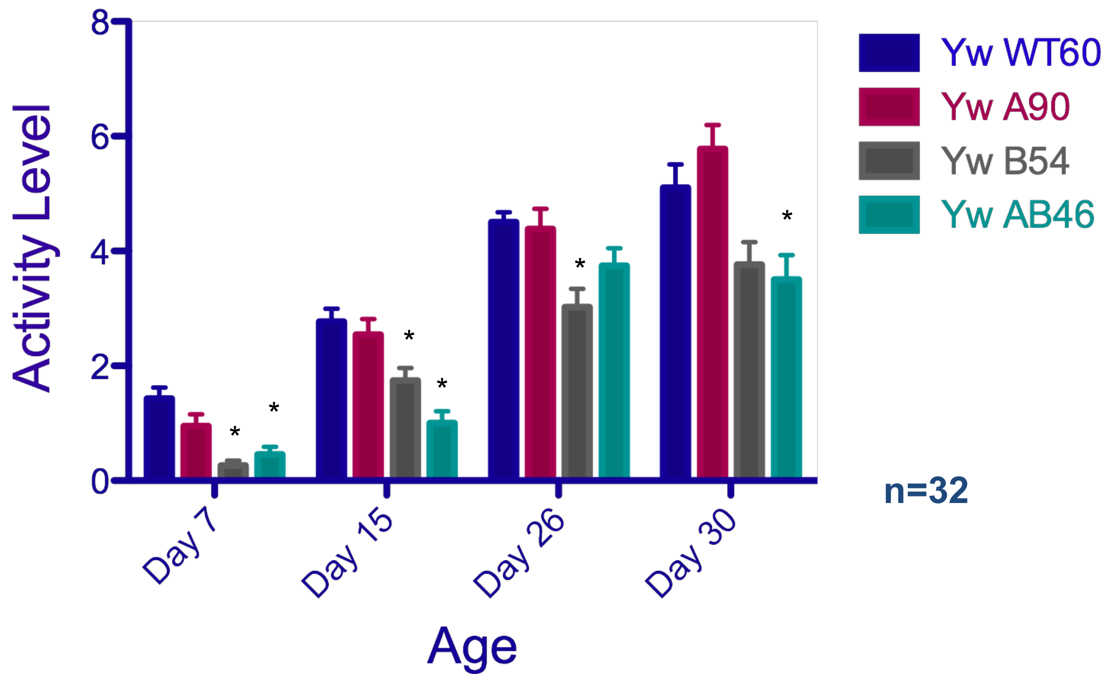

Figure 12. Walking Assay Results using the Four Msr Lines on 2mM Paraquat. The DAM system walking assay was repeated using the four Msr genotypes; all flies were raised on 2mM paraquat food to replicate chronic oxidative stress conditions. Flies were tested in the same manner previously outlined for flies not

under stress. A two-way ANOVA with Tukey's post hoc HSD test was performed to assess the significance of each independent variable as well as any significant differences between data sets (see Tables 7 and 8 in the Appendix). An asterisk indicates that a data set is significantly lower than wild type.

Flies raised on food containing 2mM paraquat exhibited a very similar mobility pattern as compared to flies raised on normal aging food. Activity levels of young and middle-aged flies raised on paraquat were much lower than activity of flies not under stress. Wild type and MsrA null flies displayed similar activity levels at all ages. *The two fly lines lacking MsrB (MsrB LOF and MsrAB LOF) were very similar at every age class and consistently lower than wild type and MsrA null flies.* On the last day of testing, day 30, activity levels were highest for wild type and MsrA null flies; activity of MsrB null and MsrAB double null flies was similar between days 26 and 30 and significantly lower than wild type and MsrA null flies. Flies were unable to be tested beyond day 30 as the majority of individuals were dead. Tukey's post hoc HSD test was performed on the data obtained from the walking assay using the four Msr genotypes that had been raised on 2mM paraquat food; results can be seen in Table 7 of the Appendix. *Results of the test indicate that there was no significant difference between the wild type and MsrA null lines at any age. Activity levels of wild type flies and MsrB null flies were significantly different from youth through middle age. Mobility of the MsrAB double null flies differed from wild type flies in all age classes with*

the exception of day 26. A two-way ANOVA was performed on the data; results can be seen in Table 14 of appendix B. Extremely significant P values of <0.0001 were found for the column and row factors, representing genotype and age, respectively.

Overall, it appears that the presence of paraquat impacts mobility of young flies but does not reduce activity of old flies. All flies experienced an increase in activity with age, as was observed in all other walking assays using the four Msr lines. Furthermore, lifespans of the four Msr lines raised on food containing 2mM paraquat were significantly reduced (unpublished data from our lab) even though mobility was unaffected in senescence.

The Effects of Msr RNAi-mediated Knockdown on Walking

Mobility of the RNAi parental and single Msr gene knockdown lines was tested using the DAM system. Thirty-two flies were tested at days 5, 16, 25, 33, and 40. Results of the MsrA knock down line and respective parental lines are depicted in Figure 13 while the results of the MsrB knock down line and respective parental lines are depicted in figure 14.

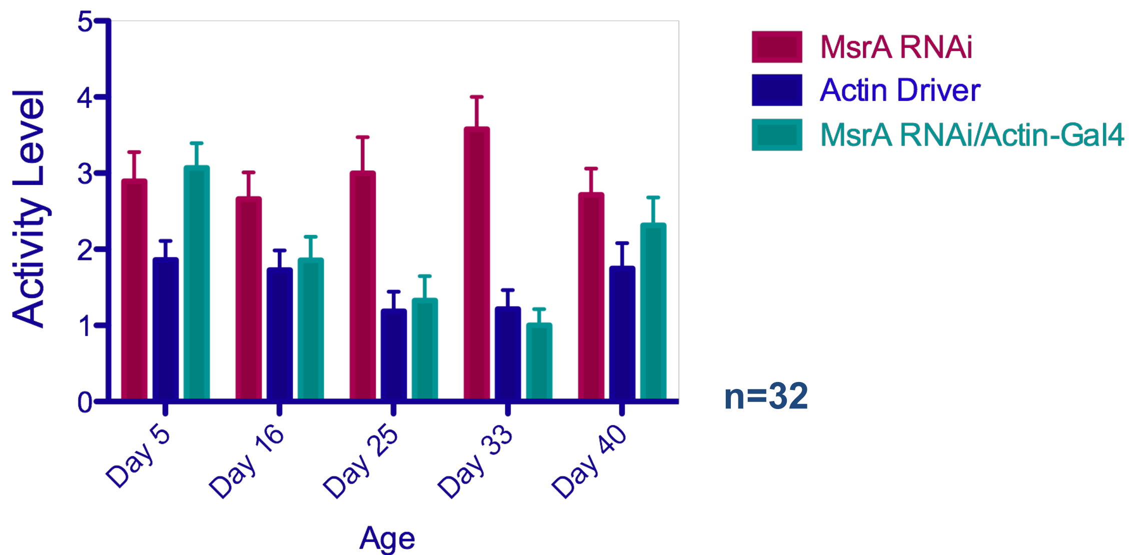

Figure 13. Walking Assay Results using MsrA RNAi Line. The walking assay was repeated in the same manner as previously described in Materials and Methods. The MsrA RNAi line was crossed with the Actin driver to produce an MsrA knock down flies. Mobility of both parental lines along with the MsrA knockdown progeny was tested. A two-Way ANOVA with Tukey's post hoc HSD test was performed to assess the significance of each independent variable as well as any significant differences between data sets (see Tables 9 and 10 in the Appendix).

Results indicate that the MsrA RNAi knockdown line exhibited activity levels equal to or exceeding the Actin driver line at all ages, indicating that absence of MsrA did not reduce mobility. Figure 14 depicts a graphical representation of the walking assay results of the MsrB RNAi knockdown line and respective parental lines.

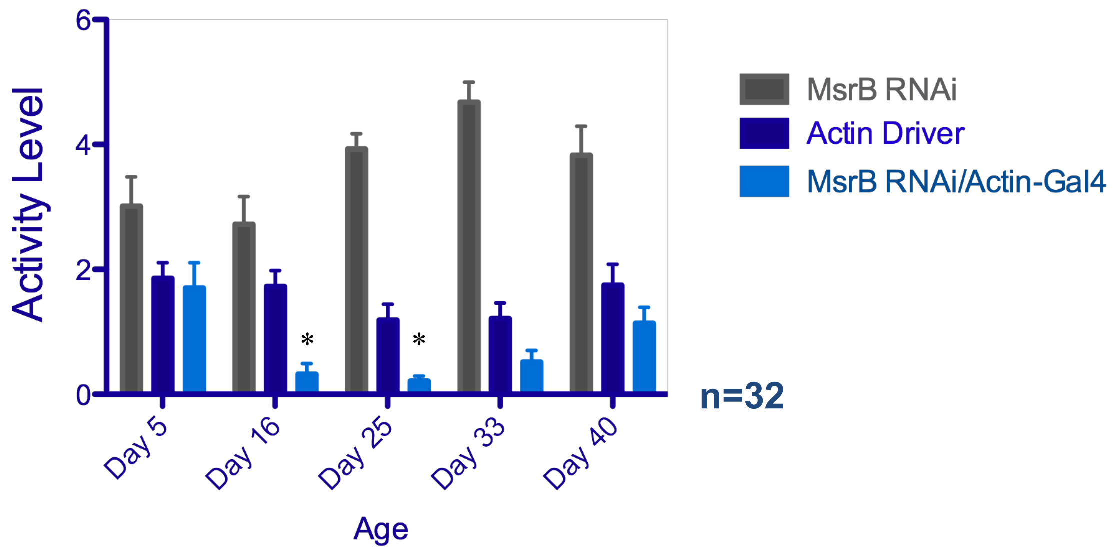

Figure 14. Walking Assay Results using MsrB RNAi Line. The walking assay was repeated in the same manner as previously stated in Materials and Methods. The MsrB RNAi line was crossed with the Actin driver to produce an MsrB null line. Mobility of both parental lines along with the MsrB knockdown progeny was tested. A two-Way ANOVA with Tukey's post hoc HSD test was performed to assess the significance of each independent variable as well as any significant differences between data sets (see Tables 9 and 10 in the Appendix).

Results indicate that the MsrB knockdown line moved significantly less than both parental lines with the exception of very young and very old flies. Furthermore, the MsrB knockdown line displayed the lowest activity levels of the five genotypes tested.

As with the other assays, Tukey's post hoc HSD test was performed on the data obtained from the walking assay using the RNAi lines (results shown in Table 9 of the Appendix). Results indicate that the MsrA RNAi knockdown flies did not differ significantly from both the MsrA RNAi parental line and the Actin driver in any of the age classes. Activity levels of the MsrB RNAi knockdown line were statistically different from the MsrB RNAi line in middle and old age; the line significantly differed from the Actin driver during middle age. A two-way ANOVA was performed on the walking assay data that tested the RNAi lines; results can be seen in Table 10 of the Appendix. Both column and row factors were statistically significant, indicating that both age and genotype affected mobility in this assay.

Overall results of this assay indicate that flies lacking MsrB experience severely decreased mobility as the MsrB RNAi knockdown line moved significantly less than both of its wild type parental lines.

Double RNAi Knockdown Line

For future experiments, it would be very useful to knock down expression of both MsrA and MsrB within the same animal. The crosses used to obtain a line that could potentially knockdown all Msr activity when crossed to a GAL4-driver line was described previously (see Table 2). To test the efficiency of this line, virgin female MsrA RNAi/MsrB RNAi flies were collected and crossed with male Actin-GAL4 flies. Cell extracts were prepared from the F1 generation of virgin males and females. Following quantification and separation, the protein

expression patterns were visualized via Western blots. Figure 15 depicts gene expression of MsrA in the double RNAi knockdown progeny when compared to the MsrA deletion line and the two parental lines. There is no detectable amount of MsrA in the MsrA RNAi-MsrB RNAi/Actin-GAL4 line.

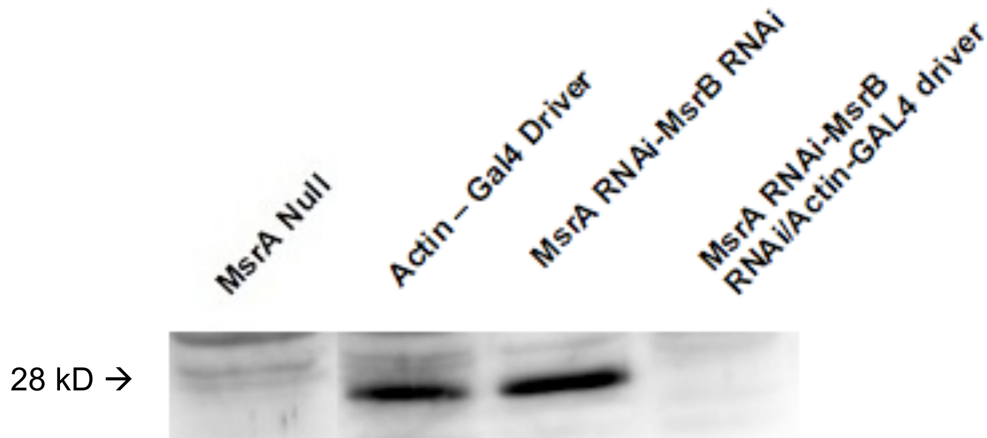

Figure 15. MsrA RNAi Double Knockout Western blot. Western blotting was performed to test the efficiency of the MsrA RNAi – MsrB RNAi line. The membrane shows the presence of MsrA protein (28 kD in size), probed with MsrA antibody (1:400). Alpha tubulin was used to provide a loading control and indicated the presence of identical levels of protein in each sample (data not shown).

To ensure that the MsrA RNAi-MsrB RNAi/Actin-GAL4 line was capable of reducing expression of MsrB, protein extracts from the progeny of the cross between the double knockdown line and Actin-GAL4 driver were separated and visualized using Western blotting. Figure 16 depicts expression of MsrB in the

two parental lines, the MsrB deletion line, and the double RNAi knockdown progeny. Expression of MsrB is severely reduced compared to the parental lines.

Figure 16. MsrB RNAi Double Knockout Western blot. Western blotting was performed to test the efficiency of the MsrA RNAi – MsrB RNAi line. The membrane shows the presence of MsrB protein (17 kD in size), probed with MsrA antibody (1:700). Alpha tubulin was used to provide a loading control and indicated the presence of identical levels of protein in each sample (data not shown).

CHAPTER 4: DISCUSSION

Using both traditional genetics and RNAi knock down experiments, the data clearly show that the absence of MsrA has no significant and consistent effect on mobility. In sharp contrast, the absence of MsrB – regardless of the presence or absence of MsrA – does significantly reduce mobility, especially as the animals age. Furthermore, there is a marked increase in mobility in the four Msr lines from youth through day 40.

Protein Regulation

The first possible explanation for the unexpected mobility results was that ATP levels were being reduced due to ROS-induced damage to mitochondria. However, it that were occurring, we would expect similar mobility results in both of the single mutant lines; this was not seen in any of the walking assays. The role of Msr in regulating proteins has been documented in both prokaryotes and eukaryotes [14, 41, 42]. In *Drosophila*, specific protein regulation due to Msr activity has been studied in both Calmodulin and voltage-gated potassium channels [21, 27]. Calmodulin, or CaM, is a calcium binding protein that modulates growth, movement, and apoptosis and is present in all eukaryotic cells [42]. Research has indicated that the loss of calcium regulation may be linked to an increase in neurodegenerative diseases. Also, mammalian research

investigating the effects of oxidation on the methionines present in CaM revealed that oxidatively modified CaM inhibits the plasma membrane (PM) Ca-ATPase that is normally activated by native CaM. Furthermore, the PM-Ca-ATPase is the main high-affinity, high-capacity transport system in cells and, by oxidatively modifying the methionines in CaM, the likelihood of changing intracellular calcium levels is highly increased [21]. Changes in intracellular calcium levels can result in neuronal excitation and muscle contraction, allowing for movement to occur [43]. If MsrB plays any role in regulation of CaM through the aforementioned system, mobility of fruit flies could be severely impeded, as was observed in my walking assays. This theory could be tested by isolating CaM from the single mutant flies and comparing the difference in the amount of oxidatively modified methionines in the MsrA mutants versus the MsrB mutants. Also, as previously mentioned, the cyclic oxidation and reduction of Msr reactions can modulate potassium channels. When specific methionines at the N-terminus are oxidized, normal N-type inactivation of the channels does not occur, thereby controlling the activity of these channels [27]. By preventing inactivation of potassium channels, nerve signaling is severely impacted and can significantly reduce or impede movement. Research has suggested that MsrA activity can control the potassium channel inactivation time course [28]; however, because so little is known about the specific roles of endogenous *Drosophila* MsrB, it is possible that it could also affect potassium channel function.

Climbing Assays

Our lab has utilized the climbing assay as the traditional manner for studying *Drosophila* mobility. However, varying results were obtained each time the assay was repeated. One major issue was that many flies walked around the bottom of the tubes without climbing or climbed halfway to the 15 cm mark then stopped, thereby not being included in the final count even though they had made significant progress up the tube. It was decided that this assay was not the most suitable for studying mobility; hence, the DAM system was utilized. A major difference being that the DAM system tests locomotion on a horizontal plane rather than a vertical plane. The DAM system collects more statistically valid data since each experiment can test 32 individual flies (n=32) over much longer periods of time.

Walking Assays Under Normal Conditions

We expected to see a decrease in mobility as flies aged due to a putative accumulation of ROS-induced cellular damage, particularly in flies raised on paraquat. Furthermore, we expected wild type flies to exhibit the highest mobility of the four Msr lines, no statistically significant difference between the single mutant flies, and the lowest mobility in the double mutants. Though our initial hypothesis regarding mobility of wild type and double mutant flies was observed in walking assays, overall, activity levels of the MsrA null flies were virtually the same as the wild type flies while MsrB null flies were most similar to the MsrAB double null flies. Flies lacking MsrB appeared to move significantly less than flies

actively producing the MsrB enzyme. This is one of the first dMsr single mutant phenotypes observed in our lab. These results were found to be true in the four Msr lines when mobility was tested in both the light and the dark in unstressed flies, in RNAi knockdown lines tested in the dark, and in flies experiencing chronic levels of oxidative stress. In the RNAi flies, the MsrB knockdown progeny moved substantially less than the parental lines and the MsrA knockdown line. Previous studies have found that climbing activity decreased with age [44], however, the observation that walking activity increased with age was consistent across the walking assay experiments using the four Msr lines. Walking on a horizontal plane exerts less energy than climbing up a vertical plane and could be a partial explanation for our results. However, personal correspondence with Dr. William Ja of the Scripps Research Institute indicated that this pattern of age-related activity increase could cease if testing were to continue beyond day 40 [45]. Future experiments will attempt to test the wild type and single mutant flies beyond this age to determine if mobility continues to increase; the MsrAB null flies will not be tested as their lifespan is limited to ~40 days. Furthermore, the age at which mobility of wild type flies resembles mobility of MsrAB double null flies at day 40 will be examined.

Oxidative Stress and Mobility

Considering the role of Msr in correcting oxidative protein damage, it was believed that chronic levels of oxidative stress would impact mobility due to reduced antioxidant activity. Results of the walking assay using flies raised on

paraquat indicate that chronic oxidative stress weakens young flies through early and middle age but does not impact older flies as activity levels were virtually the same in old flies raised on regular food and old flies raised on paraquat. Flies lacking MsrB activity experienced severely lower rates of mobility as compared to the other two genotypes. As previously mentioned, our lab has performed lifespan assays using the four Msr lines raised on 2mM paraquat food and found that, although mobility is not highly affected by low levels of paraquat, the flies do not live as long as unstressed flies, particularly the MsrB null flies and double MsrAB null flies (unpublished data). It is possible that the young flies have not had adequate time to produce sufficient levels of antioxidant enzymes and may compensate for lack of Msr by up-regulating other antioxidant systems as they age, thus, resulting in standard activity levels later in life. As previously stated, neurons present a highly susceptible target for free radical damage, resulting in neurodegenerative diseases such as Alzheimer's [46]. Since dMsr has not been extensively studied, the possibility remains that MsrB specifically acts as a buffer for oxidative stress in motor neurons due to the results obtained from this study indicating that lack of MsrB is highly detrimental to mobility while loss of MsrA is not. One way to study this theory would be to cross the RNAi lines with a motor neuron specific driver. Resulting progeny would then be tested on the DAM system to observe mobility and motor function.

Mical and Actin

An enzyme known as mical, a multidomain, cytosolic protein present in axons and involved in axon guidance [47], acts as a methionine oxidase and selectively converts Met-44 to MetO44 in actin [48]. The alteration of this specific methionine to methionine sulfoxide leads mical to disassemble individual and bundled actin filaments, also known as F-actin [49]. Furthermore, it is believed that, due to its redox activity, mical acts as a regulator of the actin cytoskeleton. Depolymerization of F-actin could undoubtedly impact and likely decrease mobility as actin filaments are involved in contracting muscle cells. However, the ability of MsrB to reduce methionine sulfoxide may reverse oxidation of the Met-44 methionine in actin, thus preventing both depolymerization of F-actin and a putative reduction in mobility. A proposed future experiment would study the amount of depolymerized actin present in wild type, MsrA null, MsrB null, and MsrAB null flies to determine if Msr indeed affects mical activity and subsequent depolymerization of actin. Results could be compared to our mobility studies in pursuit of a molecular mechanism of dMsr independent of its role in oxidative stress response.

Msr and Lenses

The roles of mammalian MsrB1, MsrB2, and MsrB3 are poorly understood. One study by Drs. Kantarow and Weissbach and their colleagues found that the three enzymes are expressed in bovine eye lenses and, when the genes were silenced, the rate of cell death due to oxidative stress increased [50].

Composed of ~750 individual eye units known as ommatidia, morphology of the *Drosophila* compound eye is distinctly different from vertebrates. However, a lens is present in each ommatidium [51], allowing the potential for oxidative-stress induced lens damage throughout the compound eye. If *Drosophila* MsrB plays any type of similar role in fruit flies, it is possible that the flies lacking MsrB may undergo some degree of vision loss, possibly contributing to their lower rates of mobility compared to the other genotypes. This could be tested by determining the amount of oxidized protein in lenses of MsrB mutant flies and comparing the results to the amount of oxidized protein in wild type fly lenses.

Summary

The main conclusions from this study are that the lack of MsrA does not reduce mobility in *Drosophila* while flies devoid of MsrB activity display a phenotype of significantly decreased mobility. Chronic levels of oxidative stress result in diminished mobility levels of young flies but do not affect movement in older flies, even though lifespan is severely reduced. One result of this research, the creation of an RNAi fly line capable of knocking down expression of both MsrA and MsrB in progeny, will allow for the study of effects resulting from tissue specific loss of both MsrA and MsrB. Future research will attempt to determine if the amount of depolymerized actin in flies lacking MsrB differs from that of wild type and MsrA null flies. Next, we plan to cross our *Drosophila* transgenic and RNAi lines with tissue specific drivers, allowing expression to be highly reduced in tissues such as muscles, motor neurons, or the central nervous system;

mobility of the resulting progeny will be tested on the DAM system. Considering the results from this research, another interesting study could be to observe any differences in coordination, gait, and speed between the four Msr lines. Also, research is underway to design a method of assaying potential differences in flight due to genotype. Furthermore, our lab will continue research in pursuit of an underlying molecular mechanism involving Msr effects on *Drosophila*.

APPENDIX

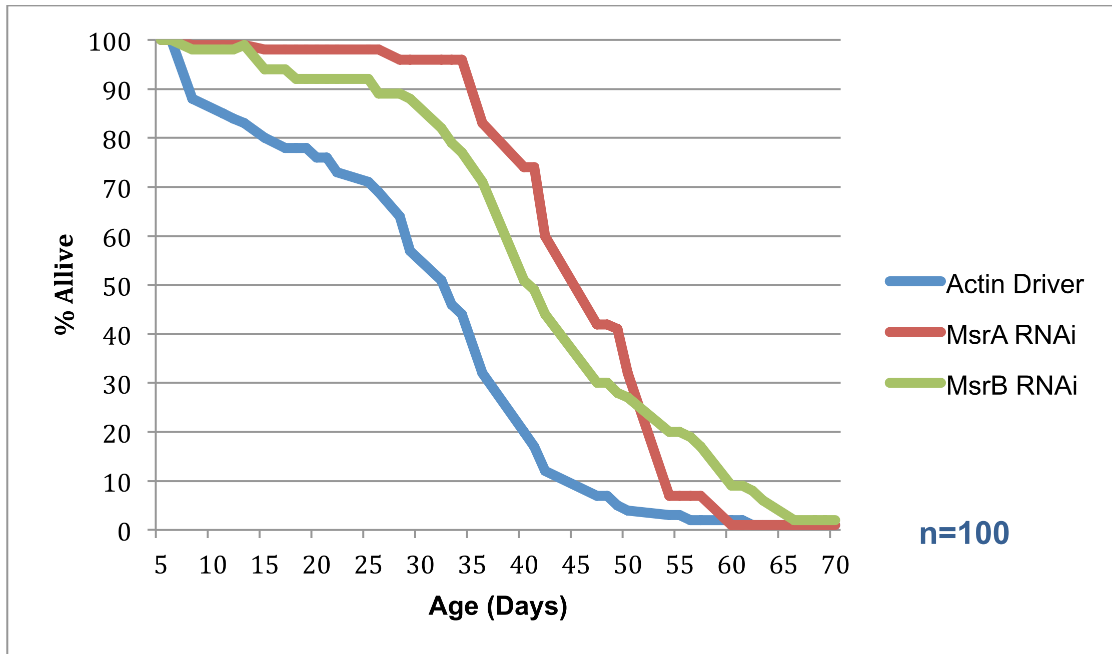

Figure A1. Survival Curve of RNAi Parental Lines. A lifespan assay was performed to assess the overall health of the two RNAi lines and the Actin driver. Male flies were collected and observed until death; each death was recorded until all flies were dead. Twice weekly, flies were transferred onto fresh food.

Tukey's Post Hoc HSD Test of Climbing Assay **RNAi Lines**				
Day 6				
Comparison	Mean Diff.	q	Significant? P < 0.05?	Summary
MsrA RNAi 26008 vs MsrB RNAi	84.44	21.45	Yes	***
MsrA RNAi 26008 vs Actin Driver	38.89	9.018	Yes	***
MsrA RNAi 26008 vs MsrA-RNAi/Actin-GAL4	9.445	2.19	No	ns
MsrB RNAi vs Actin Driver	-45.55	11.57	Yes	***
MsrB RNAi vs MsrB-RNAi/Actin-GAL4	-75.77	21.52	Yes	***
Actin Driver vs MsrA-RNAi/Actin-GAL4	-29.45	6.828	Yes	***
Actin Driver vs MsrB-RNAi/Actin-GAL4	-30.22	7.676	Yes	***
MsrA-RNAi/Actin-GAL4 vs MsrB-RNAi/Actin-GAL4	-0.7758	0.1971	No	ns
Day 16				
Comparison	Mean Diff.	q	Significant? P < 0.05?	Summary
MsrA RNAi 26008 vs MsrB RNAi	79.76	16.99	Yes	***
MsrA RNAi 26008 vs Actin Driver	57.85	11.25	Yes	***
MsrA RNAi 26008 vs MsrA-RNAi/Actin-GAL4	1.8	0.349	No	ns
MsrB RNAi vs Actin Driver	-21.91	4.66	Yes	*
MsrB RNAi vs MsrB-RNAi/Actin-GAL4	-60.5	14.4	Yes	***
Actin Driver vs MsrA-RNAi/Actin-GAL4	-56.05	10.9	Yes	***
Actin Driver vs MsrB-RNAi/Actin-GAL4	-38.59	8.218	Yes	***
MsrA-RNAi/Actin-GAL4 vs MsrB-RNAi/Actin-GAL4	17.46	3.719	No	ns
Day 23				
Comparison	Mean Diff.	q	Significant? P < 0.05?	Summary
MsrA RNAi vs MsrB RNAi	78.74	24.42	Yes	***
MsrA RNAi vs Actin Driver	52.84	14.96	Yes	***
MsrA RNAi vs MsrA-RNAi/Actin-GAL4	-8.47	2.398	No	ns
MsrB RNAi vs Actin Driver	-25.9	8.032	Yes	***
MsrB RNAi vs MsrB-RNAi/Actin-GAL4	-74.21	25.73	Yes	***
Actin Driver vs MsrA-RNAi/Actin-GAL4	-61.31	17.36	Yes	***
Actin Driver vs MsrB-RNAi/Actin-GAL4	-48.31	14.99	Yes	***
MsrA-RNAi/Actin-GAL4 vs MsrB-RNAi/Actin-GAL4	13	4.033	No	ns
Day 35				
Comparison	Mean Diff.	q	Significant? P < 0.05?	Summary

MsrA RNAi 26008 vs MsrB RNAi	69.72	22.19	Yes	***
MsrA RNAi 26008 vs Actin Driver	65.68	17.66	Yes	***
MsrA RNAi 26008 vs MsrA-RNAi/Actin-GAL4	29.67	8.617	Yes	***
MsrB RNAi vs Actin Driver	-4.04	1.174	No	ns
MsrB RNAi vs MsrB-RNAi/Actin-GAL4	-47.3	16.83	Yes	***
Actin Driver vs MsrA-RNAi/Actin-GAL4	-36.01	9.685	Yes	***
Actin Driver vs MsrB-RNAi/Actin-GAL4	-43.26	12.57	Yes	***
MsrA-RNAi/Actin-GAL4 vs MsrB-RNAi/Actin-GAL4	-7.243	2.305	No	ns

Table 3. Tukey’s Post Hoc HSD Test Results of Climbing Assay Using RNAi Lines. Climbing ability of the RNAi lines was observed through a climbing assay as outlined in Materials and Methods. Tukey’s post hoc HSD test was performed on the data to determine if any statistically significant differences existed between genotypes. Data between 0.01 and 0.05 was considered significant. Values between 0.001 and 0.01 were considered highly significant while any values of < 0.001 were extremely significant.

Two-way ANOVA Results of Climbing Assay **RNAi Lines**				
Source of Variation	% of total variation	P value		
Interaction	4.38	< 0.0001		
Column factor	79.24	< 0.0001		
Row factor	13.37	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	***	Yes		
Column factor	***	Yes		
Row factor	***	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	12	5064	422	6.057
Column factor	4	91612	22903	328.7
Row factor	3	15462	5154	73.98
Residual	75	5225	69.67	

Table 4**.** Two-Way ANOVA Results of Climbing Assay Using RNAi Lines. Climbing ability of the RNAi lines was observed through a climbing assay as outlined in Materials and Methods. A two-way ANOVA was performed to analyze the effects that the two independent variables, age and genotype, exert upon the independent variable, mobility, in this assay. Data between 0.01 and 0.05 was considered significant. Values between 0.001 and 0.01 were considered highly significant while any values of < 0.001 were extremely significant.

Tukey's Post Hoc HSD test Results of Walking Assay Four Msr Lines				
Age	**Day 4**			
Comparison	**Mean Diff.**	**q**	**Significant? P < 0.05?**	**Summary**
WT60 vs A90	-0.207	0.791	No	ns
WT60 vs B54	-0.458	1.745	No	ns
WT60 vs AB46	0.614	2.338	No	ns
A90 vs B54	-0.25	0.954	No	ns
A90 vs AB46	0.821	3.129	No	ns
B54 vs AB46	1.073	4.083	Yes	*
Age	**Day 7**			
Comparison	**Mean Diff.**	**q**	**Significant? P < 0.05?**	**Summary**
WT60 vs A90	-0.89	3.397	No	ns
WT60 vs B54	0.6806	2.577	No	ns
WT60 vs AB46	0.8863	3.383	No	ns
A90 vs B54	1.571	5.947	Yes	***
A90 vs AB46	1.776	6.78	Yes	***
B54 vs AB46	0.2057	0.779	No	ns
Age	**Day 11**			
Comparison	**Mean Diff.**	**q**	**Significant? P < 0.05?**	**Summary**
WT60 vs A90	-0.212	0.793	No	ns
WT60 vs B54	1.291	4.821	Yes	**
WT60 vs AB46	1.718	6.366	Yes	***
A90 vs B54	1.503	5.615	Yes	***
A90 vs AB46	1.93	7.154	Yes	***
B54 vs AB46	0.427	1.584	No	ns
Age	**Day 18**			
Comparison	**Mean Diff.**	**q**	**Significant? P < 0.05?**	**Summary**
WT60 vs A90	-1.492	4.411	Yes	*
WT60 vs B54	1.509	4.672	Yes	**
WT60 vs AB46	1.958	6.062	Yes	***
A90 vs B54	3.001	8.807	Yes	***
A90 vs AB46	3.45	10.12	Yes	***
B54 vs AB4	0.4487	1.378	No	ns
Age	**Day 22**			
Comparison	**Mean Diff.**	**q**	**Significant? P < 0.05?**	**Summary**
WT60 vs A90	-0.698	2.27	No	ns
WT60 vs B54	1.62	5.305	Yes	**
WT60 vs AB46	1.727	5.655	Yes	***

A90 vs B54	2.318	7.532	Yes	***
A90 vs AB46	2.425	7.879	Yes	***
B54 vs AB46	0.106	0.35	No	ns
Age	**Day 28**			
Comparison	**Mean Diff.**	**q**	**Significant? P < 0.05?**	**Summary**
WT60 vs A90	-1.032	2.919	No	ns
WT60 vs B54	0.3356	0.9497	No	ns
WT60 vs AB46	1.284	3.605	Yes	*
A90 vs B54	1.367	3.869	Yes	*
A90 vs AB46	2.316	6.501	Yes	***
B54 vs AB46	0.9487	2.663	No	ns
Age	**Day 31**			
Comparison	**Mean Diff.**	**q**	**Significant? P < 0.05?**	**Summary**
WT60 vs A90	-0.366	1.089	No	ns
WT60 vs B54	1.211	3.684	No	ns
WT60 vs AB46	1.359	4.102	Yes	*
A90 vs B54	1.578	4.681	Yes	**
A90 vs AB46	1.726	5.082	Yes	**
Age	**Day 38**			
Comparison	**Mean Diff.**	**q**	**Significant? P < 0.05?**	**Summary**
WT60 vs A90	-0.182	0.487	No	ns
WT60 vs B54	1.829	4.871	Yes	**
WT60 vs AB46	1.306	3.478	No	ns
A90 vs B54	2.012	5.401	Yes	**
A90 vs AB46	1.489	3.997	Yes	*

Table 5**.** Tukey's Post Hoc HSD Test Results of Walking Assay Using Four Msr Lines. The DAM system walking assay was performed using the four Msr genotypes using the procedure previously outlined in Materials and Methods. Tukey's post hoc HSD test was performed on the walking assay data to determine if any statistically significant differences existed between genotypes. Data between 0.01 and 0.05 was considered significant. Values between 0.001 and 0.01 were considered highly significant while any values of < 0.001 were extremely significant.

Two-way ANOVA Results of Walking Assay **Four Msr Lines**				
Source of Variation	% of total variation	P value		
Interaction	2.59	0.0009		
Column factor	10.86	< 0.0001		
Row factor	34.25	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	***	Yes		
Column factor	***	Yes		
Row factor	***	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	21	149.3	7.112	2.281
Column factor	3	626.2	208.7	66.96
Row factor	7	1976	282.2	90.54
Residual	975	3039	3.117	

Table 6**.** Two – Way ANOVA Results of Walking Assay Using Four Msr Lines. The DAM system walking assay was conducted using the four Msr genotypes using the procedure previously outlined in Materials and Methods. A two-way ANOVA was performed to analyze the effects that the two independent variables, age and genotype, exert upon the independent variable, mobility, in this assay. Data between 0.01 and 0.05 was considered significant. Values between 0.001 and 0.01 were considered highly significant while any values of < 0.001 were extremely significant.

Tukey's Post Hoc HSD Test Results of Walking Assay Four Msr Lines on 2mM Paraquat				
Day 7				
Comparison	Mean Diff.	q	Significant? P < 0.05?	Summary
WT60 – Paraquat vs A90 – Paraquat	0.4773	2.959	No	ns
WT60 – Paraquat vs B54 – Paraquat	1.166	7.227	Yes	***
WT60 – Paraquat vs AB46 – Paraquat	0.9751	6.045	Yes	***
A90 – Paraquat vs B54 – Paraquat	0.6885	4.235	Yes	*
A90 – Paraquat vs AB46 – Paraquat	0.4978	3.062	No	ns
B54 – Paraquat vs AB46 – Paraquat	-0.1906	1.173	No	ns
Day 15				
Comparison	Mean Diff.	q	Significant? P < 0.05?	Summary
WT60 – Paraquat vs A90 – Paraquat	0.2316	1.002	No	ns
WT60 – Paraquat vs B54 – Paraquat	1.026	4.441	Yes	*
WT60 – Paraquat vs AB46 – Paraquat	1.769	7.657	Yes	***
A90 – Paraquat vs B54 – Paraquat	0.7944	3.439	No	ns
A90 – Paraquat vs AB46 – Paraquat	1.537	6.654	Yes	***
B54 – Paraquat vs AB46 – Paraquat	0.7428	3.216	No	ns
Day 26				
Comparison	Mean Diff.	q	Significant? P < 0.05?	Summary
WT60 – Paraquat vs A90 – Paraquat	0.12	0.4033	No	ns
WT60 – Paraquat vs B54 – Paraquat	1.478	5.142	Yes	**
WT60 – Paraquat vs AB46 – Paraquat	0.7602	2.601	No	ns
A90 – Paraquat vs B54 – Paraquat	1.358	4.564	Yes	**
A90 – Paraquat vs AB46 – Paraquat	0.6402	2.118	No	ns
B54 – Paraquat vs AB46 – Paraquat	-0.7182	2.457	No	ns
Day 30				
Comparison	Mean Diff.	q	Significant? P < 0.05?	Summary
WT60 – Paraquat vs A90- Paraquat	-0.672	1.636	No	ns
WT60 – Paraquat vs B54 – Paraquat	1.339	3.234	No	ns
WT60 – Paraquat vs AB46 – Paraquat	1.604	3.904	Yes	*
A90- Paraquat vs B54 – Paraquat	2.011	4.895	Yes	**
A90- Paraquat vs AB46 – Paraquat	2.276	5.584	Yes	***
B54 – Paraquat vs AB46 – Paraquat	0.2645	0.6439	No	ns

Table 7**.** Tukey's Post Hoc HSD Test Results of Walking Assay using Four Msr Lines on 2mM Paraquat. The DAM system walking assay was performed using

the four Msr genotypes raised on food containing 2mM paraquat while following the procedure previously outlined in Materials and Methods. Tukey's post hoc HSD test was performed to determine if any statistically significant differences existed between genotypes. Data between 0.01 and 0.05 was considered significant. Values between 0.001 and 0.01 were considered highly significant while any values of < 0.001 were extremely significant.

Two-way ANOVA Results of Walking Assay **Four Msr Lines on 2mM Paraquat**				
Source of Variation	% of total variation	P value		
Interaction	1.77	0.0409		
Column factor	7.38	< 0.0001		
Row factor	42.61	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	*	Yes		
Column factor	***	Yes		
Row factor	***	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	9	46.49	5.165	1.97
Column factor	3	194.2	64.72	24.69
Row factor	3	1122	373.8	142.6
Residual	485	1271	2.622	

Table 8. Two – Way ANOVA Results of Walking Assay using Four Msr Lines on 2mM Paraquat. The DAM system walking assay was conducted using the four Msr genotypes raised on food containing 2mM paraquat while following the procedure previously outlined in Materials and Methods. A two-way ANOVA was performed to analyze the effects that the two independent variables, age and genotype, exert upon the independent variable, mobility, in this assay. Data between 0.01 and 0.05 was considered significant. Values between 0.001 and

0.01 were considered highly significant while any values of < 0.001 were extremely significant.

Tukey's Post Hoc HSD Test Results of Walking Assay RNAi Lines				
Day 5				
Comparison	Mean Diff.	q	Significant? P < 0.05?	Summary
Actin Driver vs. MsrA RNAi	-1.034	2.766	No	ns
Actin Driver vs. MsrB RNAi	-1.154	3.088	No	ns
Actin Driver vs. MsrA RNAi/Actin-GAL4	-1.209	3.235	No	ns
Actin Driver vs. MsrB RNAi x Actin	0.1553	0.4155	No	ns
MsrA RNAi vs. MsrA RNAi/Actin-GAL4	-0.175	0.4682	No	ns
MsrB RNAi vs. MsrB RNAi/Actin-GAL4	1.31	3.504	No	ns
MsrA RNAi/Actin-GAL4 vs. MsrB RNAi/Actin-GAL4	1.364	3.65	No	ns
Day 16				
Comparison	Mean Diff.	q	Significant? P < 0.05?	Summary
Actin Driver vs. MsrA RNAi	-0.935	2.922	No	ns
Actin Driver vs. MsrB RNAi	-0.9981	3.119	No	ns
Actin Driver vs. MsrA RNAi/Actin-GAL4	-0.1284	0.4014	No	ns
Actin Driver vs. MsrB RNAi/Actin-GAL4	1.402	4.382	Yes	*
MsrA RNAi vs. MsrA RNAi/Actin-GAL4	0.8066	2.521	No	ns
MsrB RNAi vs. MsrB RNAi/Actin-GAL4	2.4	7.502	Yes	***
MsrA RNAi/Actin-GAL4 vs. MsrB RNAi/Actin-GAL4	1.531	4.784	Yes	**
Day 25				
Comparison	Mean Diff.	q	Significant? P < 0.05?	Summary
Actin Driver vs. MsrA RNAi	-1.813	5.828	Yes	***
Actin Driver vs. MsrB RNAi	-2.739	8.804	Yes	***
Actin Driver vs. MsrA RNAi/Actin-GAL4	-0.1394	0.4789	No	ns
Actin Driver vs. MsrB RNAi/Actin-GAL4	0.9726	3.342	No	ns
MsrA RNAi vs. MsrA RNAi/Actin-GAL4	1.674	5.752	Yes	***
MsrB RNAi vs. MsrB RNAi/Actin-GAL4	3.712	12.75	Yes	***
MsrA RNAi/Actin-GAL4 vs. MsrB RNAi/Actin-GAL4	1.112	4.127	Yes	*
Day 33				
Comparison	Mean Diff.	q	Significant? P < 0.05?	Summary

Actin Driver vs. MsrA RNAi	-2.365	8.116	Yes	***
Actin Driver vs. MsrB RNAi	-3.466	11.89	Yes	***
Actin Driver vs. MsrA RNAi/Actin-GAL4	0.2098	0.72	No	ns
Actin Driver vs. MsrB RNAi/Actin-GAL4	0.6956	2.387	No	ns
MsrA RNAi vs. MsrA RNAi/Actin-GAL4	2.574	8.836	Yes	***
MsrB RNAi vs. MsrB RNAi/Actin-GAL4	4.161	14.28	Yes	***
MsrA RNAi/Actin-GAL4 vs. MsrB RNAi/Actin-GAL4	0.4858	1.667	No	ns
Day 40				
Comparison	Mean Diff.	q	Significant? P < 0.05?	Summary
Actin Driver vs. MsrA RNAi	-0.9653	2.69	No	ns
Actin Driver vs. MsrB RNAi	-2.08	5.798	Yes	***
Actin Driver vs. MsrA RNAi/Actin-GAL4	-0.567	1.58	No	ns
Actin Driver vs. MsrB RNAi/Actin-GAL4	0.6107	1.688	No	ns
MsrA RNAi vs. MsrA RNAi/Actin-GAL4	0.3983	1.11	No	ns
MsrB RNAi vs. MsrB RNAi/Actin-GAL4	2.691	7.44	Yes	***
MsrA RNAi/Actin-GAL4 vs. MsrB RNAi/Actin-GAL4	1.178	3.256	No	ns

Table 9. Tukey's Post Hoc HSD Test Results of Walking Assay using RNAi Lines. While following the procedure previously outlined in Materials and Methods, the DAM system walking assay was performed using the parental RNAi lines, Actin driver, and subsequent progeny. Tukey's post hoc HSD test was performed to determine if any statistically significant differences existed between genotypes. Data between 0.01 and 0.05 was considered significant. Values between 0.001 and 0.01 were considered highly significant while any values of < 0.001 were extremely significant.

Two-way ANOVA Results of Walking Assay RNAi Lines				
Source of Variation	% of total variation	P value		
Interaction	5.36	< 0.0001		
Column factor	22.08	< 0.0001		
Row factor	1.3	0.0088		
Source of Variation	P value summary	Significant?		
Interaction	***	Yes		
Column factor	***	Yes		
Row factor	**	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	16	192.5	12.03	3.524
Column factor	4	793.3	198.3	58.08
Row factor	4	46.7	11.68	3.42
Residual	750	2561	3.414	

Table 10. Two – Way ANOVA of Walking Assay using RNAi Lines. While following the procedure previously outlined in Materials and Methods, the DAM system walking assay was conducted using the parental RNAi lines, Actin driver, and subsequent progeny. A two-way ANOVA was performed to analyze the effects that the two independent variables, age and genotype, exert upon the independent variable, mobility, in this assay. Data between 0.01 and 0.05 was considered significant. Values between 0.001 and 0.01 were considered highly significant while any values of < 0.001 were extremely significant.

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