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Measuring mtDNA in Drosophila

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A look at DNA content and depletion in *Drosophila* expressing a mitochondrially targeted nuclease

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Introduction

Role of the mitochondria

The mitochondrion is the organelle responsible for ATP production, a molecule broken down for energy inside all cells. It produces ATP via oxidative phosphorylation, which consists of a sequence of redox reactions (Srivastava, 2017). The mitochondrion also has its own genome made of mitochondrial DNA (mtDNA) which is stored in small circular chromosomes. This genome mostly codes for proteins involved in cellular metabolism (Salminen, 2020). Mitochondria are often studied in relation to aging due to their importance to cells' functionality, and due to their dysfunction being linked to shortened lifespan and increased aging (Salminen, 2020) (Cho, 2011).

Mitochondrial dysfunction

Mitochondrial dysfunction, or stress, can be caused in several ways. During metabolism, reactive oxygen species (ROS) are produced as a by-product, and as these accumulate over time, they can lead to increased oxidative stress which causes cellular and DNA damage (Cho, 2011) (Salminen, 2020). Dysfunction can also arise from the accumulation of mtDNA mutations and mtDNA damage, which both affect the proteins encoded by mtDNA, which are essential for the organelle's functioning (Ide, 2001) (Srivastava, 2017).

The effects of mitochondrial dysfunction are diverse. Mitochondrial dysfunction can lead to compromised oxidative phosphorylation, which further increases the release of ROS, resulting in widespread cellular damage and augmented mitochondrial stress (Cho, 2011) (Salminen, 2017). Resulting oxidative stress and mtDNA damage can lead to a decreased copy number of mtDNA, which further impairs the organelle (Ide, 2001) (Salminen, 2017). Damaged mtDNA can leak into the cytoplasm and trigger the cell's innate immune response, which, when

chronically activated, is linked to shortened lifespan and increased neurodegeneration and aging (West, 2015) (Kounatidis, 2017).

Artificial mitochondrial dysfunction

Mitochondrial dysfunction can be artificially induced through damage or degradation of mtDNA to cause stress. One method to deplete mtDNA is by expressing a nuclease that specifically targets and degrades mtDNA. One such nuclease, UL12.5, comes from the herpes simplex virus 1 genome and uses its mitochondrial matrix localization sequence to traffic to the mitochondria. Once in the mitochondria, the nuclease begins to degrade mtDNA (Corcoran, 2009). It has been shown that UL12.5 expression leads to mtDNA depletion in osteosarcoma cells, which means that the nuclease's attack on mtDNA decreases the amount of mtDNA in cells (Saffran, 2007). UL12.5 avoids nuclear DNA by steering clear of the nucleus entirely and remaining mostly in the cytoplasm or mitochondria (Reuven, 2004).

Using UL12.5 to study aging

Because UL12.5 causes mtDNA degradation and depletion, we can use its expression in model organisms to study the effects of mtDNA damage on aging (Figure 1). Our lab has investigated the downstream effects of UL12.5 expression on several age-related factors, namely lifespan and innate immunity. We found that upregulated UL12.5 expression leads to an uptick in the production of diptericin in flies, an antimicrobial peptide (AMP) produced during the innate immune response (Garschall, 2022) (Wang, 2019). This suggests that increased UL12.5 expression leads to heightened innate immune activity. Due to other research indicating that mtDNA damage causes upregulated immune activity via mtDNA leakage into the cytoplasm where it is recognized as foreign by the cell, it is likely that UL12.5-mediated mtDNA damage is

causing this same leakage, thus triggering the innate immune response of the cell (West, 2015) (Kounatidis, 2017) (Ide, 2001) (Salminen, 2017).

We have also looked at the impact UL12.5 expression has on lifespan, and we found that increased UL12.5 expression was correlated with shortened lifespan (Wang, 2019). Due to our previous finding that UL12.5 expression results in a heightened innate immune response, and other research showing that chronically heightened innate immunity is linked to shortened lifespan, it stands to reason that UL12.5 shortens lifespan in flies via an upregulated innate immune response (West, 2015) (Kounatidis, 2017).

Lastly, we are currently investigating the effects of UL12.5 expression on flies' resistance to bacterial challenge. Research has shown that upregulation of certain pathways involved in the innate immune response protects flies against bacterial infection but decreases lifespan when no bacterial challenge is presented (Sciambra, 2021). Our previous research shows that UL12.5 expression is linked to an upregulated innate immune response, and we are looking to see if flies that have a UL12.5-mediated hyperactive innate immune response are also protected against bacterial infection.

In order to study the downstream effects of UL12.5 expression, it is important to validate the upstream steps to verify that UL12.5 is having the molecular effects we expect. The effects we expect are the degradation of mtDNA leading to depletion of mtDNA content as it leaks out of the mitochondria (Figure 1). In this paper, we will be discussing the assay developed to validate the depletion of mtDNA resulting from UL12.5 expression.

To what extent does UL12.5 expression deplete mtDNA?

In this study, we aimed to develop an assay to determine the extent of mtDNA depletion as a result of UL12.5-mediated mtDNA degradation in *Drosophila*. We used quantitative-PCR to

compare copy number of a mitochondrially encoded gene between flies that had varying levels of UL12.5 expression to see if increased expression of the nuclease correlated to decreased mtDNA content. These results will be useful in the investigation of other downstream effects of mtDNA degradation, which we use to better understand the effects of mitochondrial dysfunction on aging.

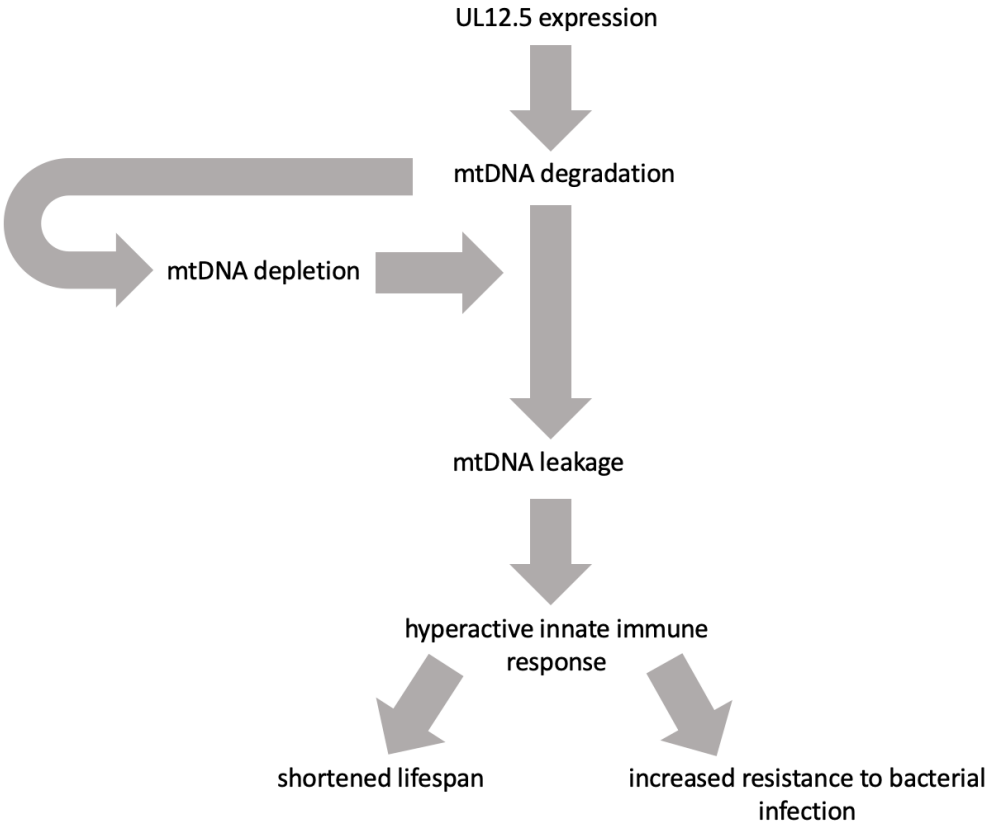

Figure 1. Flow-map of the effects of UL12.5 expression in *Drosophila*. UL12.5 degrades mtDNA causing depletion and leakage of this DNA into the cytoplasm of cells. This triggers an upregulated innate immune response because the cell recognizes this mtDNA as a foreign molecule and destroys it as it leaks out. Chronically heightened innate immunity has been linked to worsened aging, or shortened lifespan, as well as improved resistance in the face of bacterial challenge.

Methods

Fly model for temperature dependent UL12.5 expression

We first created flies that had varying levels of UL12.5, from which we would later measure mtDNA content. Because expression of UL12.5 is developmentally lethal when expressed in most tissues in fruit flies, we controlled its expression so that it was only expressed after flies had developed. To do so, we utilized the *hsp70*-GAL4 system to make the nuclease temperature dependent, and more specifically, synched to the expression of a heat shock protein, *hsp70*. *Hsp70* is a protein-folding chaperone that is expressed in *Drosophila* at temperatures higher than 25C, that serves as a response to heat shock or excessive heat (Lindquist, 1980). *Hsp70* is produced in response to temperature because it has a heat-sensitive promoter region. We took advantage of this by using the *hsp70* promoter region to control expression of UL12.5, rendering UL12.5 expression temperature dependent. In this system, one parental fly has the promoter region of *hsp70* controlling the transcriptional activator GAL4. GAL4 is a protein found in yeast. The other parental fly has an upstream activation sequence (UAS) that controls a gene of interest, which in this case, is the UL12.5 nuclease. Crossing these two parental flies together yields offspring containing all four of these components, with the genotype: UAS_UL12.5/*hsp70*-GAL4. In the offspring, heat will trigger the activation of the heat-sensitive *hsp70* promoter, which initiates the expression of GAL4. The GAL4 protein binds to UAS to promote expression of the gene it is controlling: UL12.5. In this way, temperature is indirectly controlling UL12.5 expression via the *hsp70* promoter (Figure 2). Linking UL12.5 expression to the heat-sensitive *hsp70* promoter allows the expression of the nuclease to be regulated by incubating the flies at different temperatures. Flies were reared and allowed to develop at 25C where only negligible *hsp70* promoter activity occurred, and therefore only negligible amounts

of UL12.5 were produced. They were then aged-out at a warmer temperature, allowing for increased expression of UL12.5 and the degradation of mtDNA to begin.

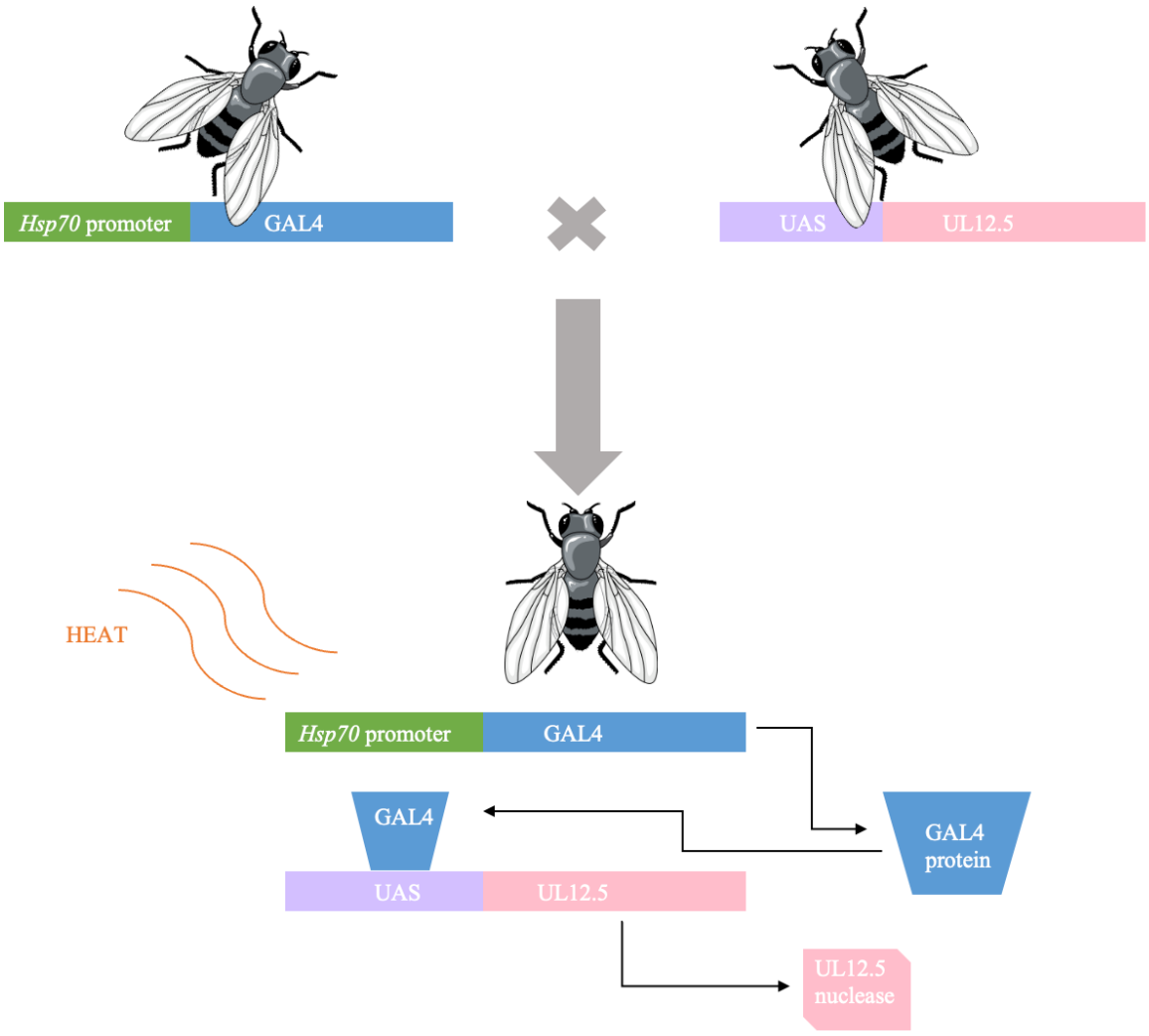

Figure 2. *Hsp70*-GAL4 system. This diagram shows how the *hsp70*-GAL4 system works to make UL12.5 expression temperature-sensitive. The male parental fly has the *hsp70* promoter controlling GAL4, and the female parental fly has UAS controlling UL12.5. When these flies mate, the resulting offspring have UL12.5 under the indirect control of the *hsp70* promoter region, making the nuclease's expression temperature dependent.

Creating and treating flies

We created our flies by crossing *hsp70-GAL4* males with UAS_UL12.5 females. We repeated this with two control genotypes, one of which expressed a catalytically dead version of the UL12.5 nuclease (UL12.5_dead), and the other, which was wildtype (W1118). These crosses were kept at 25C for 13 days to keep UL12.5 expression minimal. Then, in 48hr increments, eclosing flies were collected into mating vials where they were mated for 24 hours, before being sorted by sex and phenotype. We considered flies to be 1 day old on the day of collection (Figure 3). Flies were separated into groups to be aged out to 10-11 days old at the following appropriate temperatures. We exposed flies of all three genotypes to three different temperature treatments: 29C for 10-11 days and heat shock on the 10th-11th day (29C+HS), 29C for 10-11 days (29C), and 25C for 10-11 days (25) (Figure 4). Heat shock consisted of a 55-minute, 37C water bath. Flies were transferred into empty vials and placed in the water bath while water temperature was monitored (it oscillated between 36-38.5C). Heat shocked flies were allowed to recover at 25C on fresh Caltech food vials for about 3 hours, and any flies that had died during the heat shock were removed. Then, regardless of heat treatment, all flies were flash frozen with liquid nitrogen and dry ice. UL12.5/*hsp70*-GAL4 flies aged out at 25C were expected to have negligible amounts of UL12.5 expression due to low temperature, whereas 29C UL12.5/*hsp70*-GAL4 flies were expected to have higher UL12.5 expression levels due to higher temperature. Heat shocking the UL12.5/*hsp70*-GAL4 flies served to show extreme upregulation of *hsp70* promoter activity and thus extreme upregulated UL12.5 expression. All UL12.5_dead/*hsp70*-GAL4 flies and W1118/*hsp70*-GAL4 flies were expected to have no UL12.5 expression.

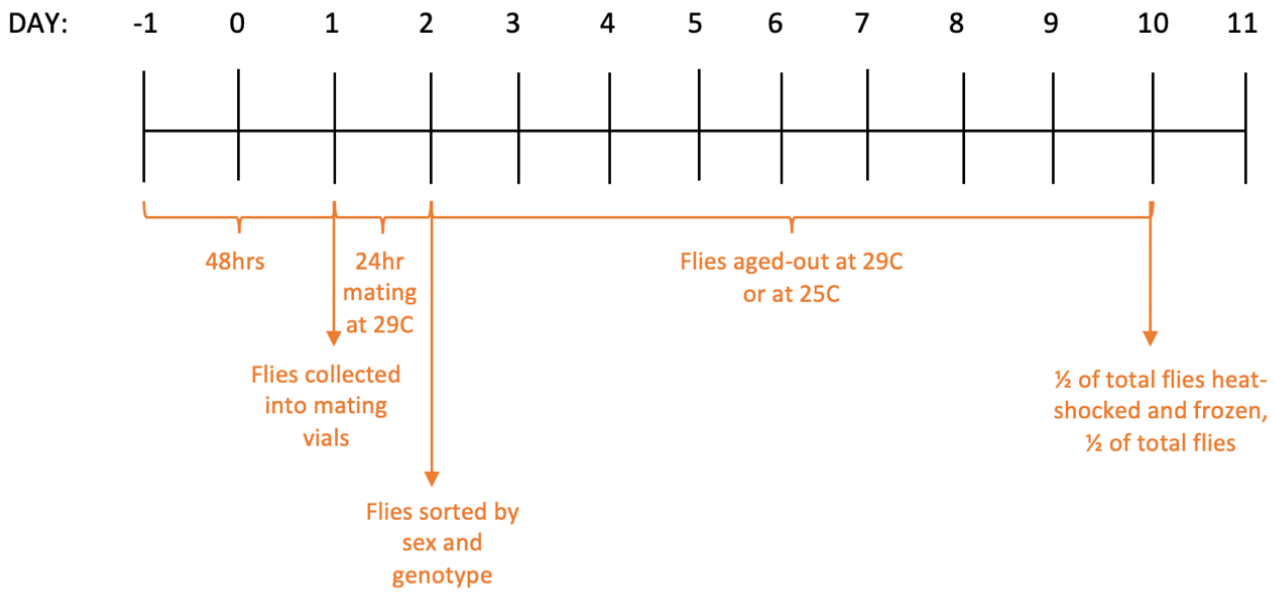

Figure 3. Timeline of fly collections. This timeline shows how old flies were (in days) at every step of our process: collection, mating, sorting, and freezing.

Heat Treatment	Sex	Age	Genotype		
29C for 10-11 days + HS on 10th or 11th day	♀	10-11 days	UL12.5 *Hsp70*-GAL4	UL12.5_dead *Hsp70*-GAL4	W1118 *Hsp70*-GAL4
29C for 10-11 days	♀	10-11 days	UL12.5 *Hsp70*-GAL4	UL12.5_dead *Hsp70*-GAL4	W1118 *Hsp70*-GAL4
25C for 10-11 days	♀	10-11 days	UL12.5 *Hsp70*-GAL4	UL12.5_dead *Hsp70*-GAL4	W1118 *Hsp70*-GAL4

Figure 4. Heat treatments of fly samples. This table shows how we split up our three genotypes of flies (UL12.5/*hsp70*-GAL4, UL12.5_dead/ *hsp70*-GAL4, and W1118/ *hsp70*-GAL4) into three groups to be subjected to three different temperature treatments. Flies were incubated for 10-11 days at the temperatures indicated under "Heat Treatment." Heat shock (HS) consisted of a 55-minute 37C water bath, and a 3-hour post-heat shock recovery period before freezing. All flies were frozen on day 10-11 regardless of heat treatment.

Obtaining samples

To extract template DNA for qPCR, samples were thawed, and DNA extracted by squishing one single frozen female fly into a squishing buffer (10mM Tris (pH 8.0), 1mM EDTA, 25mM NaCl, DI water) and pipetting out the supernatant. A NanoDrop was used to quantitate the DNA and ensure acceptable concentration and purity (at least 150ng/uL, and at least 1.30 Abs260/Abs280). This DNA was diluted to 10ng/uL using sterile water and used in the following qPCR assay.

Primers

Our goal was to compare mtDNA content between samples by using qPCR to target a mitochondrially encoded gene called cytochrome c oxidase subunit I (COXI). We chose to use COXI as our reporter gene because this gene is highly expressed in all cells in *Drosophila*, as it codes for a subunit of the cytochrome c oxidase complex, which is an essential part of the electron transport chain (Huynh, 2020). To compare mtDNA content across the different DNA samples, we needed to normalize each sample to a reference gene that was expressed in all cells. This gene also needed to be nuclearly encoded so that it would be safe from UL12.5 nuclease activity and degradation. We chose to use Tubulin as our normalization gene because it fit both requirements. We ran two separate qPCR reactions on the same plate, one that targeted COXI, our mtDNA reporter gene, and one that targeted Tubulin, the constitutively expressed protein that we used for normalization. The first set of primers targeted the mitochondrially encoded gene, COXI. The sequence of the forward primer is: 5'-GAGCTCATCATATATTTACCGTTGG-3', and the reverse is: 5'-CAACTCCTGTTAATCCTCCTACTG-3'. These primers anneal from base pair 2330 to base pair 2536 of the COXI gene to create a 207 base pair amplicon. We rested our analysis on the

assumption that levels of COXI expression would be representative of total mtDNA content and that this gene would be degraded equally to the rest of the mitochondrial genome. Our second set of primers targeted the nuclearly encoded gene Tubulin, which is the constitutively expressed protein we used for normalization of samples. The sequence of the forward primer is: 5'-CCTTCCCACGTCTTCACTTC-3', and the reverse is: 5'-TTCTTGGCATCGAACATCTG-3'. These primers anneal from base pair 5573 to base pair 5690 of the Tubulin gene to create a 118 base pair amplicon.

qPCR reaction

Both qPCR reactions contained the same set of samples, and only differed in the primers used (COXI-targeting or Tubulin-targeting). We used a 96-well optical plate to run a total of 90 10uL reactions (45 using the COXI master mix, and 45 using the Tubulin master mix). Each sample consisted of one single female fly from which DNA had been extracted earlier. The samples were all run in technical triplicates and represented all three genotypes (UL12.5/*hsp70*-GAL4, UL12.5_dead/*hsp70*-GAL4, W1118/*hsp70*-GAL4) and all three heat treatments (29C+HS, 29C, 25C). We also ran no-template control (NTC) triplicates for both sets of primers and a serially diluted W1118/*hsp70*-GAL4 25C DNA sample triplicate that served as a standard curve calibration, and was diluted in the following way: 1:20, 1:40, 1:80, 1:160, and 1:320. Both master mixes were made from 1X KAPA SYBER FAST One-Step qRT-PCR Master Mix (2X), 10uM appropriate forward primers, 10uM appropriate reverse primers, 10ng of template DNA, 1X 50X Low ROX, and PCR-grade water. The plate was run using the following thermocycler settings: 95C 10-minute initial denaturation, 95C 15-second denaturation, 57C 20-second annealing, 72C 1-second extension, and 72C, 5-minute final extension. Denaturation, annealing, and extension were repeated 40 times. We used the QuantStudioTM 3 Real-Time PCR System to

run a relative standard curve, SYBR Green, standard qPCR. Results were analyzed using the QuantStudio 3 Design and Analysis software.

Results

Mean quantification cycle (Cq) values, which are the number of qPCR cycles needed for the signal to cross a threshold of background noise, were obtained from the QuantStudio 3 Design and Analysis software. Standard deviation (SD) for each sample was calculated and triplicates omitted if they were more than 0.30 SD from the mean Cq value of the sample. A total of three wells were censored, and mean Cq values were recalculated for all samples. Next, we constructed standard curves for both sets of primers (Figure 5). The slopes of the linear trendline of these curves were used to calculate the primer efficiencies for each set of primers using the following formula: $\text{Efficiency}=-1+10^{(-1/\text{slope})}$. The efficiency of the COXI primers was 52.74% and the efficiency of the Tubulin primers was 66.81%. Next, each sample's COXI Cq value was normalized to Tubulin. To do this, we used the Pfaffl method, which is appropriate for relative quantification between sets of primers with efficiencies more than 5% apart. The formula used was the following: $(\text{Efficiency\_COXI}^{\text{Cq\_COXI}})/(\text{Efficiency\_Tubulin}^{\text{Cq\_Tubulin}})$. These ratios were plotted in Figure 6.

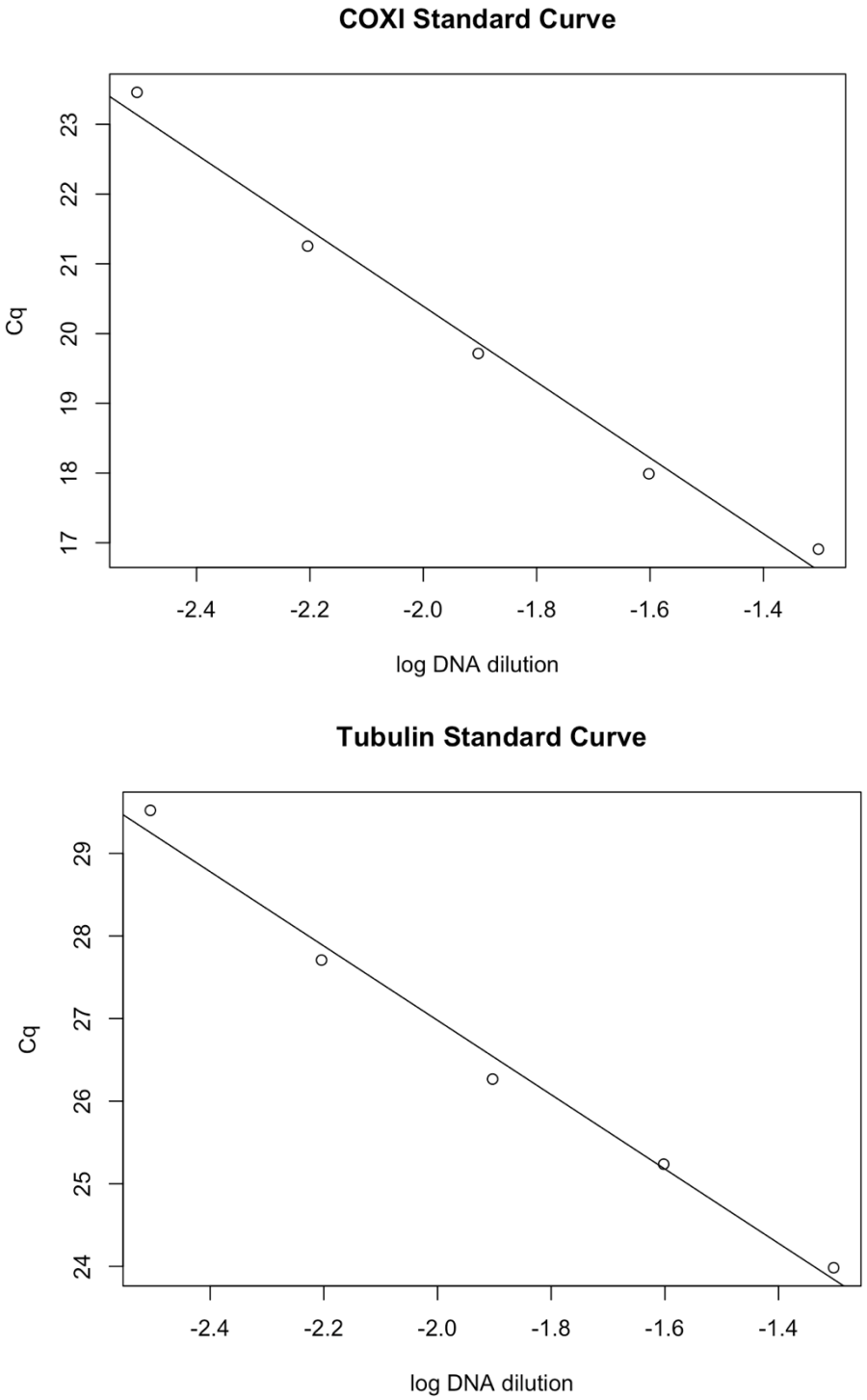

Figure 5. COXI and Tubulin standard curves. These figures represent the standard curves constructed by plotting the Cq values of the serially diluted 25C W1118/*hsp70*-GAL4 sample against the logarithm of the dilution of the sample: 1:20, 1:40, 1:80, 1:160, 1:320. A linear trendline was added using RStudio, and the slope of this line was used to calculate the efficiencies of both sets of primers using this equation: Efficiency=$-1+10^{(-1/\text{slope})}$. The efficiency of the COXI primers was 52.74% and the efficiency of the Tubulin primers was 66.81%.

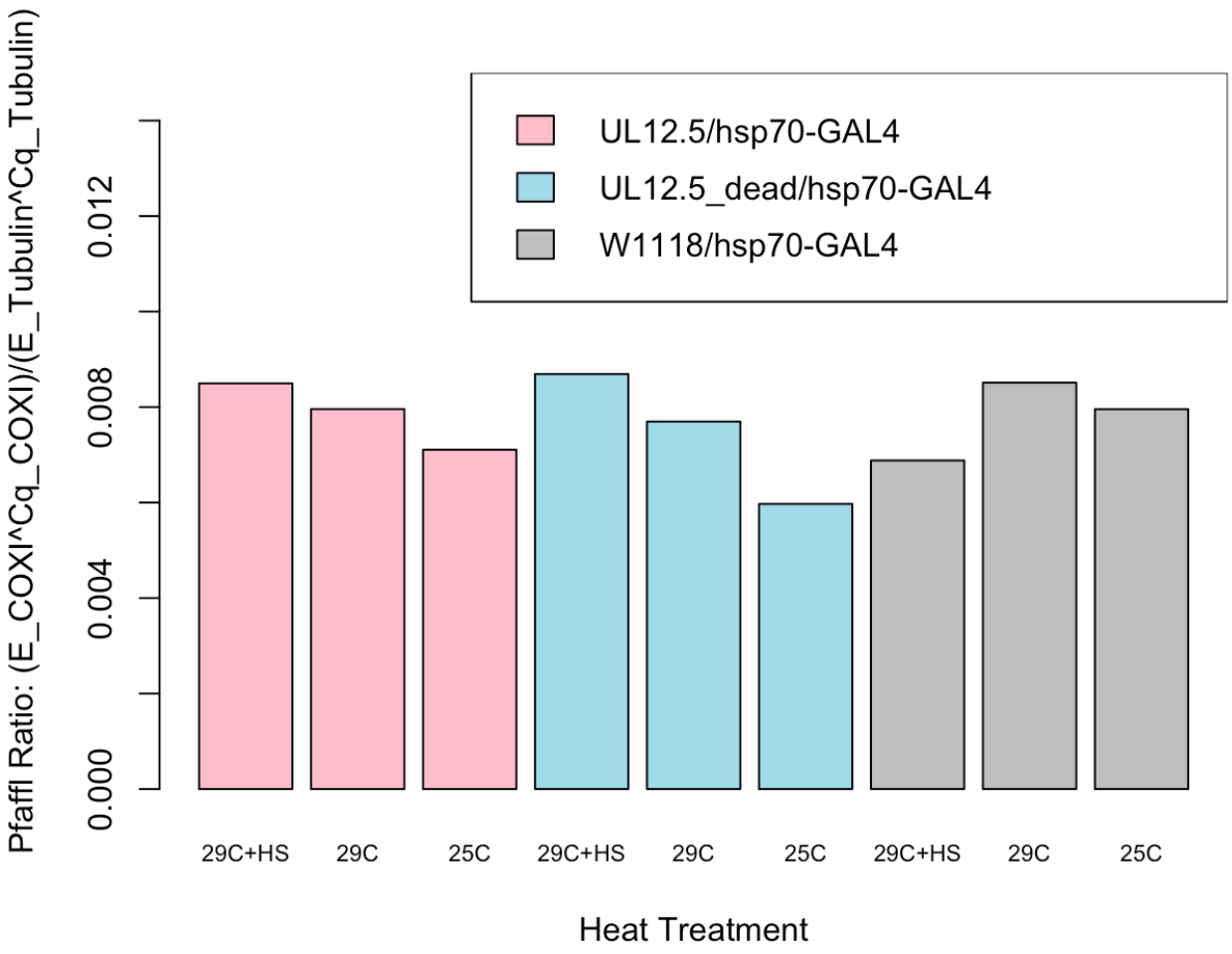

Figure 6. Bar plot of Pfaffl ratios to compare COXI content in *Drosophila* expressing varying levels of UL12.5. This figure shows the Pfaffl ratios normalizing COXI Cq values to Tubulin Cq values to compare levels of COXI across flies of the following three genotypes: UL12.5/*hsp70*-GAL4, UL12.5_dead/*hsp70*-GAL4, W1118/*hsp70*-GAL4, treated with the indicated heat treatments: 29C for 10-11 days + 55-minute heat shock at 37C followed by 3-hour recovery period and freezing, 29C for 10-11 days and freezing, 25C for 10-11 days and freezing. Figure made in RStudio.

A one-way ANOVA (analysis of variance) was run to test for significant differences between any of our nine samples, and the resulting p-value was 0.14. To be significant, this value must be less than 0.05. We expected that the 29C+HS UL12.5/*hsp70*-GAL4 29C sample would have extreme levels of UL12.5 expression, and thus significantly decreased mtDNA content compared to all 25C samples and UL12.5_dead/*hsp70*-GAL4 and W1118/*hsp70*-GAL4 samples, which we expected to have little or no UL12.5 expression, and thus high, healthy levels of mtDNA. We expected that the 29C UL12.5/*hsp70*-GAL4 flies would have levels of UL12.5 expression and mtDNA that were somewhere in between the previous two groups. We predicted this based on the idea that UL12.5 expression would increase as temperature increased, whether this temperature change was during heat shock, or through incubation. Instead, we observed no significant differences in COXI content in any of our samples.

Discussion

Optimizing qPCR

There are many pieces to discuss as to why our results deviated from our expectations. Our primer efficiencies did not fall within the desired range of 90%-110%, suggesting that our qPCR did not work. Because our efficiencies were so much lower than the optimal range, this means that amplification of our target genes, COXI and Tubulin, was not ideal and the results of the qPCR are not reliable. Low qPCR efficiency means that the assay needs to be optimized, and there are two main ways to approach this. First, it is likely that our annealing temperature was suboptimal, and this resulted in poor primer annealing and poor amplification as a result. To correct this, future work might consist of running a gradient qPCR, which would test a wide range of annealing temperatures to find the best one. Another way to approach this problem is to

try a different set of primers to see if using differently structured primers can improve the efficiency of the qPCR. Therefore, our next step is to rerun this qPCR using primers that target another mitochondrially encoded gene called cytochrome c oxidase subunit III, or COXIII. If trying the COXIII primers does not improve qPCR efficiency, a gradient qPCR will be run to optimize annealing temperature.

Lengthening post-heat shock recovery time

Our results suggest that our assay was not able to capture a change in mtDNA content as a result of UL12.5 expression. Our 29C+HS UL12.5/*hsp70*-GAL4 sample did not demonstrate the extreme drop in mtDNA content we had anticipated. We had predicted this because this sample should have had the most UL12.5 expression as a result of being aged out at the warmest temperature and being heat shocked, and therefore, the most mtDNA degradation. The fact that we didn't see this is consistent with the qPCR not being sensitive enough to detect a change (see above), but an alternative explanation could be that UL12.5-mediated mtDNA degradation and resulting depletion is a more time-consuming process than we originally thought. After being heat shocked, flies were allowed to recover for 3 hours before being frozen, but 3 hours may not have been enough time for increased UL12.5 expression to exert observable effects. In the future, this protocol will be repeated with two longer recovery periods, 6 hours and 24 hours, to see if this allows us to capture UL12.5-mediated depletion of mtDNA.

There are also a couple of less likely explanations for our unexpected results. One approach to explain our results is that there may exist a strong compensatory mechanism that allows *Drosophila* experiencing mtDNA degradation to regenerate DNA faster than we can capture the initial depletion. A slightly more likely explanation is that our UL12.5 flies are not actually expressing the nuclease we think they are expressing. To verify that they are, we could

run another qPCR targeting UL12.5 and measure the levels of expression in our differently treated flies to see if the nuclease is present and how those levels vary based on temperature.

Conclusion

In conclusion, our results suggest that our assay was not able to detect a change in mtDNA content as a result of UL12.5 expression. Current efforts to amend our assay consist of re-running the qPCR with different primers that target COXIII, as well as extending the post-heat shock recovery period to 6 hours and 24 hours. If these efforts don't result in an improved qPCR efficiency as well as the ability to detect a decrease in mtDNA in heat shocked UL12.5/*hsp70*-GAL4 flies, we will investigate whether UL12.5 is actually present in our flies and explore the possibility of a compensatory mechanism. Doing so will further our endeavor to develop an assay that can measure mtDNA depletion as a result of UL12.5 expression, which we will use to validate projects looking at the further downstream effects of UL12.5 on aging.

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