

INVESTIGATION OF THE RELATIONSHIP BETWEEN *METHUSELAH* AND STRESS GRANULES  
IN YOUNG *DROSOPHILA MELANOGASTER*

By

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## ABSTRACT (~200 words)

*mth<sup>1</sup>*, a longevity mutation found in fruit flies, has been previously found to improve stress resistance. Stress granules are complexes of misfolded mRNA and protein which form when an organism is stressed, and then dissolve afterward. While connections between stress granule dynamics and longevity mutations have been found in other organisms, the possible connection between *mth<sup>1</sup>* and stress granules has not yet been explored. In this study, I generated a recombinant line of *mth<sup>1</sup>-rin-GFP* flies which could be used to visualize stress granules, conducted lifespan studies on these flies to determine whether the recombinant was long-lived, and conducted heat shock experiments on young *mth<sup>1</sup>-rin-GFP* flies to determine if the recombinant formed more or less stress granules when stressed. The lifespan study found that the *mth<sup>1</sup>* flies lived significantly longer than *w<sup>1118</sup>* control flies, with a large increase in maximum lifespan and a small increase in average lifespan. However, the *mth<sup>1</sup>-rin-GFP* flies did not live longer compared to the same control. All heat shocked flies formed stress granules, and the *mth<sup>1</sup>-rin-GFP* flies formed larger stress granules than did the *rin-GFP/rin-GFP* control. These findings support the hypothesis that the *mth<sup>1</sup>* mutation affects stress granule dynamics in early life.

## 1. INTRODUCTION

In recent years, a class II G-protein coupled receptor (GPCR) in *Drosophila melanogaster* has garnered attention due to its effects on lifespan. The GPCR, appropriately dubbed

“methuselah” (*mth*), regulates insulin sensitivity (Gimenez et al., 2013). *mth<sup>1</sup>*, a hypomorphic mutation of this gene generated by a P-element insertion on the third chromosome, has been found to increase lifespan, improve resistance to a variety of stresses, and affect various sensorimotor functions in an age-dependent fashion (Lin et al., 1998; Pandey et al., 2015; Petrosyan et al., 2014; Petrosyan et al., 2007; Shukla et al., 2014). Although agonists and downstream effectors of *mth* have been heavily investigated, the mechanism by which it produces these effects are still not clearly understood (see Cvejic et al., 2004; Delanoue et al., 2016; Heo et al., 2008; Ja et al., 2009; Ja et al., 2007; Kim et al., 2006; Song et al., 2002).

One possible method may be regulation of stress granules. Stress granules (SGs) are transient aggregates of mRNA and protein that form in response to a wide variety of stresses, including oxidative stress and heat shock (Farny et al., 2009). “Recent evidence suggests that the recruitment of specific proteins to SGs inhibits apoptosis and affects cellular proliferation,” and SGs have been found in many different eukaryotes, including mammalian, fungal, and plant cells, which attests to their biological importance (Farny et al., 2009).

It has been observed that SGs in *C. elegans* behave differently in old age: the components are different, and they assemble and disassemble less readily (Cao et al. 2020). The aggregation of certain components which are highly aggregated in old age “is associated with smaller animal size, reduced fitness, and shortened lifespan,” while long-lived mutants did not see a similar age-related change in composition (Cao et al., 2020). Thus, a link between SGs and lifespan is well-established in *C. elegans*.

The precedent set by the connection between SG dynamics and long-lived mutations in *C. elegans* is promising. It suggests a new hypothesis for the mechanisms of long-lived

mutations in other organisms, and the investigation of these connections may further our understanding of human aging, since stress granules also form in humans (Farny et al., 2009). *mth* is a long-lived mutation in *Drosophila melanogaster*, and it has been found to positively affect resistance to heat shock and oxidative stress (Lin et al., 1998). Given the overlap in the observed effects of this mutation and SG formation, there is reason to believe that *mth*<sup>1</sup> may alter SG dynamics, the hypothesis being that *mth*<sup>1</sup> preserves the healthy functioning of stress granules in old age.

This thesis investigates the impact of the *mth*<sup>1</sup> mutation on SG assembly in 2-day-old flies in response to heat shock. The flies were placed in a hot water bath for 1 hour. After 30 minutes of recovery, the fly brains were dissected, stained, and sent for imaging. SGs were visualized using a GFP tagged Rasputin, which was recombined onto the third chromosome with the *mth*<sup>1</sup> mutation. Images were then compared in terms of the size of the SGs. We found that heat shock induced the formation of SGs, and the response was larger in the *mth*<sup>1</sup> flies compared to the control. This supports the hypothesis that *mth*<sup>1</sup> alters SG dynamics, even in early life.

## 2. METHODOLOGY

Lab work consisted of three components. The first was breeding a fly line suitable for examining SGs in the context of *mth1*. The second was lifespan studies to verify that the *mth1* flies I used exhibited extended lifespan. The third was heat shock experiments, followed by dissection, to determine the difference in stress granule (SG) formation between *mth1* and wild type flies.

### **2.1. Generate fly stocks with the experimental genotype, *mth1-rin-GFP/mth1-rin-GFP***

There were two stocks I needed to generate to run experiments. The first was the experimental stock, with the longevity mutant *mth1* and a GFP tagged Rasputin (Rin, a protein found in SGs). The second was the control stock, with only the GFP tagged Rasputin (abbreviated as RIN-GFP).

Crosses involve placing female virgin and male flies in a vial with 5 mL of food. It is crucial that females are virgins, to ensure that all offspring are the result of the cross one has deliberately set up, rather than prior mating events. All the females are genetically identical, and all the males are identical as well, but the females and males have different genotypes (otherwise, it is just a stock). The parents are transferred into a new vial every 2-3 days, and the old vial they were in is kept so that the offspring can be collected once they emerge. After 15 days, the parents are discarded entirely, to prevent collecting any offspring that may have resulted from the original parents' offspring mating. In cases when the parents are not producing many offspring, a small amount of yeast is applied to the side of the vial, which encourages mating and egg laying.

Diagrams for all of the crosses are shown in figures 1-6. Figures 1 and 2 show the generation of the experimental stock, while figures 3-6 show the generation of the control stock (the purpose of which is described in section 2.2). The summary of the two genotypes and the desired offspring are shown on top, with the full Punnett square beneath. Blue text denotes male flies, red text denotes female flies, gray cells are nonviable genotypes, and yellow highlighted genotypes are the desired product of the cross.

In the experimental stock, *mth1* and *rin-GFP* are both located on the third chromosome. This means that I needed to generate a line with *mth1* over *rin-GFP* (denoted *mth1/rin-GFP*) and then select offspring in which recombination had occurred, putting *mth1* and *rin-GFP* on the same chromosome. The first cross was between the original longevity mutant, *mth1/mth1*, and the *ds-Red/ds-Red; rin-GFP/rin-GFP* stock (Figure 1). All offspring were *mth1/rin-GFP*, so offspring could be collected without the need to screen for particular phenotypes.

$$\frac{mth^1}{mth^1} \times \frac{rin-GFP}{rin-GFP} = \frac{mth^1}{rin-GFP}$$

<b>Experimental Cross 1</b>		
	<i>rin-GFP</i>	<i>rin-GFP</i>
<i>mth<sup>1</sup></i>	<i>mth<sup>1</sup></i> <i>rin-GFP</i>	
<i>mth<sup>1</sup></i>	<i>mth<sup>1</sup></i> <i>rin-GFP</i>	

Figure 1. Virgin females were collected from the offspring. Highlighted cells are the offspring collected.

These *mth1/rin-GFP* offspring were then crossed with another stock, *mth<sup>Δ6</sup>/TM6B* (Figure 2). *rin-GFP* confers green fluorescence all over the fly's body, but this can be difficult to separate from a low level of fluorescence normally found in the fly's stomach. It is easier to

discern the presence of GFP when screening larvae rather than adult flies, so I screened for fluorescence in larvae, moving any fluorescent larvae to a new vial. *TM6B* is a balancer, which means that it prevents recombination for the chromosome it is on (in this case, the third chromosome), is lethal if homozygous, and has a dominant phenotype that can be seen and thus selected for or against (in this case, a “tubby” body that is short and wide). *TM6B*’s tubby phenotype is easiest to see in pupa, so I selected for tubby offspring at the pupal stage. This involved removing any non-tubby pupa from the vial using a metal spatula once a day every weekday. I did not collect offspring on Mondays since screening for tubby pupa did not take place over the weekend. Once the pupa emerged, I screened the adults for red eyes. The *rin-GFP* flies had white eyes, and the *mth<sup>1</sup>* mutation was the result of a P element insertion in a white eyed genetic background (generally shortened to “white background”). The P element carried the wild type gene for white eyes (which produces red eyes), which meant that flies with the P element insertion had red eyes and *mth<sup>1</sup>*. Thus, red eyes were proof that *mth<sup>1</sup>* is present.

$$\frac{mth^1}{rin-GFP} \times \frac{mth^{\Delta 6}}{TM6B} = \frac{mth^1-rin-GFP}{TM6B}$$

Experimental Cross 2		
	<i>mth</i> <sup>Δ6</sup>	<i>TM6B</i>
<i>mth</i> <sup>1</sup> - <i>rin</i> -GFP	$\frac{mth^1 - rin - GFP}{mth^{\Delta 6}}$	$\frac{mth^1 - rin - GFP}{TM6B}$
<i>mth</i> <sup>1</sup>	$\frac{mth^1}{mth^{\Delta 6}}$	$\frac{mth^1}{TM6B}$
<i>rin</i> -GFP	$\frac{mth^{\Delta 6}}{rin - GFP}$	$\frac{rin - GFP}{TM6B}$
+	$\frac{mth^{\Delta 6}}{+}$	$\frac{TM6B}{+}$

Figure 2. Male offspring were collected after being screened for tubby, GFP, and red eyes, discarding any with red fluorescing eyes. Highlighted cells are the offspring collected.

The original *rin*-GFP flies had a construct on the second chromosome which could have potentially interfered with experiments. This construct conferred red fluorescence in the eyes as well as increased sensitivity to heat shock (the stress I intended to use to induce SG formation). Thus, I also screened adult flies to ensure they did not have red fluorescence in their eyes. These flies were crossed with each other, establishing the stock from which I would eventually collect the experimental flies. The stock produced two types of offspring, those which were balanced over *TM6B* and those which were not. The ones I used for the experiment were not balanced, so that they would be *mth*<sup>1</sup>-*rin*-GFP homozygous in a white background.

## 2.2. Crosses to get the control genotype, +/+; *rin*-GFP/*rin*-GFP

To compare stress granules in the experimental and control flies, I needed control flies with *rin*-GFP as well. As mentioned in the previous section, the *rin*-GFP stock used to derive the

experimental flies had a construct on the second chromosome which would prevent the use of heat shock as a stress. Thus, the control flies also needed to be bred from *rin*-GFP stock to remove the construct. Because this involves the second chromosome, I crossed flies from the *rin*-GFP stock with another stock, *Sp/CyO; Sb/TM3 Ser*, with a balancer on both the second and third chromosomes (Figure 3). Curly of Oster (*CyO*) is a balancer on the second chromosome which harbors a dominant mutation that causes the wings of a fly to curl upward. TM3 is a balancer on the third chromosome that harbors a dominant mutation, Serrate (*Ser*), which causes small notches on the inner edges of the fly's wings. The other mutations in this stock also produce selectable phenotypes, making it very convenient to work with. Sternopleural (*Sp*) alters the number and/or orientation of hairs on the fly's body, while Stubble (*Sb*) decreases the length and increases the width of the fly's bristles.

$$\frac{Sp}{cyO}; \frac{Sb}{TM3-Ser} \times \frac{rin-GFP}{rin-GFP} = \frac{dS-Re}{cyO}; \frac{rin-GFP}{TM3-S}$$

$$\frac{Sp}{cyO}; \frac{Sb}{TM3-S} \times \frac{rin-GFP}{rin-GFP} = \frac{dS-Red}{Sp}; \frac{rin-G}{TM3}$$

<b>Control Cross 1</b>		
	<b><i>dS-Red; rin-GFP</i></b>	<b><i>dS-Red; rin-GFP</i></b>
<b><i>Sp; Sb</i></b>	$\frac{dS-Red}{Sp}; \frac{rin-GFP}{Sb}$	$\frac{dS-Red}{Sp}; \frac{rin-GFP}{Sb}$
<b><i>Sp; TM3-Ser</i></b>	$\frac{dS-Red}{Sp}; \frac{rin-GFP}{TM3-Ser}$	$\frac{dS-Red}{Sp}; \frac{rin-GFP}{TM3-Ser}$
<b><i>cyO; Sb</i></b>	$\frac{dS-Red}{cyO}; \frac{rin-GFP}{Sb}$	$\frac{dS-Red}{cyO}; \frac{rin-GFP}{Sb}$
<b><i>cyO; TM3-Ser</i></b>	$\frac{dS-Red}{cyO}; \frac{rin-GFP}{TM3-Ser}$	$\frac{dS-Red}{cyO}; \frac{rin-GFP}{TM3-Ser}$

Figure 3. Virgin female offspring were collected after being screened for curly wings and serrated wings. Male offspring were collected after being screened for sternopleural and serrated wings. Highlighted cells are the offspring collected.

From Control Cross 1, I screened virgin females for curly and serrated wings, and I screened males carrying *Sp* and *Ser*. These two genotypes (*ds-Red/CyO; rin-GFP/TM3 Ser* and *ds-Red/Sp; rin-GFP/TM3 Ser*, respectively) were selected for the parents of the second cross (Figure 4). Male and virgin female offspring from this cross were screened for GFP as larvae, and then sternopleural and curly wings as adults (Figure 4). Any flies with serrated wings were discarded. From the collected offspring, I made a stock.

$$\frac{dS-Re}{Sp}; \frac{rin-GFP}{TM3-Ser} \times \frac{dS-Re}{cyO}; \frac{rin-G}{TM3-S} = \frac{Sp}{cyO}; \frac{rin-GFP}{rin-G}$$

Control Cross 2				
	<i>dS-Red; rin-GFP</i>	<i>dS-Red; TM3-Ser</i>	<i>Sp; rin-GFP</i>	<i>Sp; TM3-Ser</i>
<i>dS-Red; rin-GFP</i>	$\frac{dS-Red}{dS-Red}; \frac{rin-GFP}{rin-GFP}$	$\frac{dS-Red}{dS-Red}; \frac{rin-GFP}{TM3-Ser}$	$\frac{dS-Red}{Sp}; \frac{rin-GFP}{rin-GFP}$	$\frac{dS-Red}{Sp}; \frac{rin-GFP}{TM3-Ser}$
<i>dS-Red; TM3-Ser</i>	$\frac{dS-Red}{dS-Red}; \frac{rin-GFP}{TM3-Ser}$	$\frac{dS-Red}{dS-Red}; \frac{TM3-Ser}{TM3-Ser}$	$\frac{dS-Red}{Sp}; \frac{rin-GFP}{TM3-Ser}$	$\frac{dS-Red}{Sp}; \frac{TM3-Ser}{TM3-Ser}$
<i>cyO; rin-GFP</i>	$\frac{dS-Red}{cyO}; \frac{rin-GFP}{rin-GFP}$	$\frac{dS-Red}{cyO}; \frac{rin-GFP}{TM3-Ser}$	$\frac{Sp}{cyO}; \frac{rin-GFP}{rin-GFP}$	$\frac{Sp}{cyO}; \frac{rin-GFP}{TM3-Ser}$
<i>cyO; TM3-Ser</i>	$\frac{dS-Red}{cyO}; \frac{rin-GFP}{TM3-Ser}$	$\frac{dS-Red}{cyO}; \frac{TM3-Ser}{TM3-Ser}$	$\frac{Sp}{cyO}; \frac{rin-GFP}{TM3-Ser}$	$\frac{Sp}{cyO}; \frac{TM3-Ser}{TM3-Ser}$

Figure 4. Male and virgin female offspring were collected after being screened for curly wings and sternopleural, discarding any with red fluorescence in the eyes or serrated wings. Highlighted cells are the offspring collected.

This stock had introduced two mutations on the second chromosome (*Sp* and *CyO*) which were not present in the experimental flies. To act as a proper control, the control flies would need to be wild-type homozygous on the second chromosome. Therefore, we took female virgins from the *Sp/CyO; rin-GFP/rin-GFP* stock I created and crossed them with males from another stock which was wild-type on the second chromosome, *+/+; Sb/TM3 Ser act GFP* (hereafter abbreviated to *TM3 Ser* because the *act GFP* is irrelevant to the crossing scheme, Figure 5). From the offspring, males and virgin females were screened for sternopleural and serrated wings. Any flies with curly wings or stubble were discarded.

$$\frac{+}{+}; \frac{Sb}{TM3-Ser} \times \frac{Sp}{cyO}; \frac{rin-GFP}{rin-GFP} = \frac{Sp}{+}; \frac{rin-GFP}{TM3-S}$$

Control Cross 3		
	<i>Sp; rin-GFP</i>	<i>cyO; rin-GFP</i>
<b>+</b> ; <i>Sb</i>	$\frac{Sp}{+}; \frac{rin-GFP}{Sb}$	$\frac{cyO}{+}; \frac{rin-GFP}{Sb}$
<b>+</b> ; <i>TM3-Ser</i>	$\frac{Sp}{+}; \frac{rin-GFP}{TM3-Ser}$	$\frac{cyO}{+}; \frac{rin-GFP}{TM3-Ser}$

Figure 5. Male and virgin female offspring were collected after being screened for sternopleural and serrated wings, discarding any with curly wings or stubble. Highlighted cells are the offspring collected.

These offspring were then crossed with each other, and male and virgin female offspring were selected which did not have sternopleural or serrated wings (Figure 6). These offspring could then be crossed with each other to grow a stock to be used as control flies in the heat shock experiments, differing from the experimental flies only in the presence or absence of the *mth<sup>1</sup>* mutation.

$$\frac{Sp; rin-GFP}{+; TM3-S} \times \frac{Sp; rin-GFP}{+; TM3-S} = \frac{+; rin-G}{+; rin-G}$$

Control Cross 4				
	<i>Sp; rin-GFP</i>	<i>Sp; TM3-Ser</i>	<i>+</i> ; <i>rin-GFP</i>	<i>+</i> ; <i>TM3-Ser</i>
<i>Sp; rin-GFP</i>	$\frac{Sp; rin-GFP}{Sp; rin-GFP}$	$\frac{Sp; rin-GFP}{Sp; TM3-Ser}$	$\frac{Sp; rin-GFP}{+; rin-GFP}$	$\frac{Sp; rin-GFP}{+; TM3-Ser}$
<i>Sp; TM3-Ser</i>	$\frac{Sp; rin-GFP}{Sp; TM3-Ser}$	$\frac{Sp; TM3-Ser}{Sp; TM3-Ser}$	$\frac{Sp; rin-GFP}{+; TM3-Ser}$	$\frac{Sp; TM3-Ser}{+; TM3-Ser}$
<i>+</i> ; <i>rin-GFP</i>	$\frac{Sp; rin-GFP}{+; rin-GFP}$	$\frac{Sp; rin-GFP}{+; TM3-Ser}$	$\frac{+; rin-GFP}{+; rin-GFP}$	$\frac{+; rin-GFP}{+; TM3-Ser}$
<i>+</i> ; <i>TM3-Ser</i>	$\frac{Sp; rin-GFP}{+; rin-GFP}$	$\frac{Sp; TM3-Ser}{+; TM3-Ser}$	$\frac{+; rin-GFP}{+; TM3-Ser}$	$\frac{+; TM3-Ser}{+; TM3-Ser}$

Figure 6. Male and virgin female offspring were collected after discarding any with sternopleural or serrated wings. Highlighted cells are the offspring collected.

### 2.3. Lifespan

The purpose of these studies was to confirm that the *mth<sup>1</sup>* flies we used to derive the *mth<sup>1</sup>-rin-GFP* stock were indeed longer lived, as well as to confirm that the *mth<sup>1</sup>-rin-GFP* recombinants were still long lived. To this end, I conducted lifespan studies on four genotypes. The first was white (*w<sup>1118</sup>*), serving as the baseline against which to compare the other results. The second was *mth<sup>Δ6</sup>/TM6B*, a line in which a P-element was used to insert the *mth<sup>1</sup>* gene and then excised. The third was *mth<sup>1</sup>*, the long-lived mutant. The fourth was *mth<sup>1</sup>-rin-GFP*, the experimental genotype.

Flies were collected using the selection method of virgining. This method involves screening flies for a black spot on the abdomen and a large, white body. Any such flies are too young to have mated yet, ensuring they are virgins. Flies were stored in vials with a low volume

of food, in a 25°C incubator. Once every weekday, the flies were counted and the number alive in each vial was recorded. At the conclusion of the study, the data was transferred from physical tables to Prism. When entering the data in Prism, if a fly was found dead after a period without data (for example, on Monday after not recording on the weekend), the fly was always assumed to die at the earliest possible time (in the example, Saturday). The data for each of the genotypes was then compared in Prism to determine if there were significant differences.

#### **2.4. Heat Shock**

The water bath used for heat shock was heated to 37°C before placing any flies in it. I used two vials, one for the control genotype (+/+; *rin*-GFP/*rin*-GFP), and one for the experimental genotype (*mth*<sup>1</sup>-*rin*-GFP/*mth*<sup>1</sup>-*rin*-GFP). To prevent the flies from getting stuck inside semi-melted food, the vials were empty of food (a tradeoff, because this adds starvation as an additional stress during the experiment). The cotton stopper was pushed down until the top was level with the top of the vial to ensure that flies could not climb above the water level to escape the high temperature. A weight was necessary to keep the vials from floating, and additional empty vials were needed to provide a stable surface for it. The temperature of the bath was monitored periodically during the experiment. When necessary, adjustments were made to the heat settings, ice was added (and stirred to make sure the temperature was even across the bath), and water was removed to avoid getting the cotton plugs wet.

The vial of control flies was placed in the bath first. It was accompanied by a few other empty vials to provide a stable platform for the weight used to keep the vials submerged. The vial of control flies was taken out of the bath once it had been heated for 1 hour. It was then

moved to a 25°C incubator for 30 minutes to let the flies recover before dissection. After the control flies had been in the bath for 30 minutes, the vial of experimental flies was added to the bath, and all steps for those flies were thus staggered by 30 minutes, allowing for timely dissections.

## **2.5. Dissection and Fixation**

All flies from one treatment were knocked out on the CO<sub>2</sub> pad until it was the individual fly's turn to be dissected. A few drops of hemolymph-like saline (HL3) were pipetted onto a petri dish, forming a single, larger drop. A fly was then transferred from the CO<sub>2</sub> pad to the liquid, and then dissected using two forceps. When the brain was isolated, it was placed in 4% paraformaldehyde in Phosphate Buffer Saline (PBS), which was kept in 1.5 mL microcentrifuge tubes in an ice bath for the duration of dissections. All the brains for a single treatment went into the same tube, but there were different tubes for different treatments. After 30 minutes had passed since the first brain was placed in 4% paraformaldehyde, any flies which had not been dissected were discarded, and the collected brains were placed in the rotator to fix for another 30 minutes. There was a separate 30 minute period for dissecting each of the treatments. The "30 minute period" was defined slightly differently between the heat shocked and non-heat shocked flies. For the non-heat shocked flies, the timer began only once the first successfully removed brain was put in 4% paraformaldehyde. The timer was started earlier for the heat shocked flies, beginning as soon as we removed them from the incubator, which meant that the timer included the duration of the first dissection. All this affected was the number of flies that could be dissected in the time, not the duration of washing or staining.

## 2.6. Washes

After fixing, the brains were washed. Unless otherwise stated, all washing and staining took place at room temperature. Washes consisted of using a plastic pipette to remove as much of the previous liquid as possible without accidentally removing a brain, then adding the next liquid. The volume per wash was not measured precisely, usually filling about 3/4 of the tube. Once the next liquid was added, the tube was vortexed briefly to keep the brains from sticking to the walls. In between most washes, the fly brains were kept on the rotator, also to prevent sticking. When the next wash was started immediately, without leaving the fly brains on the rotator, I will refer to it as a “quick wash.” The first wash was a quick wash in PBS. The next 3 washes also used PBS, 10 minutes apart.

## 2.7. Permeabilization

After the washes were completed, the brains were transferred to a clear 24-well plate using a glass pipette. All the brains for a single treatment went in one well, but there were different wells for different treatments. Any PBS that was transferred over with them was removed, and then the well was filled with 0.1% Triton in PBS (PBS-TX) to permeabilize the brains. The well plate was then placed on a shaker to preventing sticking. Because all the brains for all of the treatments could not be handled at the same time, and all of them should be stained at once to minimize variation, the process stalled here until the later brains were at this step. Once all the brains for the four treatments were in the PBS-Tx, they were left on the shaker for another 30 minutes. Then the 0.1% PBS-Tx was removed, and the brains were incubated in 0.25% PBS-Tx for 10 minutes.

## **2.8. Blocking**

From this point until mounting, the brains stayed in the well plate, and on the shaker when the liquids were not being changed. When liquids were swapped out, the process was done under the microscope to prevent sucking out a brain.

Blocking solution is used to adsorb to the nonspecific binding sites, reducing the background noise when imaging. The blocking solution was 5% Normal Goat Serum (NGS) and 2% Bovine Serum Albumin (BSA) in 0.25% PBS-Tx. It was mixed on the day of use. The 0.1% PBS-Tx was removed from the wells, and 333  $\mu$ L of block were added in its place. The well plate was then left on the shaker for 45 minutes.

## **2.9. Staining with anti-GFP-FITC**

Between blocking and staining, I did four washes with 0.25% PBS-Tx. The first was a quick wash, and the latter 3 were 15 minutes each. The anti-GFP-FITC rabbit polyclonal antibody was used to visualize GFP when imaging. It was mixed at a concentration of 1:300 in the blocking solution from section 2.6, and then spun down at 12 x 100 RPM for 5 minutes. The block was removed from the wells, and 300  $\mu$ L of the antibody solution were added in its place. The well plate was wrapped completely in aluminum foil to prevent light from bleaching the FITC fluorophore. The brains were incubated in the antibody solution overnight (about 15 hours) at 4°C. For all future steps, I tried to minimize the amount of time the brains spent uncovered by the foil.

## 2.10. Staining with Hoechst

Between the previous antibody and this one, I did three washes with 0.25% PBS-Tx for 15 minutes each, and then 2 washes with PBS for 10 minutes each. Hoechst stains the DNA, providing a marker for the location of the nucleus. It was mixed at a concentration of 1:100 in PBS, but the initial Hoechst solution used was already diluted to 1:100, so the resulting concentration was 1:10,000. It was then spun down at 120 x 100 RPM for 5 minutes. The PBS was removed from the wells, and 300  $\mu$ L of the Hoechst was added to each in its place. The well plate was incubated for 25 minutes. Then I did a final wash in PBS for about 40 minutes.

## 2.11. Mounting

The brains were placed on glass slides and sealed in place using cover slips to prepare for imaging. The brains from two treatments could be placed on a single glass slide. I cut an opening in several hole reinforcers (stickers shaped like donuts), and then stuck two of them to each glass slide, on opposite ends of the glass slide. All the brains from a single treatment were transferred using a glass pipette to the empty center of a single sticker, so two glass slides were necessary for the four treatments. Almost all of the PBS that was transferred with the brains was removed from the glass slide, and then a drop of antifade mounting media was applied as quickly as possible to prevent the brains from drying out. Forceps were used to carefully place the cover slide atop the sticker. The hole reinforcers were used to support the cover slide, rather than having it crush the brain. The opening cut in the hole reinforcer allowed excess liquid, such as the mounting media, to escape without lifting the cover slip. Once all the brains

were transferred and cover slips were applied, I dabbed a small amount of nail polish on the corners of each of the cover slips to hold them in place.

## 2.12. Imaging

The brains were imaged on a Zeiss 800 Inverted Confocal Microscope using a Plan-Apochromat 20x/0.8 objective (Figures 8-11). Two filter sets were used on each image. All images used one for GFP to visualize the stress granules (in green). For the images at 63x magnification, a DAPI filter was used to visualize the nuclei stained by Hoechst (in magenta, Figures 10-11). The brains were imaged at 5-micron optical section thickness for the images at 20x magnification, and at less than 1-micron optical section thickness for the 63x images at 63x magnification.

## 3. RESULTS AND CONCLUSIONS

### 3.1. Crosses

By the time that I collected the experimental flies, the mixed stock seemed to have gone almost completely homozygous, *mth<sup>1</sup>-rin-GFP/mth<sup>1</sup>-rin-GFP*. This could be discerned from the difference in eye color. When the flies were balanced over *TM6B*, there was only one allele for red eye color, on the homolog with *mth<sup>1</sup>*. When the stock became homozygous, the flies had red eyes. These were the ones I used for heat shock experiments.

The control flies did not behave as expected. The offspring from Control Cross 3 (Figure 5) had red fluorescence in the eyes, despite the homologs from chromosome 2 in those flies coming from a stock without *ds-Red*. In addition, offspring from Control Cross 4 (Figure 6) had a

mixture of white and red eyes, and the white eyed flies had red fluorescence in the eyes while the red eyed ones did not. In an effort to create a stock of only flies without *ds-Red*, we crossed the red-eyed offspring from Control Cross 4. Some of the parents used to do this were not virgins, and that predictably produced mixed results in terms of eye color and the presence of *ds-Red*. However, even when virgin offspring without red fluorescence in their eyes were taken from this cross and mated, the mixture persisted in the resultant offspring. Due to time constraints, the flies used as controls in this experiment were taken from such mixed crosses, though all the flies were screened to ensure they did not have red fluorescence in the eyes and only flies with red eye color were used. The unusual results of these crosses suggest that *ds-Red* may not actually be located on the second chromosome.

### 3.2. Lifespan

The results are summarized in Figure 7. This is based on 60 *mth<sup>1</sup>* flies (30 male, 30 female), 57 *w<sup>1118</sup>* flies (30 male, 27 female), 50 *mth<sup>Δ6</sup>/TM6B* (all female), 32 *mth<sup>1</sup>-rin-GFP* flies (30 male, 32 female). All significance values were calculated in Prism using the Mantel-Cox test.

## Survival proportions: Survival of Data 1

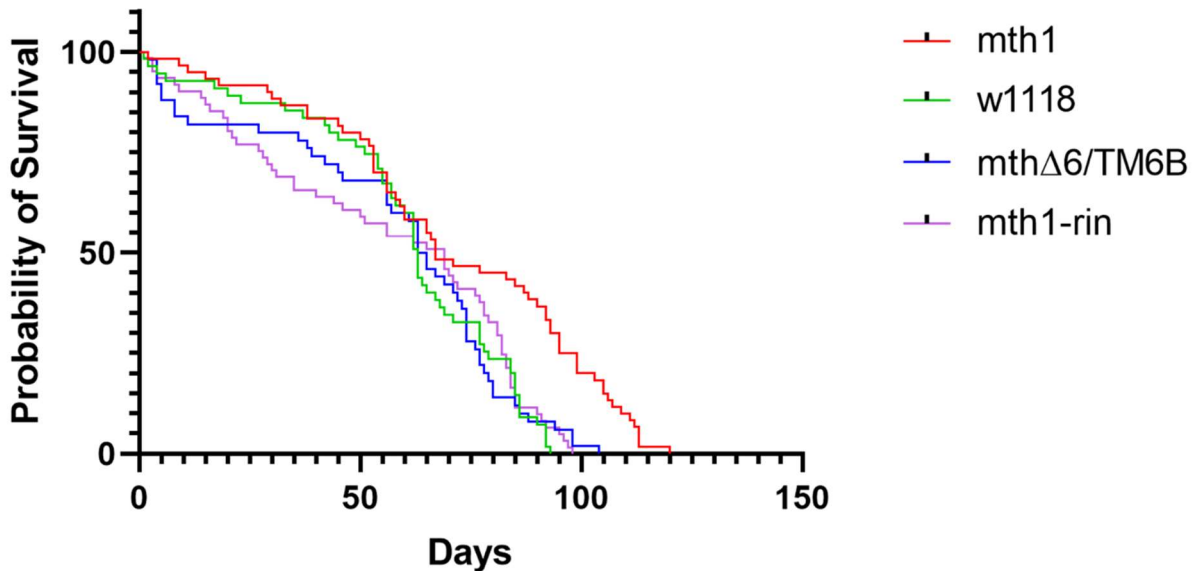


Figure 7. Lifespan data for four genotypes.

*mth*<sup>1</sup> flies live longer than *w*<sup>1118</sup> flies, with median survival increasing from 63 to 67 days and maximum survival increasing from 93 to 120 days (\*\*\*) . *mth*<sup>1</sup>-*rin*-GFP flies and *mth* <sup>$\Delta$ 6</sup>/*TM6B* flies, however, do not live significantly longer than *w*<sup>1118</sup> flies. This is not surprising for the *mth* <sup>$\Delta$ 6</sup>/*TM6B* flies, which were the product of imprecise excisions of the P-element used to insert *mth*<sup>1</sup> into the genome. These flies not only do not possess the longevity mutation, but also had DNA adjacent to the insertion deleted (Lin et al. 1998). These results are surprising, however, for the *mth*<sup>1</sup>-*rin*-GFP flies, which *do* possess the longevity mutation. These results indicate that the presence of *rin*-GFP influences the effects of *mth*<sup>1</sup>, consistent with the fact that the correct control by which to evaluate the longevity of *mth*<sup>1</sup>-*rin*-GFP flies are *rin*-GFP flies. It remains to be seen whether the *mth*<sup>1</sup>-*rin*-GFP flies are long-lived under this comparison.

The *mth*<sup>1</sup> flies seem to diverge from the wild type flies in later life, remaining largely indistinguishable until approximately 60 days old. This is not consistent with past findings,

which saw an increase in survival as early as 30 days old (Lin et al. 1998). By contrast, *mth<sup>1</sup>-rin-GFP* flies underperform in early life, and then begin to outlive the wild type flies in later life, also approximately 60 days old. It is possible that the *rin-GFP* line lives less long than wild type flies at all time points, and the shift at 60 days old is due to the *mth<sup>1</sup>* mutation.

### 3.3. Stress Experiments

I collected 30 flies with the experimental genotype, *mth<sup>1</sup>-rin-GFP/mth<sup>1</sup>-rin-GFP*. The control genotype, *+/+; rin-GFP/rin-GFP*, were collected from a cross, the offspring of which were varied regarding eye color and the presence of *dsRed*. Thirty-one males were collected with the control genotype, screening against the red fluorescence which indicates the presence of *dsRed*. Of these 31 flies, 24 had red eyes and 7 had orange eyes. Only the red-eyed flies were used. Twelve each of the control/experimental flies were set aside to be dissected without applying heat shock, while another 12 of both genotypes were set aside for heat shock. However, only about 7-8 brains from each treatment were successfully dissected in the 30-minute window.

When the flies had been allowed to recover from heat shock for 30 minutes, there was a visible difference between the experimental and control genotypes. Previous work found that *mth<sup>1</sup>* flies survived heat shock for longer on average, but it did not comment on activity levels (Lin et al., 1998). I would have expected that the experimental flies would be more active after the heat shock as a sign of superior recovery rate, but reduced movement of the experimental flies may also be a coping mechanism. All except for two of the control flies were moving, and some were even climbing over halfway up the sides of the vial. By contrast, none of the

experimental flies were very active, the most energetic of which were still on their backs, only swaying their legs subtly. This is especially striking because the temperature fluctuated far more at first, when only the control flies were in the bath, sometimes reaching as high as 39°C. When the experimental flies were in the bath, the temperature was more consistently within the range of 36-37°C. This would indicate that the heat shock was slightly more extreme for the control flies. As for overall survival rates, it was difficult to discern the number of living flies at such low levels of activity, but it is possible that the unmoving ones were still alive.

The brain images are shown in figures 8-11. While there is no difference among the brains apparent at 63x magnification, there is a clear difference at 20x magnification. The heat shocked flies for both the experimental and control genotypes show more green signal, which corresponds to the presence of *rin*-GFP. The increase in *rin*-GFP is consistent with prior knowledge about SGs: when placed under stress, proteins found in SGs (such as *rin*-GFP) should aggregate, resulting in more signal.

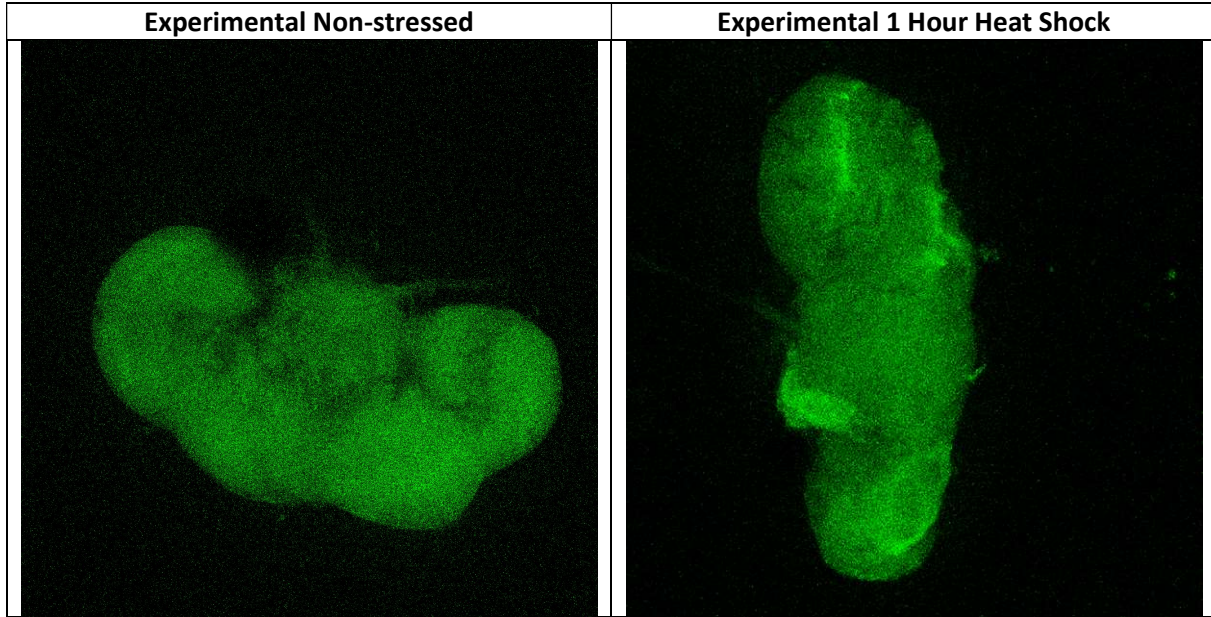


Figure 8. *mth<sup>1</sup>-rin-GFP/mth<sup>1</sup>-rin-GFP* fly brains imaged at 20x magnification. Green is GFP, and pink is A568.

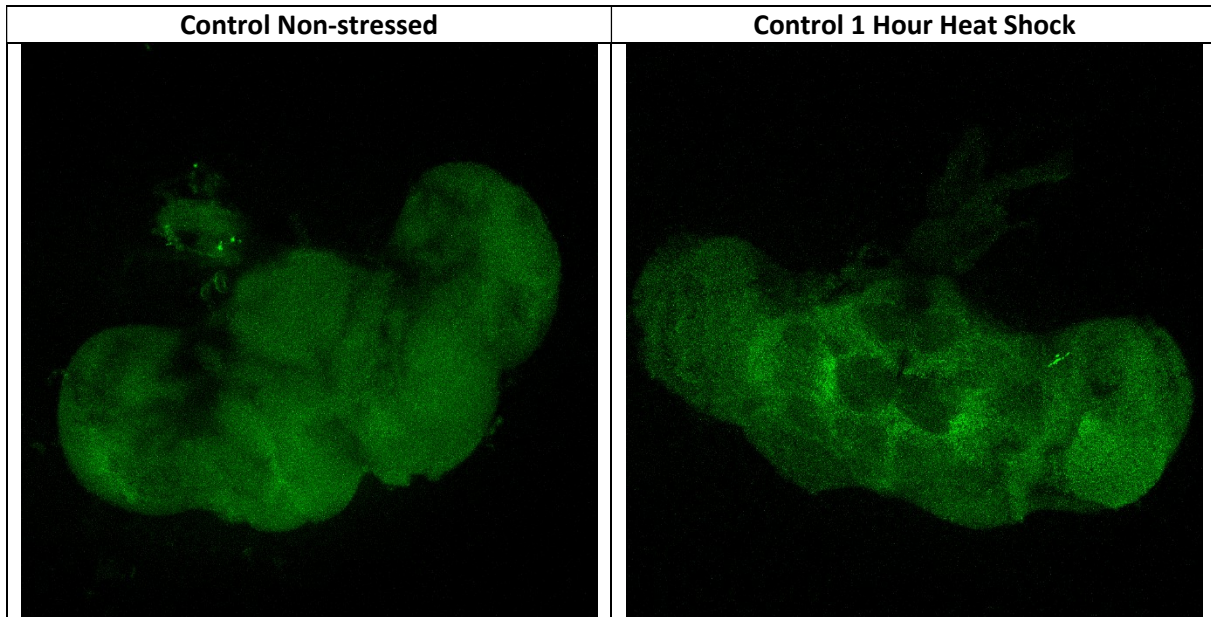


Figure 9. *+/+; rin-GFP/rin-GFP* fly brains imaged at 20x magnification. Green is GFP, and pink is A568).

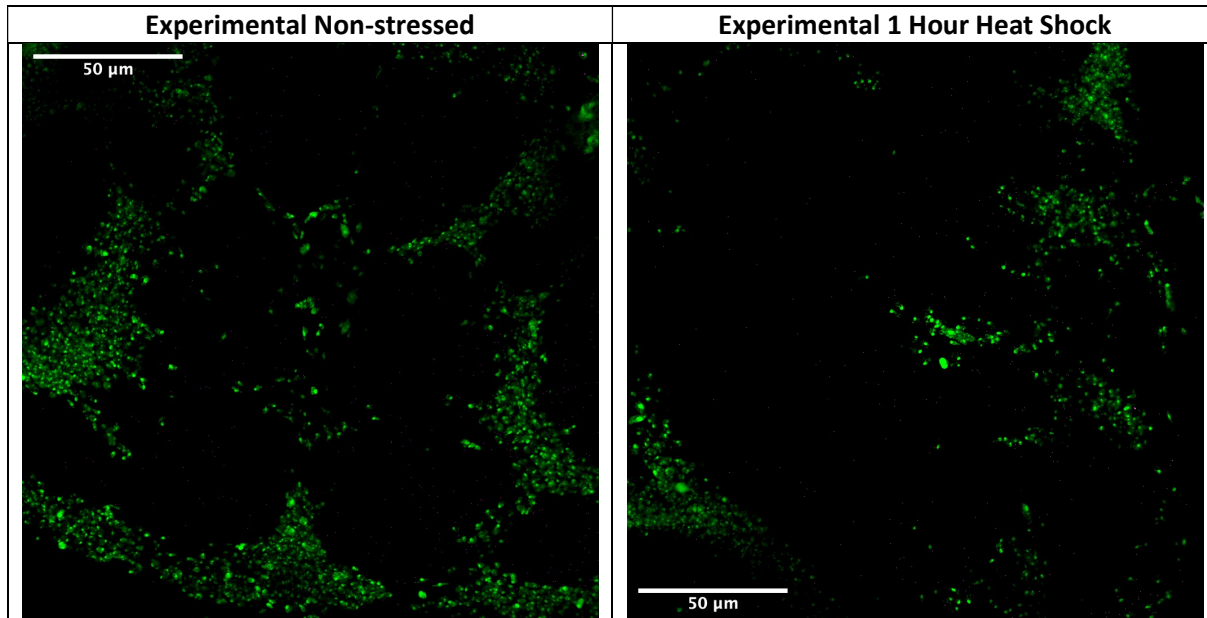


Figure 10. *mth<sup>1</sup>-rin-GFP/mth<sup>1</sup>-rin-GFP* fly brains imaged at 63x magnification. Green is GFP, and pink is Hoechst (nuclei).

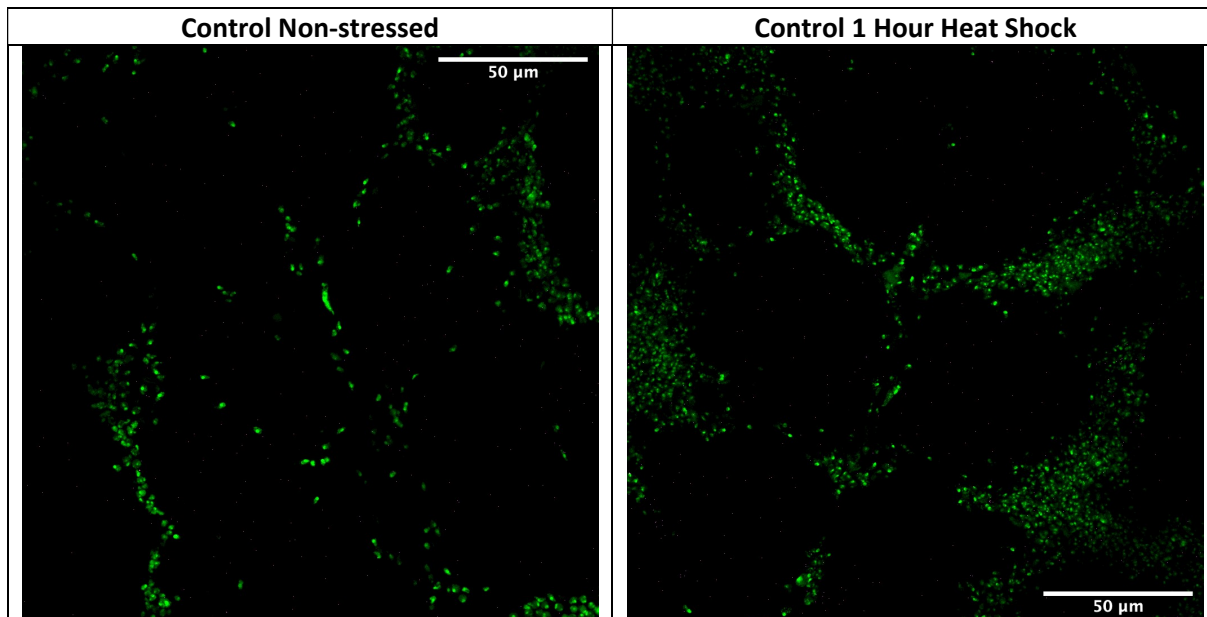


Figure 11. *+/+; rin-GFP/rin-GFP* fly brains imaged at 63x magnification. Green is GFP, and pink is Hoechst (nuclei).

The images at 20x magnification also support the hypothesis that *mth<sup>1</sup>* influences SG dynamics. While both genotypes see a response to heat shock, the experimental flies show a greater response than the control flies. One interesting aspect of this finding is that these flies

are only 2 days old. As seen in the lifespan study above, *mth<sup>1</sup>* produces extension of lifespan not by increasing average or early survival, but rather at the end. This may initially lead one to suppose that *mth<sup>1</sup>* increases fitness at the end of the fly's lifespan, such as preventing age-related decline in function. However, prior studies have had similar findings to this one: *mth<sup>1</sup>* improves sensorimotor function in young flies and actually causes a decrease in function compared to controls at old age (Petrosyan et al., 2007).

This thesis found that *mth<sup>1</sup>* flies live significantly longer than *w<sup>1118</sup>* flies. This increase stems mostly from an increase in survival late in life as opposed to a marked increase in average lifespan. While it is unclear if *mth<sup>1</sup>* flies survive heat shock at greater rates, these flies were more lethargic 30 minutes after recovery. Both *mth<sup>1</sup>* flies and the control saw an increase in GFP in response to heat shock, indicating the formation of SGs, and the *mth<sup>1</sup>* flies saw a larger increase, indicating altered SG dynamics. It remains unclear if this increase in SGs is protective.

There are many avenues for further research. To confirm whether the recombinant *mth<sup>1</sup>-rin-GFP* flies are long-lived, a lifespan study should be conducted on *rin-GFP* flies without DsRed and then compared to the results for the *mth<sup>1</sup>-rin-GFP* flies. In light of previous findings about *mth<sup>1</sup>* age-dependent effect on sensorimotor function, the heat shock experiments should be repeated at middle-aged and old-aged time points to discern if *mth<sup>1</sup>* still has the same (or any) effect later in life (Petrosyan et al., 2007). The overall size of SGs is also only one aspect of SG dynamics. Given past findings that *mth<sup>1</sup>* alters the composition of SGs in *C. elegans* in old age, SG composition in *mth<sup>1</sup>* should be compared to controls at different points in life (Cao et al., 2020).

#### 4. LAB SET-UP FIGURES

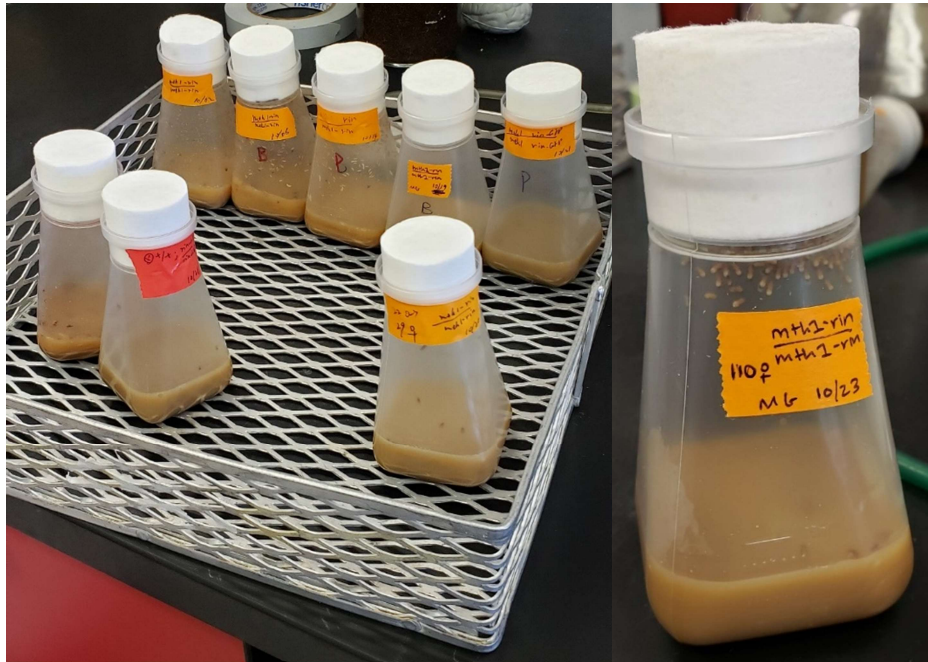


Figure 12. Bottles with 50 mL of food, used for storing a large number of flies.



Figure 13. Anesthesia CO<sub>2</sub> blow gun (left) and CO<sub>2</sub> tank (right).

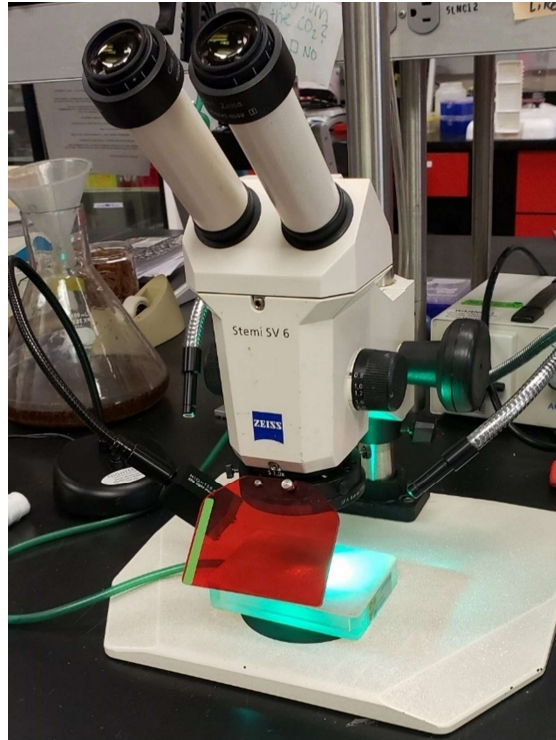


Figure 14. The set-up for screening flies for the presence of red fluorescence in the eyes using a NIGHTSEA Stereo Microscope Fluorescence Adapter with a green filter.

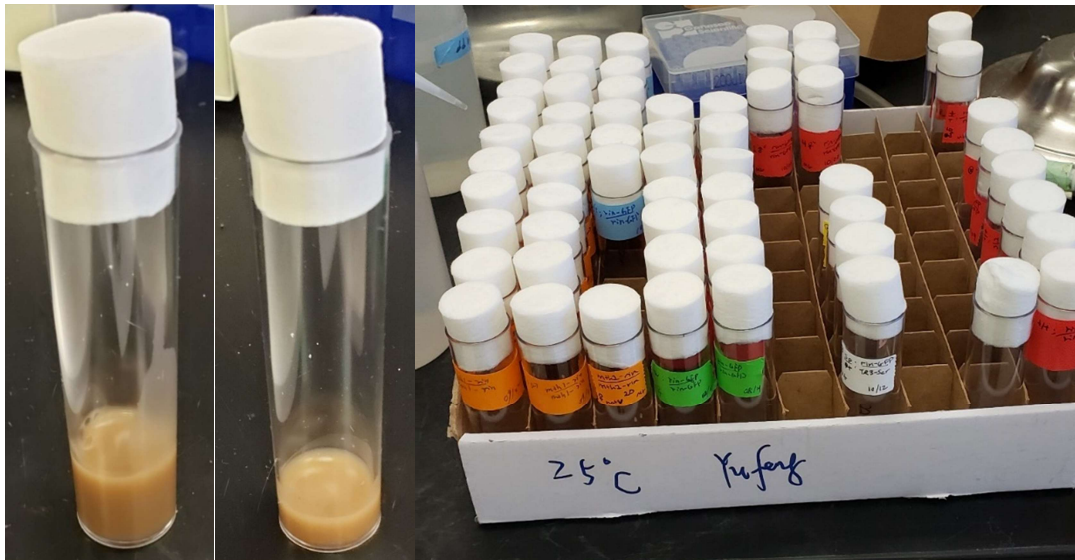


Figure 15. Vial with 5 mL of food, used for crosses and stocks (left); vial with 3 mL of food, used for storing a small number of flies (center); tray of vials (right).

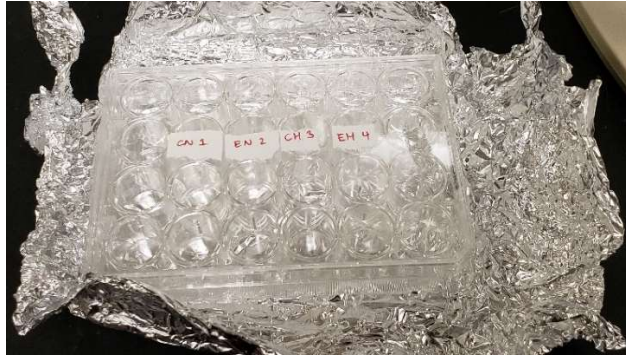


Figure 16. Labelled 24-well-plate which contains the brains during blocking and staining. Also shown is the aluminum foil it is wrapped in between steps.



Figure 17. Forceps used for brain dissections, as well as adjusting the positioning of the brains when necessary during subsequent washing, blocking, staining, and mounting.



Figure 18. Dissection set-up. Petri dish is illuminated beneath the microscope; flies are knocked out on the CO<sub>2</sub> pad to the left, and fly brains are transferred to microcentrifuge tubes in the ice bath to the right. Forceps are placed on a Kim wipe when not in use to prevent contamination.

## 5. GROUP WORK

MyDuyen Tran dissected a portion of the brains, assisted in setting up the heat shock experiments, and provided guidance on washing, staining, and mounting.

Yufeng Liu set up, and collected from, Control Crosses 3 and 4.

Rebekah Keating Godfrey imaged the fly brains and provided guidance on staining.

Erik Michael Lehmkuhl assisted with lifespan for the *mth<sup>1</sup>-rin-GFP/mth<sup>1</sup>-rin-GFP* and *mth<sup>1</sup>* flies when the pandemic limited access to the lab, both collecting a portion of the virgins and counting the collected flies.

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