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The Effects of MsrA and MsrB in Anoxia Tolerance in Aging Drosophila melanogaster

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Abstract. Anoxia occurs when cells are deprived of oxygen. Throughout the animal kingdom, organisms respond differently to anoxia. *Drosophila melanogaster* (fruit fly) enters a protective coma that allows it to withstand hours of anoxia. The greatest oxidative stress occurs during the period called reoxygenation. A burst of reactive oxygen species (ROS) is produced when oxygen is reintroduced to the cells. In humans, ROS cause oxidative damage to critical cellular molecules, which contributes to aging and development of age-related neurodegenerative diseases. Methionine, a common amino acid in proteins, is especially sensitive to oxidation by ROS. Two methionine sulfoxide reductases (MsrA and MsrB) effectively reduce the methionine sulfoxide residues back to functional methionine. Our lab is investigating a link between the *Msr* genes and anoxia recovery using *Drosophila*. Currently, little is known about how the absence of Msr activity affects the ability of *Drosophila* to recover from anoxia. In this study, *MsrA* and *MsrB*, single deletion mutants, were exposed to one hour of anoxia and the Drosophila Activity Monitor (DAM) recorded their recovery times. RNA interference (RNAi) lines were used to mimic the effect of these deletion lines by ubiquitously knocking down Msr expression. **My current data indicates that there was a significant difference in recovery time for the *MsrA* and *MsrB* single loss-of-function genetic mutants during middle age, but not near senescence.** Insight into the role(s) of Msr genes under anoxic stress could lead to a better understanding of how these genes contribute to aging.

Introduction

The response of organisms to oxygen deprivation, often referred to as anoxia, differs throughout the animal kingdom. Mammals only tolerate anoxia for a few minutes before undergoing irreversible brain damage [1]. In contrast, the fruit fly, *Drosophila melanogaster* enters a protective coma that allows it to withstand hours of anoxia [2]. Interestingly, the most severe damage from anoxia occurs during reoxygenation. This is the period when oxygen is reintroduced into the system, which leads to a burst of reactive oxygen species (ROS), such as superoxide anion (0_2^-) and hydrogen peroxide (H_20_2) [3]. ROS is also produced during normal activities of the cell that can lead to cellular damage, especially to lipids, DNA, RNA and proteins [4]. ROS and their oxidized protein products are found to be more common in older individuals, especially those with age-related neurodegenerative disorders like Alzheimer's disease [5]. According to the Free Radical Theory of Aging, the accumulation of oxidative damage with time is a major contributor to the aging process [6].

ROS is capable of oxidizing several different amino acids, although the two sulfur containing amino acids, methionine and cysteine, are most sensitive. Methionine and cysteine are distinct from other amino acids because their oxidation is reversible [7]. Oxidized methionine, methionine sulfoxide, has two different enantiomers since the sulfur atom is asymmetrical within the methionine [5].

When methionine is oxidized by ROS to form methionine sulfoxide, it can be reduced back to functional methionine by methionine sulfoxide reductase (Msr). The enzyme encoded by the *MsrA* gene specifically reduces the S enantiomer (met-S-(o)) while the enzyme encoded by the *MsrB* gene specifically reduces the R enantiomer (met-R-(o)) of methionine sulfoxide [8]. Both of these genes were originally found in the bacterium *E.* coli [9]. In addition to repairing oxidative damage to methionine, both the MsrA and

MsrB enzymes can function as efficient antioxidants by reducing methionine sulfoxide created by a reaction with an ROS back to functional methionine [10]. Thus, the ROS can be destroyed before they are able to damage any cellular components.

The genesis of the research interest of the lab was the demonstration that overexpression of the bovine *MsrA* gene selectively within the central nervous system (CNS) of Drosophila resulted in a substantial increase in life span [8]. It was thus predicted that overexpression of *MsrB* should have a similar effect. However, an in-depth analysis showed that overexpression of *MsrB* in *Drosophila* had no effect on lifespan or any other phenotype that was examined [11].

Our lab found that overexpression studies of *Msr*A and *Msr*B were unproductive. Therefore, we changed our experimental approach to use classical genetic mutations. A series of *Msr*A and *Msr*B mutations were created in the lab. These mutations are fairly large deletions of the promoter and portions of the transcription unit, thereby leading to complete loss-of-function (LOF) alleles (unpublished data). More specifically, strains of *Drosophila* are available that are homozygous for wild-type alleles of both *Msr*A and *Msr*B, homozygous for the *Msr*A LOF allele, homozygous for the *Msr*B LOF and homozygous for both *Msr*A and *Msr*B LOF alleles. This last strain is, to our knowledge, the only developmentally complex eukaryotic model organism that is completely deficient in all known *Msr* activity. Not surprisingly, the phenotype of *Msr*A and *Msr*B mutants is usually more severe in the presence of oxidative stress. Our goal is to use the genetic and molecular tools available for *Drosophila* to better understand the underlying molecular mechanisms. My experiments will focus on the response of the *Msr* mutants to anoxic stress. Previous work by Danielle Howard showed that overall, single mutations in either *Msr*A or *Msr*B or mutations in both genes resulted in a longer recovery from the protective coma induced by anoxia. The average movements of both the wild-type and *MsrA/MsrB* double LOF flies were seen to increase with age, but the *MsrA/MsrB* double LOF flies showed varying mobility. Overall, the *MsrA/MsrB* double LOF flies have a higher recovery rate in comparison to wildtype flies right after anoxic stress was applied.

I extended these studies by using single deletions lines of either the *MsrA* or *MsrB* mutants to determine if these flies show increased susceptibility to anoxia as they approach senescence (60-65 days). I also used RNAi lines of *Drosophila* that selectively knock-down expression of either *Msr*A or *Msr*B in a tissue-specific manner. The RNAi lines are designed to mimic the single deletion lines. For example, I knocked down expression of *MsrA* and/or *MsrB* ubiquitously in the flies. The results of these experiments provided insight into how oxidative stress over the life of the organism can contribute to aging and possibly development of age-related neurodegenerative diseases.

Data

The wildtype line (WT60) recover significantly later as they approach senescence, while the MsrA and MsrB mutants reach maximum recovery time at middle age (40-45 days old).

ROS concentration significantly increases during the period after anoxic stress [3], thus the role of *MsrA* and *MsrB* genes in recovering from anoxic stress. The line containing both *MsrA* and *MsrB* genes (WT60) and single LOF (A90 and B54) at three age groups were stressed with anoxia for one hour. Their average recovery time was analyzed. From the data collected, the WT60 line recovers significantly later from anoxic stress as the fly approaches senescence **(Fig 1.A - 1.C).** There is no significant difference in average recovery time at young age (20-25 days) between $MsrA^{+}MsrB^{+}$ and either single deletion Msr mutant **(Fig 1.D).** There is a significant difference in average recovery time between the 40-45 day old Msr single deletion mutant and $MsrA^{+}MsrB^{+}$ **(Fig 1.E).** At 60-65 days, there is no significant difference in average recovery time between the Msr single deletion mutants and WT60 **(Fig 1.F). There is no significant difference in average recovery time after anoxic stress between the Msr single deletion mutants, as they approach senescence (60-65 days old).**

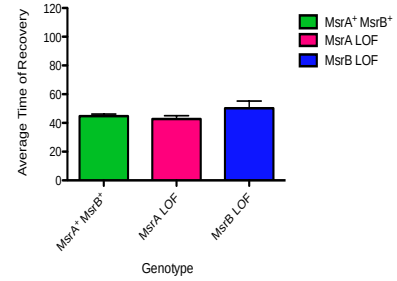
Preliminary data from a previous Honors student, Danielle Howard, has shown that there is

no significant difference in average recovery time after anoxic conditions between the 35-39 day old Msr single deletion mutants. We wanted to see whether this insignificance in average recovery time is seen after anoxic conditions as the fly approaches senescence. The MsrA and MsrB single deletion mutants were stressed for one hour under anoxia and their average recovery time was recorded via the DAM system. As the fly approached senescence there was no significant difference observed in the Msr single deletion mutants' average recovery time when they were 20-25 days old, 40-45 days old, and 60-65 days old **(2.A, 2.B, 2.C). The RNAi-A and RNAi-B knockdown lines reach maximum average recovery time after anoxic stress at middle age (40-45 days old).**

Because of the increased recovery time after anoxic conditions in the 40-45 day old Msr single deletion mutants, we began to see if this similar pattern is seen in the RNAi-A and RNAi-B knockdown lines. New genotypes were developed by crossing the RNAi-A x YW with Act x YW to make the RNAi-A x Act (RNAi-A ubiquitous knockdown). The RNAi-B x YW was crossed with Act x YW to make the RNAi-B x Act (RNAi-B ubiquitous knockdown). The RNAi-A and RNAi-B knockdown lines showed no significant difference in average recovery time at 20-25 days, similar to the 20-25 day old single deletion lines. The RNAi-A and RNAi-B knockdown lines also show no significant difference in average recovery time at 40-45 days, contrary to the 40-45 day old deletion lines. There is a significant difference in average recovery time seen between the RNAi-A and RNAi-B knockdown lines at 60-65 days, contrary to the 60-65 day old deletion lines. **The RNAi-A and RNAi-B knockdown lines show a significant difference in average recovery time as they approach senescence (60-65 days old).**

Since we did not see a significant difference in average recovery time after anoxic stress between the Msr single deletion mutants, we assumed the RNAi-A and RNAi-B knockdown lines would display a similar pattern. Only the 20-25 day old RNAi-A and RNAi-B knockdown lines did not show a significance in average recovery time, similar to the Msr single deletion lines. However, the 40-45 day old and 60-65 day old RNAi knockdowns contradicted the results obtained from the single deletion lines. There was no significant difference in average recovery time between the two 20-25 day old RNAi knockdown lines. There was a significant difference seen between the single MsrA and MsrB knockdown lines of 40-45 and 60-65 days old.

A.



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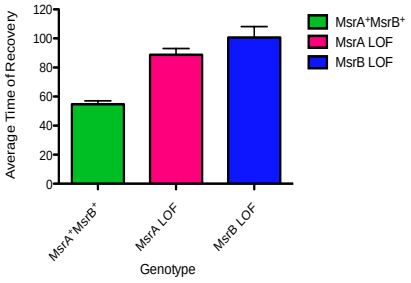

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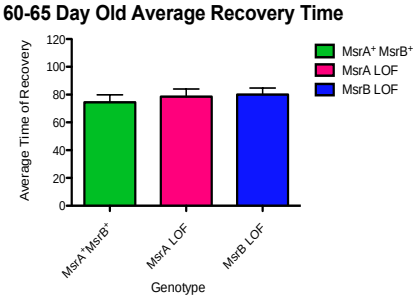


D.

MsrA⁺MsrB⁺ vs. MsrA LOF	MsrA⁺MsrB⁺ vs. MsrB LOF	MsrA LOF vs. MsrB LOF
p=0.4720	p=0.2936	p=0.1791

E.

MsrA⁺MsrB⁺ vs. MsrA LOF	MsrA⁺MsrB⁺ vs. MsrB LOF	MsrA LOF vs. MsrB LOF
p<0.0001	p<0.0001	p=0.1713

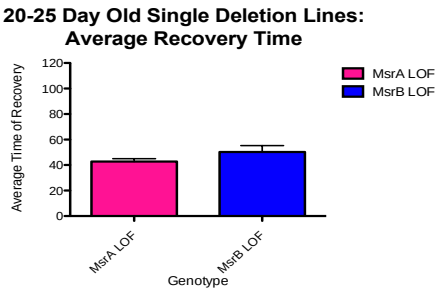
F.

MsrA⁺MsrB⁺ vs. MsrA LOF	MsrA⁺MsrB⁺ vs. MsrB LOF	MsrA LOF vs. MsrB LOF
p=0.1124	p=0.5190	p=0.8899

Figure 1 Average Recovery Time for MsrA$^+$MsrB$^+$ (WT60), MsrA Loss of Function (A90), and MsrB Loss of Function (B54) Flies Following Anoxic Stress.

The wildtype line (WT60) contained expression of both the MsrA and MsrB genes and served as the control line. The A90 line had knocked down expression of the MsrA gene and the B54 line contained knockdown expression of the MsrB gene. All three lines were stressed for one hour under anoxic conditions, after which their recovery time was recorded via the DAM system. There is no significant difference in average recovery time at young age (20-25 days) between MsrA$^+$MsrB$^+$ line and either single deletion Msr mutant (1.D). A significance of p<0.0001 is observed between the 40-45 day old Msr single deletion mutants and the wildtype line (1.E). At 60-65 days, there is no significant difference in recovery time between the Msr single deletion mutants and wildtype line (1.F). 32 flies that were 20-25 days old (1.A), 40-45 days old (1.B), and 60-65 days old (1.C) were tested for each genotype.

A.



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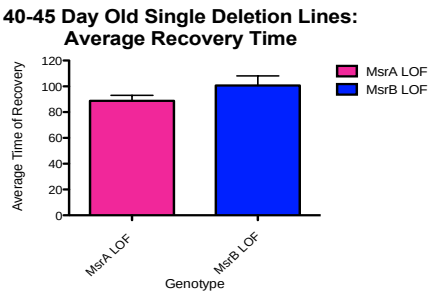

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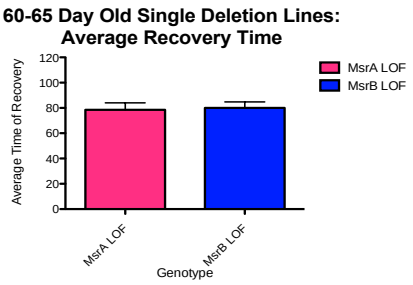

Figure 2 Average Recovery Time for MsrA Loss of Function (A90), MsrB Loss of Function (B54) Flies Following Anoxic Stress.

MsrA LOF (A90) and MsrB LOF (B54) were stressed with an hour of anoxic conditions, after which their recovery rates were recorded via the DAM system. There was no significance seen between the A90 and B54 lines at 20-25 days (p=0.1791), 40-45 days p=0.1713, or at 60-65 days p=0.8899 (2.A-2.C). 32 flies which were 20-25 days old, 40-45 days old, and 60-65 days old were tested for each genotype.

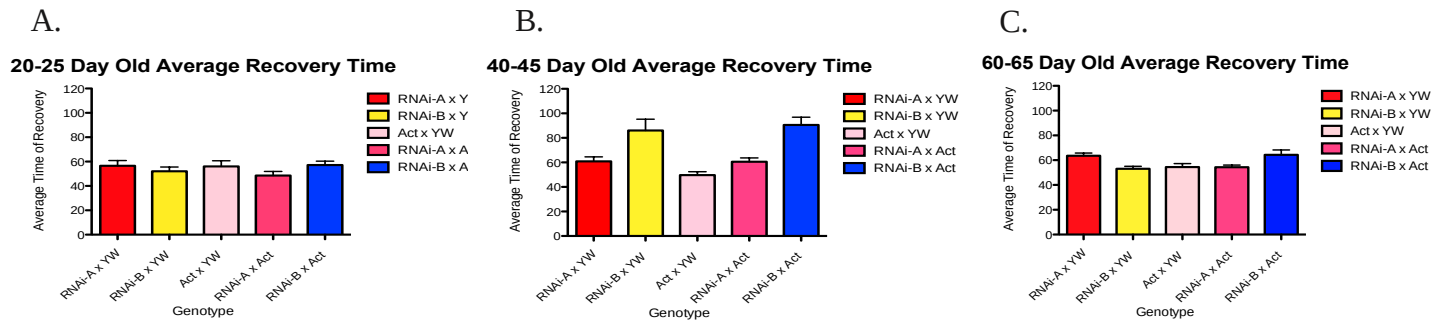

Figure 3 Average Recovery Time for RNAi-A x YW, RNAi-B x YW, Act x YW, RNAi-A x Act (ubiquitous knockdown of MsrA gene expression), and RNAi-B x Act (ubiquitous knockdown of MsrB gene expression) Flies Following Anoxic Stress.

All five lines were stressed under anoxic conditions for one hour, after which they were placed in the DAM system to record their average recovery time. There is no significant difference in average recovery time when comparing the 20-25 day old RNAi-A x Act and RNAi-B x Act lines with their respective parents, p>.0650 (3.A). There is no significant difference in average recovery time when comparing the 40-45 day old RNAi-A x Act and RNAi-B x Act lines with their respective parents, p>0.5550 (3.B).There is no significant difference in average recovery time when comparing the 60-65 day old RNAi-A x Act and RNAi-B x Act lines with their respective parents, p>0.0500 (3.C). 32 flies that were 20-25 days old, 40-45 days old, and 60-65 days old were tested for each genotype. There were roughly equal numbers of countries in each group.

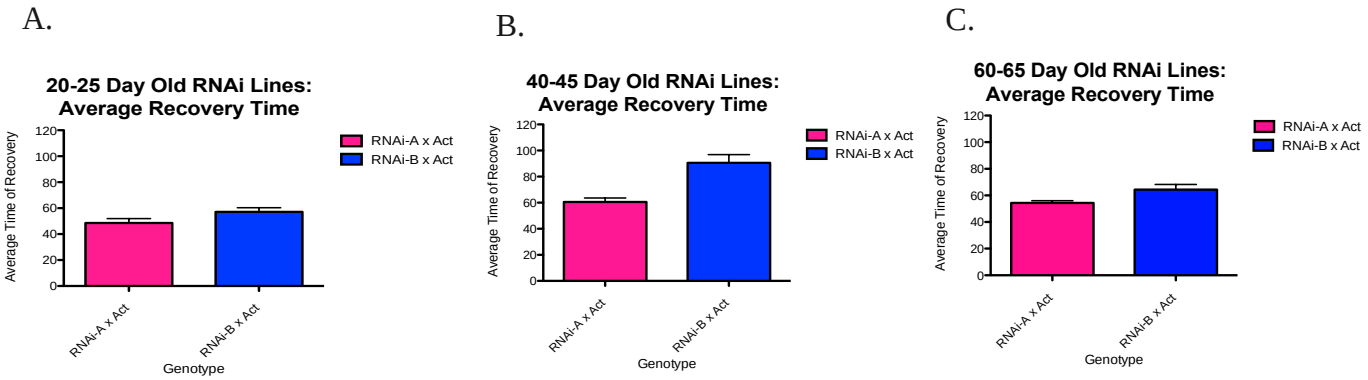

Figure 4 Average Recovery Time for RNAi-A x Act and RNAi-B x Act knockdown lines Following Anoxic Stress.

Both lines were stressed under anoxic conditions for one hour, after which they were placed in the DAM system to record their average recovery time. The 20-25 day old RNAi knockdown lines showed no significance in average recovery time, p=0.0832. The 40-45 day old RNAi knockdown lines display a significance of p<0.0001 while the 60-65 day old RNAi knockdown lines display a significance of p=0.0133. 32 flies that were 20-25 days old, 40-45 days old, and 60-65 days old were tested for each genotype.

Hypothesis and Methodology

The groups of flies that were used have the following genotypes: *MsrA* and *MsrB* present (wild-type) line, *MsrA* single LOF line, and *MsrB* single LOF line. The following procedures were done to determine the average recovery time after anoxic stress for these single deletion lines during normoxia, anoxia, and reoxygenation. Three genotypes of fly were used in this experiment. One wildtype line (WT60), one MsrA Loss-of-Function (LOF) line (A90), and one MsrB LOF line (B54). Flies for each genotype were put into bottles and allowed to grow for about ten days. The flies were then cleared. After five days, the male flies were collected and their age was determined to be one to five days old. Only males were used for experiments at 20-25 days old, 40-45 days old, and 60-65 days old. Flies used in the "normoxia" samples were not exposed to anoxia or reoxygenation. Flies used as "anoxia" and "reoxygenation" samples will be exposed to 1 hour of anoxia in the anoxia chamber. Average recovery time for each fly line were averaged using the Drosophila Activity Monitor. The *Drosophila* were aged from 5 days (young) to senescence. Five genetic crosses were performed. The three parental lines, RNAi-MsrA, RNAi-MsrB, and yellow white (yw) were used to produce the five genetic crosses. The RNAi-*Msr*A was outcrossed to yellow white (*yw*) as the first control line. This cross produced the RNAi-MsrA control line, for expression of the RNAi-MsrA transgene. The RNAi-*Msr*B was maintained over a balancer chromosome, to prevent genetic recombination, and was crossed with *yw* to serve as the second control for the "leaky" expression of only the RNAi-*Msr*B transgene. This cross produced the RNAi-MsrB second control line. The actin Gal4-driver line was crossed with *yw* to serve as the third control for expression of only the actin driver. Then, the last two lines were primarily used to knockout the expression of either the MsrA or MsrB gene. The RNAi-*Msr*A line was crossed with an actin Gal4-driver line to ubiquitously knock down expression of the *Msr*A gene. The same effect is applied to RNAi-*Msr*B when crossed with an actin driver. The five genetic lines containing the three control lines with expression of either MsrA or MsrB gene and the two expression knockdown lines of either gene were placed under normoxia, 1 hour of anoxia, and reoxygenation conditions before their average recovery times were averaged via the Drosophila Activity Monitor. The flies in each respective tube were placed into the anoxia tank. The flies were stressed in an anoxia tank containing only nitrogen gas for 1 hour. The Drosophila Activity Monitoring system was composed of 32 small tubes with holes drilled on one of the sides. The purpose of the small holes is for the gases to freely flow through the tube. The monitor has 32 holes in total, one hole for each tube. Every minute, the monitor recorded the animal's movements in each tube using an infrared (IR) beam. The DAM system was used to record the exact time the flies recovered from anoxic stress. The first time during reoxygenation when a fly broke through the IR beam, that individual fly is considered "recovered." Flies were monitored for 5 hours of reoxygenation.

Table 1: Msr Single Loss of Function Lines

Line	Genotype	MsrA	MsrB
RNAi-MsrA Control	w; UAS-RNAi-MsrA/ +;+	Present	Present
RNAi-MsrB Control	w; UAS_RNAi-MsrB/ +;+	Present	Present
Act-Gal4 Control	w; Act5c-Gal4/ +;+	Present	Present
Ubiquitous MsrA Knockdown	w; Act5c-Gal4/UAS-RNAi-MsrA;+	Not Present	Present
Ubiquitous MsrB Knockdown	w; Act5c-Gal4/UAS-RNAi-MsrB;+	Present	Not Present

Table 2: RNAi Lines

Genotype	MsrA	MsrB
WT60	Present	Present
A90	Not Present	Present
B54	Present	Not Present

Results

Our results obtained indicate the *MsrA* and *MsrB* genes do play a role in recovering from anoxic stress, especially in aging. Previous testing on the WT60 and single Msr deletion mutants were performed under anoxic conditions. The fly lines were aged to 35-39 days and displayed no significant difference in average recovery time between the *MsrA* deletion line and *MsrB* deletion line. Thus, this was the first time our lab has aged fly lines past 40 days old. Our findings indicate that there was no significant difference in average recovery time seen between the single deletion mutants under anoxic stress, as the flies approach senescence **(Figs. 2A-2C).** However, the wildtype line increases in recovery time as the fly approaches senescence **(Figs. 1A-1C).** This effect may be seen because as the fly ages, the fly becomes more susceptible to anoxic stress. Thus, this susceptibility causes the fly to take longer to recover from this anoxic stress with increasing age [14].

Our lab wanted to continue this testing to determine whether the Msr single deletion mutants display this increase in average recovery time after anoxic stress as the fly approaches senescence (60-65 days old). The lab used two approaches, the single deletion lines and the RNAi lines. The single deletion lines are known to be accurate as this system takes out the entire gene, either the *MsrA* or *MsrB*. The RNAi lines knock down the expression of the gene, however this system may not completely eliminate **all** of the corresponding Msr activity. Our findings show that the 20-25 day old Msr single deletion lines do not show a significant difference in average recovery time in comparison to the wildtype. This is most likely due to the flies being young and least susceptible to anoxic conditions. However, when the flies were middle-age (40-45 days old), there was a significant difference in average recovery time between the single Msr deletion mutants and the wildtype line. This indicates during the middle-age time period, the flies are more susceptible to anoxia and therefore take longer to recover from this anoxic stress. The *MsrA* and *MsrB* genes however do seem to be playing a role by causing the flies to recover from the protective coma, rather than dying following the anoxic stress. Contrary to our predictions, the 60-65 day old Msr single deletion lines recovered faster than the 40-45 day old Msr single deletion lines, but did not show a significant difference in average recovery time when compared to the wildtype line **(Figs 1A-1C).** We presume that this decrease in recovery time may be due to genes other than Msr being activated during this time frame. Evolutionary, most of these flies are known to not survive past 65 days. Thus, this mechanism of activating other genes to recover from the anoxic stress and prevent death may be the reason the single deletion mutant flies recover faster.

Although the wildtype and Msr single deletion lines displayed a significant difference in average recovery time at 40-45 days old, there was no significant difference seen near senescence (60-65 days). However, when comparing the RNAi-A and RNAi-B ubiquitous knockdown lines with their parents, a significant difference in average recovery time is seen at 60-65 days instead of 40-45 days **(Figs 3A-3C).** The inconsistency with the RNAi lines is most probably due to there being residual forms of the MsrA or MsrB activity being present in the fly, as only the expression of the gene is knocked down. This knockdown does not guarantee a 100% removal of the genes, thus showing the limitations of using the RNAi lines. When comparing the RNAi-A and RNAi-B ubiquitous knockdown lines with each other, there is a significant difference in average recovery time as the flies approach senescence. This result contradicts the data attained from the deletion lines, proving that the RNAi lines are not an accurate system to use. For future work, the RNAi lines will be improved with a stronger ubiquitous driver called ArmDa.

Issues with the anoxia tank and a lack of time limited the opportunity to retest the RNAi lines with the actin driver as well as other drivers

such as ArmDa (ubiquitous), GAWB (muscle), and OK6 (motor neurons).

Conclusion

Overall, the results obtained from this study show that MsrA and MsrB do play a role in protecting against oxidative stress in aging. It is known that MsrA and MsrB behave as antioxidants to reduce methionine sulfoxide (nonfunctional form of methionine from ROS oxidation) to the functional form of methionine [2]. Our findings from the single deletion lines indicate that MsrA and MsrB continue to play the role as the fly approaches senescence. Further testing with the RNAi lines is needed to reconfirm the data obtained from the deletion lines. This study could lead us to a better understanding of how these genes affect aging. Our long-term goal is to apply this knowledge to more human-based areas by designing therapeutic drugs around these genes in relation to anoxia, as in cases of stroke.

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