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FUNCTIONAL STUDY OF TWO TESTIS-SPECIFIC NUCLEAR-ENCODED MITOCHONDRIAL GENE DUPLICATES IN DROSOPHILA MELANOGASTER

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FUNCTIONAL STUDY OF TWO TESTIS-SPECIFIC NUCLEAR-ENCODED MITOCHONDRIAL GENE DUPLICATES IN *DROSOPHILA MELANOGASTER*

by

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April 20, 2018

ABSTRACT

FUNCTIONAL STUDY OF TWO TESTIS-SPECIFIC NUCLEAR-ENCODED MITOCHONDRIAL GENE DUPLICATES IN DROSOPHILA MELANOGASTER

Mohammad Rashik, B.S. Biology

The University of Texas at Arlington, 2019

Faculty Mentor: Esther Betrán

This project involved the study of the fertility effects of lowering the amount of transcript (i.e., knocking down) two testis-specific duplicated genes in *Drosophila melanogaste*r (commonly known as the fruit fly) to understand their function. The Betrán lab discovered that many genes duplicated from parental genes with mitochondria related function. The lab also found that these duplicated genes acquired testis-specific expression by studying the function of these genes. The study of two specific genes can help elucidate the reason for the duplication. The first gene was *cytochrome c distal* (a.k.a *CG13263*). The second gene was *cytochrome c1 like* (a.k.a *CG14508*). RNA interference (RNAi) technology was used to knock down the genes at two different temperatures (25ºC and 27ºC). This was achieved in a single fly cross. The progeny of these flies were obtained and crossed with a standard laboratory strain (w^{1118}) and the number of progeny was

counted. Flies that did not have the Gal 4 driver were mated with w^{1118} flies, and this cross was considered the control group. Males from the *CG14508* and *CG13263* knockdowns showed significantly reduced fertility at 25ºC and complete sterility at 27ºC for the gene libraries that were tested (GD library for *CG13263* and KK library for *CG14508*). The results revealed that these genes are important for male reproduction. Furthermore, the study helped the understanding of the selective pressures that lead to the duplication of those genes. For *CG13263*, the RNAi served to confirm that the observed effects recapitulate the phenotype of a P-element insertion confirming RNAi usefulness as an approach to study gene function in spermatogenesis (i.e., serving as a positive control of the approach for the lab).

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CHAPTER 1

INTRODUCTION

1.1 Mitochondria and Testis-Specific Gene Duplicates

The mitochondrion is a very important organelle in the eukaryotic cell because it plays a major role in the production of Adenosine Triphosphate (ATP; Tait and Douglas, 2012). Therefore, it is generally considered as the powerhouse of the cell. Another unique characteristic of the mitochondria is that it contains its own DNA, which is called mitochondrial DNA (mtDNA; Giles et al., 1980). According to the endosymbiotic theory, the mitochondrion was originally obtained from bacteria, which invaded a prokaryotic cell around 1.5 billion years ago (Martin et al., 2015). The bacterial genomes are bigger than the mitochondrial genome because some of the genes from the mitochondria were lost during evolution and some transferred to the nucleus that are now part of the nuclear DNA (Martin et al., 2015). Currently, around 1500 genes are in the nucleus and have mitochondria function. These are called nuclear-encoded mitochondrial genes (N-mt genes). These genes encompass other genes derived from ancestral mitochondria genes that were transferred to the nucleus and additional genes that evolved mitochondrial function (Martin et al., 2015).

It is known that N-mt genes have duplicates in *D. melanogaster* (Gallach et al., 2010) and humans (Eslamieh et al., 2017). Duplication involves the formation of a copy of

a certain gene, which could maintain parental function (function that the original genes had) or acquire a new function (Ohno et al., 1968).

The expression pattern of N-mt duplicated genes in *D. melanogaster* shows that all tissue-specific genes are testis specific (i.e., highly expressed in testis; Gallach et al., 2010). However, the parental gene is expressed in every tissue, which suggests that this gene is required for every tissue (also known as housekeeping genes). The Betrán lab discovered this pattern and wants to understand why all tissue specific duplicated genes are testis specific. Studying the function of the testis-specific duplicates can help answer this question.

While in *D. melanogaster*, most N-mt duplicated genes are highly expressed in testis and have energy-related functions (Gallach et al., 2010), this pattern is different in humans (Eslamieh et al., 2017). The N-mt duplicated genes in humans not only express highly in testis but also have high expression in liver and skeletal muscle. There are; however, many glycolysis genes that are duplicated in mammals and are testis specific (Dorus et al. 2006; Wasbrough et al. 2010).

The availability of numerous genomic tools for *D. melanogaster* makes this species a great model organism for the study of the different functions of a gene and even the evolution of the genome. We can take advantage of the tools offered by this organism to study genes shared with other species. In this project; however, the genes studied are new duplicates and *Drosophila* specific, but follow patterns that have been observed in other species and are of interest because of this.

1.2 Genes of Interest

As mentioned above, in *D. melanogaster,* most N-mt duplicated genes are highly expressed in testis and have energy-related functions (Gallach et al., 2010). One of the genes involved in this project is called *cytochrome c1 like* and is also known as *CG14508*. It has two exons and is located on the right arm of the third chromosome of the fly. It is involved in mitochondrial electron transport and is part of mitochondrial oxidative phosphorylation complex III (a process involved in energy production; Gaudet et al., 2010). This gene is highly expressed in adult testis (Graveley et al., 2011). It is also expressed in the imaginal disc, larval fat body, and accessory glands at low levels (Graveley et al., 2011) likely due to contamination by gonads in those tissues (Swayer et al., 2017).

The other gene of interest is *cytochrome c distal* and is also known as *CG13263*. It has three exons and is located on the left arm of the second chromosome of the fly. It is involved in the process that regulates the rate of programmed cell death in the compound eye retina of the fly (Mendes et al., 2006). It also takes part in packing up spermatids in separate membranes (Arama et al., 2003). This gene is highly expressed in adult testis (Graveley et al., 2011). It is also expressed in the imaginal disc, larval fat body, and accessory glands at low levels (Graveley et al., 2011) likely due to contamination by gonads in those tissues (Swayer et al., 2017).

While the two genes are present in both male and female genomes, they highly express only in testis in the males. These observations led to the selection of these genes for the project. After obtaining more information about these genes, it was inferred that the removal of these genes could have a potential effect on male fertility. For *CG13263*, the RNAi served to confirm that the observed effects recapitulate the phenotype of a P-element

insertion (Castrillon et al., 1993) confirming RNAi usefulness (i.e., serving as a positive control for the approach).

1.3 The UAS-Gal4 System and Specific Fly Strains

In order to understand the function(s) of these testis-specific genes and the reasons for their duplication, *CG14508* was knocked down through RNA interference by using the UAS-GAL4 system. Knockdown of *CG13263* was used as a positive control because fertility effects have been observed for this gene before (Castrillon et al., 1993). Gal4 is a yeast protein-coding gene, which is inserted in flies as a transgene and expressed by a tissue-specific promoter in flies. Because of this, lines containing the transgene are called 'driver' strain (Kakidani and Ptashne, 1988). Another strain of transgenic flies is called the 'responder' and carries a transgene that includes the yeast enhancer called Upstream Activation Sequence (UAS) upstream of the inverted repeats of DNA for a region of the gene under study. When the driver line is crossed with the responder line, the Gal4 protein binds to the UAS to form the UAS-Gal4 complex in the progeny (Duffy, 2002). This complex drives the transcription of the inverted repeats that form a double-stranded hairpin RNA. The hairpin eventually is cleaved into small interfering RNAs (siRNAs; Duffy, 2002). These siRNAs interact with the mRNA produced by the gene of interest and trigger the degradation of the mRNA by the cellular machinery. As a result, the protein from that specific gene cannot be produced or is produced at lower levels. This effect is referred as gene knockdown and can reveal the effect of the loss of function of a gene.

It must be mentioned that the UAS-hairpin driver was implemented on two specific types of RNA interference fly strain libraries. The first one was the GD library where the P-element (a transposable element that acts as a vector of the transgene) had random

insertion sites in the fly's genome. The second one was the KK library, where the clone had a single and defined insertion site as a phage recombinase was used to insert the transgene. In simpler words, it basically means that the KK library had the inverted repeats inserted at a specific site whereas the GD library had the repeats placed at random sites. There are no definitive answers as to which library produces more efficient results. That is why both the libraries were used in the lab. Since GD and KK libraries targets have inverted repeats for different regions of the gene, they also serve as controls for the RNA interference effects. The expectation is that each of them would provide coinciding results.

1.4 The Hypothesis

Knockdown of the testis-specific genes *cytochrome c1 like* and *cytochrome c distal* individually leads to decreased fertility in the male of *Drosophila melanogaster*. In the case of *cytochrome c distal*, this result will act as a positive result as infertility has been observed for that gene in previous studies. The spermatogenesis defects will be explored further at the cytological level by another undergraduate researcher in the lab.

CHAPTER 2

METHODOLOGY

The driver flies with the Bam-Gal4 were provided by the Dr. Michael Buszczak lab at UT Southwestern Medical Center. Bam-Gal4 flies were crossed to a fluorescent protein stock to see that it drives in the male germline. The responder flies that carry the UAS-RNAi line for the gene *cytochrome c like* (strain ID: 106229, KK library, genotype P{KK105518}VIE-260B) and *cytochrome c distal* (strain ID: 17128, GD library, genotype w1118; P{GD7970}v17128) were obtained from the Vienna Drosophila Resource Centre (Dietzl et al., 2007).

The strains of flies were kept in vials at 25ºC in an incubator with sufficient food stock in each vial. These vials were changed every thirty days in order to get the next generation of flies. Before collecting virgins, a period of 12 days was allotted for the flies to breed and lay eggs. The figures presented below depict a typical vial, a case used to keep the flies, and the incubator used to keep the flies.

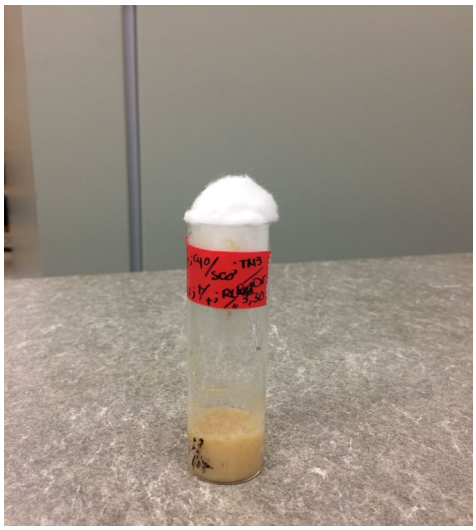

Figure 2.1: Vial Used to Keep Flies

Figure 2.2: Tray Used to Keep Vials of Flies

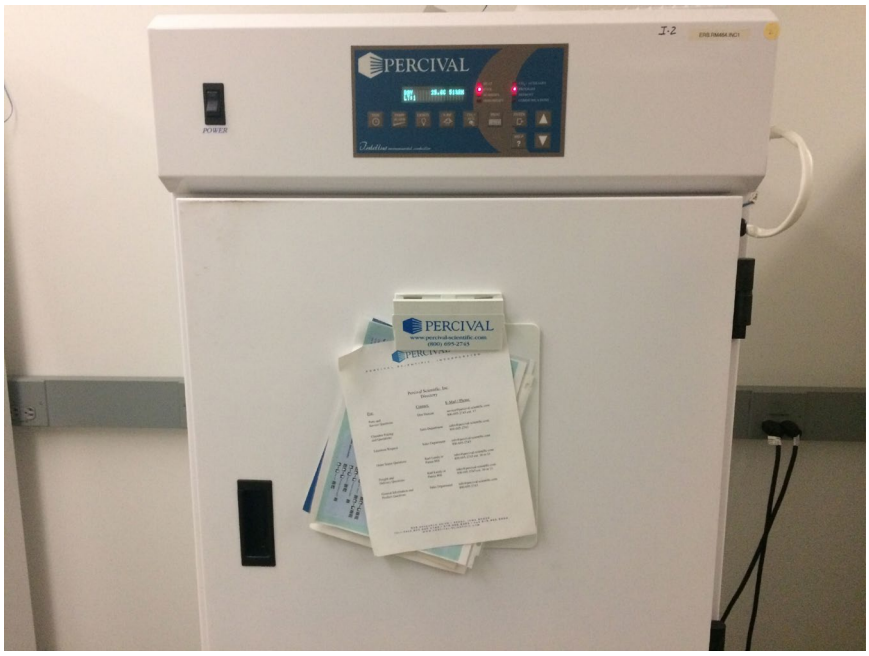

Figure 2.3: Incubator Used to Store Vials Under Control Temperature and with Day and Night Cycle of Light of 12 Hours

During the time of collection, flies were dumped out early in the morning and virgins were collected in separate vials after a period of 8 hours. This was done to separate the males and females before they mature to perform controlled crosses. Observing the differences in genitalia of the flies helped in the separation of males and females. The males have dark and round genitalia at the tip of the abdomen, whereas females have genitalia that is light and pointy. Apart from that, male flies are significantly smaller than the females. This fact was used for separation of the sexes. The flies were placed on the carbon dioxide pad and the specimens were knocked unconscious through the flow of carbon dioxide gas through the tank. After that, the pad was placed under the microscope and the genitalia was observed to separate the males from the females. The figures below depict the microscope, carbon dioxide pad, and the use of microscope in order to separate and collect flies.

Figure 2.4: Microscope and Carbon Dioxide Pad (White Pad with Tube Attached to the Base of the Microscope)

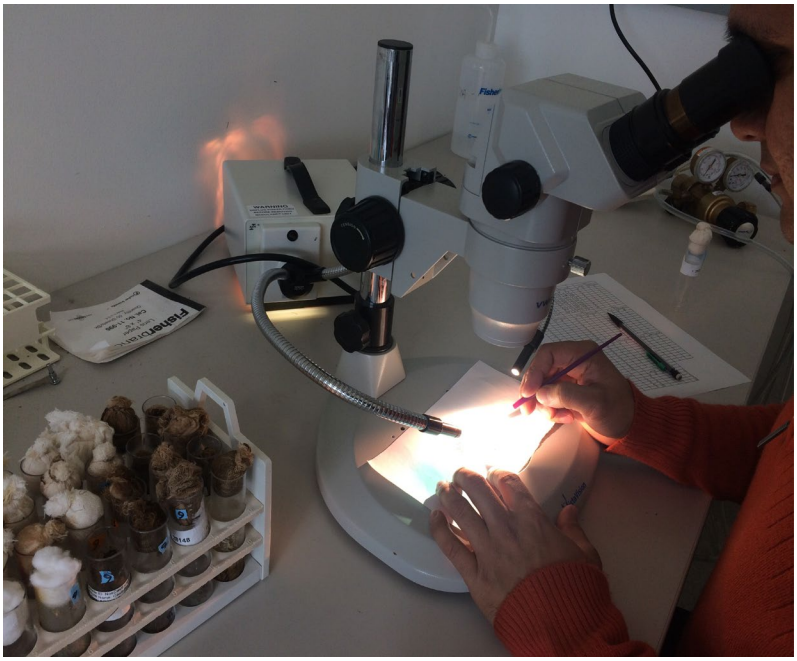

Figure 2.5: A Lab Member Using the Microscope

The crosses were done at two temperatures (25°C and 27°C). The maximum efficiency of UAS-Gal4 is at 29°C. However, this temperature has a detrimental effect on

male fertility (Ben-David et al., 2015). That is why the room temperature (25°C) and an increased temperature (27°C) were used to enhance the effects of the UAS-Gal4 RNAi.

To establish a control group (control group 1), *white* mutant flies that carry the genomic background of the VDRC lines (strain 60100 from VDRC; w^{1118}) female flies, kept at 25°C, were crossed with the males from the UAS-RNAi line. In order to complete the cross, two males were placed in a vial with three virgin females and were placed in an incubator with a temperature setting of 25ºC for a period of 11 days. After this period, the male offspring of the F1 generation were obtained and crossed with w^{1118} female flies for the two different temperature settings. The first temperature setting was 25ºC. The vials were placed in the incubator set to 25ºC for a period of 14 days. The second temperature setting was 27ºC. The vials were placed in the incubator set to 27ºC for a period of 11 days. This time, the vials contained one male and two females. The progeny of the second cross was then obtained and the total number was counted in each vial. The number of male and female offspring was individually counted. The number of days at the different temperatures was set to guarantee that most of the individuals would have emerged. For all of the trials, three replicates were done for the experimental and the control crosses. A second control group (control group 2) was created using the same procedure, but instead of using female w^{1118} flies for the cross with the male UAS RNAi line, male w^{1118} flies were crossed with the female UAS RNAi line. Then the female offspring of the F1 generation from this line were crossed with male w^{1118} flies. It should be noted that the w^{1118} flies are mutants and have white eyes and the genotype: w^{1118} w^{1118};+/+;+/+ females and w^{1118} Y;+/+;+/+ males. In contrast, the other flies do not carry the alleles for the white eyes and

are therefore red in color. These crosses are depicted in Figure 2.6 and tested males carrying one and the other transgene as controls. The transgenes carry a mini *white* reporter gene.

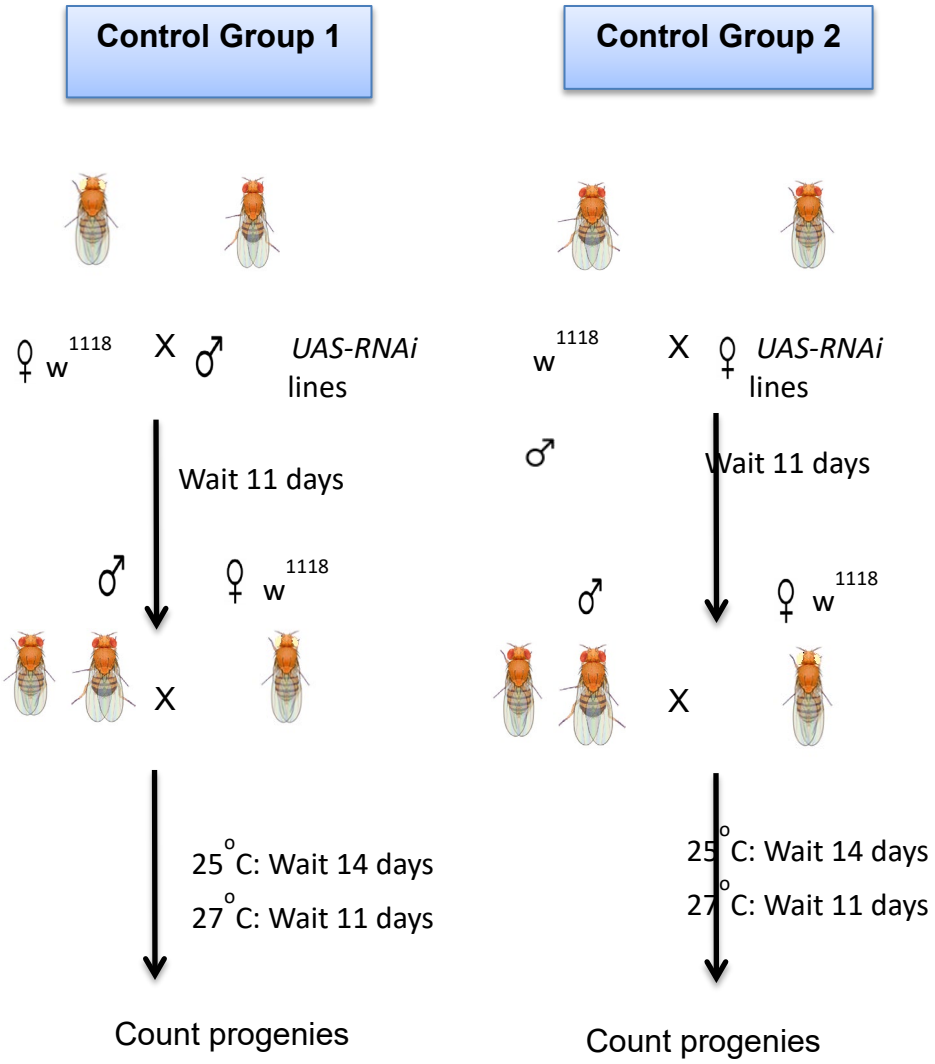

Figure 2.6: Design of the Control Crosses

To establish the experimental group, female Bam-Gal4 flies were crossed with male flies from the UAS-RNAi gene-specific line. Bam-Gal4 flies (genotype P{hs-bam.O}18d, w1118) contain a transgene that drives Gal4 with the expression pattern of Bam (i.e.,

germline specific expression; Wang et al., 2008). Two males were placed in a vial with three females and put in an incubator with a temperature setting of 25ºC. After a period of 11 days, the male progenies of the F1 generation were collected and crossed with female w^{1118} flies and received two different temperature settings. The first temperature setting was 25ºC. The vials were placed in the incubator set to 25ºC for a period of 14 days. The second temperature setting was 27ºC. The vials were placed in the incubator set to 27ºC for a period of 11 days. The progeny of the F2 generation were then obtained and the total number was counted in each vial. The number of males and female offspring was individually counted. For all of the trials, three replicates were done for the experimental groups. A second experimental group was created using the same procedure, but instead of using female Bam-Gal4 flies for the cross with the male UAS RNAi line, male Bam-Gal4 flies were crossed with the female UAS RNAi line. Then the female offsprings of the F1 generation from this line were crossed with male w^{1118} flies. This is an additional control because no fertility effect is expected. An illustration of the design of crosses is shown in Figure 2.7.

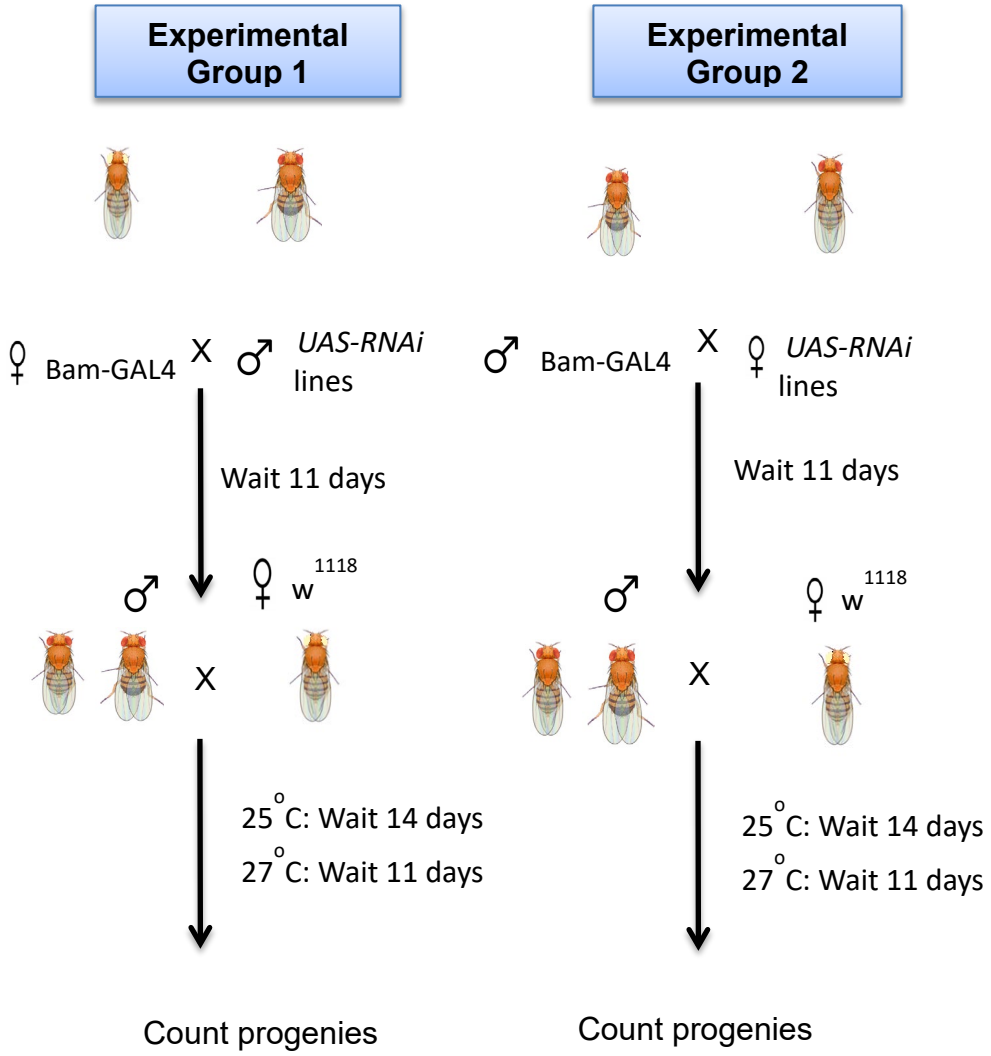

Figure 2.7: Design of the Experimental Crosses to Drive RNA Interference

After the progenies were counted for each cross, the data of all the replicates were entered in their respective tables in Microsoft Excel. Excel was used for the calculation of mean and standard error of the mean values. The data from the tables were used to construct bar graphs where the number of progenies of the experimental groups and control groups were placed next to each other. Error bars were drawn on each of the bars and the differences between the mean of the experimental and control were tested using a t-test

(Sokal and Rohlf, 1995). The arbitrary critical value used for statistical significance was 0.05.

CHAPTER 3

RESULTS

3.1 Results from *Cytochrome c Distal* (GD Library, Line 17128) RNAi at 27ºC

During experimentation, the flies containing the knockdown of the gene *CG13263* were recorded as G7 for convenience instead of writing *CG13263* for every cross. A total of four experimental and four control crosses were performed.

3.1.1 Experimental and Control Cross 1

The results from the experimental cross of female progenies from the F1 generation (female G7 crossed with male Gal4 at 27ºC F1 generation) with male w^{1118} flies have been presented below. The results from the control cross of female progenies from the F1 generation (female G7 crossed with male w^{1118} at 27ºC gave F1 generation) with male w^{1118} flies were also included.

Table 3.1: Female Fertility Test ♀ x ♂ at 27ºC

Experimental Crosses			Control Crosses		
♀ [♀G7 x ♂Gal4](27ºC) x♂ (w^{1118} 25ºC)			♀[♀G7 x ♂ w^{1118}] (27ºC) x♂ (w^{1118} 25ºC)		
Number of offspring			Number of offspring		
Male	Female	TOTAL	Male	Female	TOTAL
26	14	40	7	4	11
19	18	37	8	5	13
22	25	47	6	8	14
Mean		41.3	Mean		12.7
Standard deviation		5.1	Standard deviation		1.5
Standard error		3.0	Standard error		0.9

The control cross revealed a mean of 41.3 flies with a standard deviation of 3.0. The control cross showed a mean of 12.7 flies with a standard deviation of 1.5. A two tailed t-test was done and a P-value of 0.000752 was obtained. This is a significant value and shows that females are more fertile.

3.1.2 Experimental and Control Cross 2

The results from the experimental cross of male progenies from the F1 generation (female G7 crossed with male Gal4 at 27ºC gave F1 generation) with female w^{1118} flies have been presented below. The results from the control cross of male progenies from the F1 generation (female G7 crossed with male w^{1118} at 27ºC gave F1 generation) with female w^{1118} flies were also included.

Table 3.2: Male Fertility Test ♀ x ♂ at 27ºC

Experimental Cross			Control Cross		
♂ [♀G7 x ♂Gal4](27°C) x ♀ w^{1118} (25°C)			♂ [♀G7 x ♂ w^{1118}](27°C) x ♀ w^{1118} (25°C)		
Number of offspring			Number of offspring		
Male	Female	TOTAL	Male	Female	TOTAL
4	7	11	3	3	6
14	7	21	0	7	7
0	0	0	4	3	7
Mean		10.7	Mean		6.7
Standard deviation		10.5	Standard deviation		0.6
Standard error		7.4	Standard error		0.4

The experimental cross revealed a mean of 10.7 flies with a standard deviation of 10.5. The control cross showed a mean of 6.7 flies with a standard deviation of 0.6. A two tailed t-test was done and a P-value of 0.546 was obtained. This value is greater than 0.05, which means that no significant differences have been observed.

3.1.3 Experimental and Control Cross 3

The results from the experimental cross of female progenies from the F1 generation (male G7 crossed with female Gal4 at 27ºC gave F1 generation) with male w^{1118} flies have been presented below. The results from the control cross of female progenies from the F1 generation (male G7 crossed with male w^{1118} at 27ºC gave F1 generation) with male w^{1118} flies were also included.

Table 3.3: Female Fertility Test Reciprocal Cross (i.e., ♂ x ♀) at 27ºC

Experimental Cross			Control Cross		
♀[♂G7 x ♀Gal4] (27°C) X ♂ w^{1118} (25°C)			♀[♀G7 x ♂ w^{1118}] (27°C) X♂ w^{1118} (25°C)		
Number of offspring			Number of offspring		
Male	Female	TOTAL	Male	Female	TOTAL
13	11	24	6	4	10
9	9	18	19	7	26
6	8	14	12	10	22
Mean		18.7	Mean		19.3
Standard deviation		5.0	Standard deviation		8.3
Standard error		2.9	Standard error		4.8

The experimental cross revealed a mean of 18.7 flies with a standard deviation of 5.0. The control cross showed a mean of 19.3 flies with a standard deviation of 8.3. A two tailed t-test was done and a P-value of 0.911 was obtained. This value is greater than 0.05, which means that no significant differences have been observed.

3.1.4 Experimental and Control Cross 4

The results from the experimental cross of male progenies from the F1 generation (male G7 crossed with female Gal4 at 27ºC gave F1 generation) with female w^{1118} flies have been presented below. The results from the control cross of male progenies from the

F1 generation (male G7 crossed with male w^{1118} at 27ºC gave F1 generation) with female w^{1118} flies were also included.

Table 3.4: Male Fertility Test Reciprocal Cross (i.e., ♂ x ♀) at 27ºC

Experimental Cross			Control Cross		
♂[♂G7 x ♀Gal4] (27°C) X ♀ w^{1118} (25°C)			♂[♂G7 x ♀ w^{1118}] (27°C) X ♀ w^{1118} (25°C)		
Number of offspring			Number of offspring		
Male	Female	TOTAL	Male	Female	TOTAL
7	14	21	21	17	38
2	13	15	19	16	35
6	5	11	22	18	40
Mean		15.7	Mean		37.7
Standard deviation		4.2	Standard deviation		2.5
Standard error		3.0	Standard error		1.8

The experimental cross revealed a mean of 15.7 flies with a standard deviation of 4.2. The control cross showed a mean of 37.7 flies with a standard deviation of 2.5. A two tailed t-test was done and a P-value of 0.00248 was obtained. This value is smaller than 0.05, which means that significant differences are observed and experimental male flies show reduced fertility in comparison to the controls.

3.1.5 Summary of Results

The results were combined in a bar graph represented below. The experimental group (blue bar) was placed next to its corresponding control group (red bar). The data showed a significant decrease in fertility for experimental males in comparison to control males for experimental and control cross 4 (♂ [♂G7 x ♀Gal4] (27°C) X ♀ w^{1118} (25°C)) and control cross 4 (♂ [♂G7 x ♀ w^{1118}] (27°C) X ♀ w^{1118} (25°C)). However, there was a significant increase in fertility for experimental males in comparison to control males for

experimental cross 2 (♂ [♀G7 x ♂Gal4] (27°C) x ♀ w^{1118} (25°C)) and control cross 2 (♂ [♀G7 x ♂ w^{1118} (27°C) x ♀ w^{1118} (25°C)).

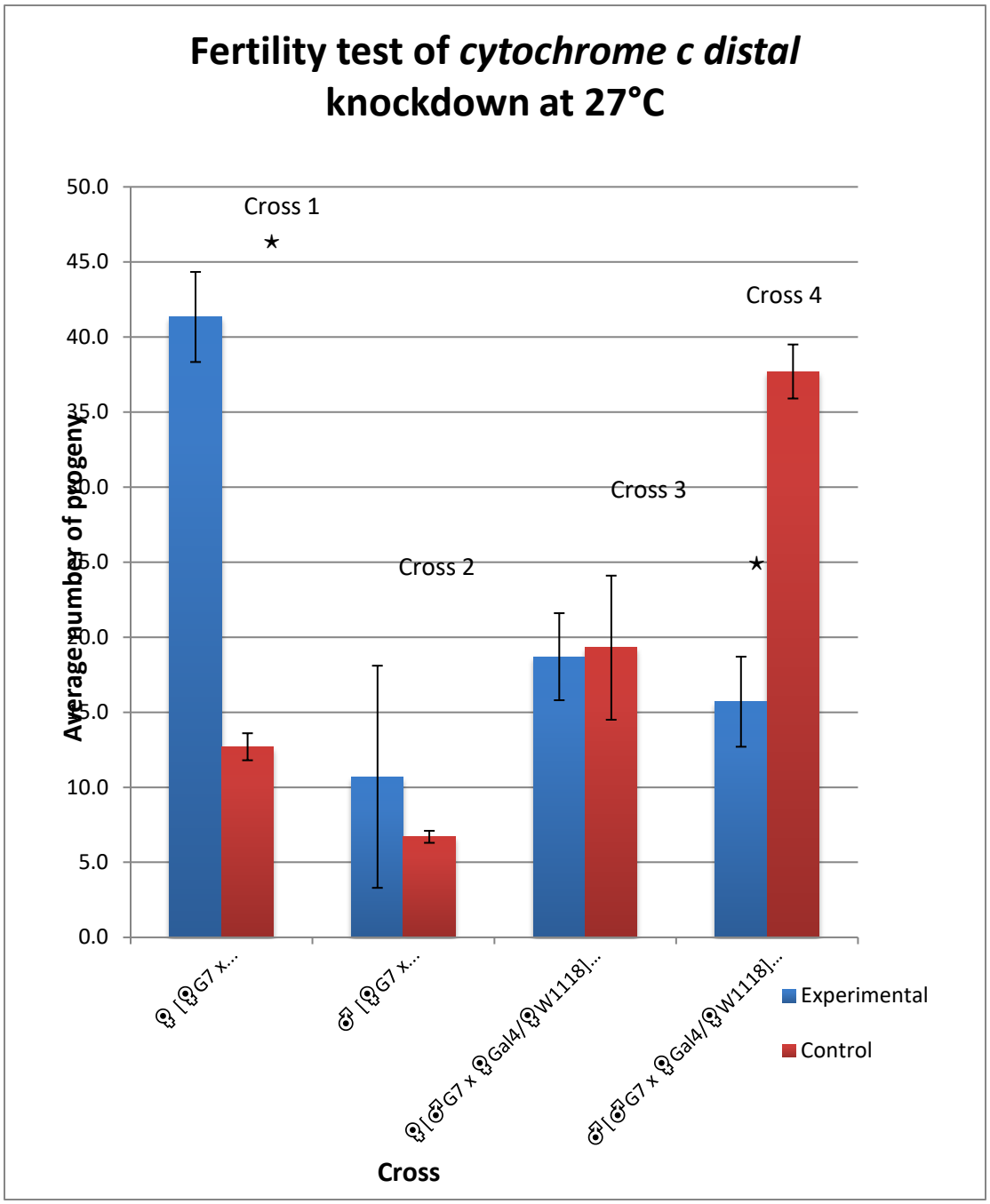

Figure 3.5: Bar Graph of the Number of Progeny vs Identity of Cross for *Cytochrome c Distal* Knockdown at 27°C (GD Library). Stars Above Bars Indicate Significant Result

3.2 Results from *Cytochrome c1 Like* (KK Library, Line 106229) at 27ºC

During experimentation, the flies containing the knockdown of the gene *CG14508* were recorded as K8 for convenience instead of writing *CG14508* for every cross. A total of four experimental and four control crosses were performed.

3.2.1 Experimental and Control Cross 1

The results from the experimental cross of female progenies from the F1 generation (female K8 crossed with male Gal4 at 27°C gave F1 generation) with male w^{1118} flies have been presented below. The results from the control cross of female progenies from the F1 generation (female K8 crossed with male w^{1118} at 27°C gave F1 generation) with male w^{1118} flies were also included.

Table 3.6: Female Fertility Test ♀ x ♂ at 27ºC

Experimental Cross			Control Cross		
♀ [♀K8 x ♂Gal4](27°C) x ♂ w^{1118} (25°C)			♀[♀K8 x ♂ w^{1118}] (27°C) x♂ w^{1118} (25°C)		
Number of offspring			Number of offspring		
Male	Female	TOTAL	Male	Female	TOTAL
18	12	30	30	37	67
9	7	16	26	22	48
8	17	25	20	15	35
Mean		23.7	Mean		57.5
Standard deviation		7.1	Standard deviation		13.4
Standard error		4.1	Standard error		7.8

The experimental cross revealed a mean of 23.7 flies with a standard deviation of 15.6. The control cross showed a mean of 57.5 flies with a standard deviation of 13.4. A two tailed t-test was done and a value of 0.0605 was obtained. This value is greater than 0.05, which means that the results are not significant.

3.2.2 Experimental and Control Cross 2

The results from the experimental cross of male progenies from the F1 generation (female K8 crossed with male Gal4 at 27°C gave F1 generation) with female w^{1118} flies have been presented below. The results from the control cross of male progenies from the F1 generation (female K8 crossed with male w^{1118} at 27°C gave F1 generation) with female w^{1118} flies were also included.

Table 3.7: Male Fertility Test ♀ x ♂ at 27ºC

Experimental Cross			Control Cross		
♂ [♀K8 x ♂Gal4](27°C) x ♀ w^{1118} (25°C)			♂ [♀K8 x ♂Gal4](27°C) x ♀ w^{1118} (25°C)		
Number of offspring			Number of offspring		
Male	Female	TOTAL	Male	Female	TOTAL
0	0	0	17	11	6
0	0	0	4	7	7
0	0	0	11	11	7
Mean		0.0	Mean		20.3
Standard deviation		0.0	Standard deviation		8.6
Standard error		0.0	Standard error		6.1

The experimental cross revealed a mean of 0.0 flies with a standard deviation of 0.0. The control cross showed a mean of 20.3 flies with a standard deviation of 8.6. A two tailed t-test was done and a value of 0.0000369 was obtained. This value is much lower than 0.05, which means that male flies show significantly reduced fertility in comparison to the controls as a result of the knockdowns.

3.2.3 Experimental and Control Cross 3

The results from the experimental cross of female progenies from the F1 generation (male K8 crossed with female Gal4 at 27ºC gave F1 generation) with male w^{1118} flies have been

presented below. The results from the control cross of female progenies from the F1 generation (male K8 crossed with female w^{1118} at 27ºC gave F1 generation) with male w^{1118} flies were also included.

Table 3.8: Female Fertility Test Reciprocal Cross (i.e., ♂ x ♀) at 27ºC

Experimental Cross			Control Cross		
♀[♂K8 x ♀Gal4] (27°C) X ♂ w^{1118} (25°C)			♀[♂K8 x ♀ w^{1118}](27°C) X ♂ w^{1118} (25°C)		
Number of offspring			Number of offspring		
Male	Female	TOTAL	Male	Female	TOTAL
68	68	136	14	11	25
68	71	139	19	22	41
45	57	102	17	4	21
Mean		125.7	Mean		29.0
Standard deviation		20.6	Standard deviation		10.6
Standard error		11.9	Standard error		6.1

The experimental cross revealed a mean of 125.7 flies with a standard deviation of 20.6. The control cross showed a mean of 29.0 flies with a standard deviation of 10.6. A two tailed t-test was done and a value of 0.00193 was obtained. This is a significant value and shows that females, which act as controls, are more fertile and tend to do better than males due to the knockdown.

3.2.4 Experimental and Control Cross 4

The results from the experimental cross of male progenies from the F1 generation (male K8 crossed with female Gal4 at 27ºC gave F1 generation) with female w^{1118} flies have been presented below. The results from the control cross of male progenies from the F1 generation (male K8 crossed with female w^{1118} at 27ºC gave F1 generation) with female w^{1118} flies were also included.

Table 3.9: Male Fertility Test Reciprocal Cross (i.e., ♂ x ♀) at 27ºC

Experimental Cross			Control Cross		
♂[♂K8 x ♀Gal4] (27°C) X ♀ w^{1118} (25°C)			♂[♂K8 x ♀ w^{1118}] (27°C) X ♀ w^{1118} (25°C)		
Number of offspring			Number of offspring		
Male	Female	TOTAL	Male	Female	TOTAL
0	0	0	28	17	45
0	0	0	15	18	33
0	0	0	10	17	27
Mean		0.0	Mean		35.0
Standard deviation		0.0	Standard deviation		6.5
Standard error		0.0	Standard error		6.0

The experimental cross revealed a mean of 0.0 flies with a standard deviation of 0.0. The control cross showed a mean of 35.0 flies with a standard deviation of 6.5. A two tailed t-test was done and a value of 0.00271 was obtained. This value is much lower than 0.05, which means that male flies show significantly reduced fertility in comparison to the controls as a result of the knockdowns.

3.3 Results from *Cytochrome c1 Like* (KK Library, Line 106229) at 25ºC

During experimentation, the flies containing the knockdown of the gene *CG14508* were termed as K8 for convenience. A total of four experimental and four control crosses were done.

3.3.1 Experimental and Control Cross 1

The results from the experimental cross of female progenies from the F1 generation (female K8 crossed with male Gal4 at 25ºC gave F1 generation) with male w^{1118} flies have been presented below. The results from the control cross of female progenies from the F1

generation (female K8 crossed with male w^{1118} at 25ºC gave F1 generation) with male w^{1118} flies were also included.

Table 3.10: Female Fertility Test ♀ x ♂ at 25ºC.

Experimental Cross			Control Cross		
♀ [♀K8 x ♂Gal4](25°C) x ♂ w^{1118} (25°C)			♀[♀K8 x ♂ w^{1118}] (25°C) x♂ w^{1118}25°C)		
Number of offspring			Number of offspring		
Male	Female	TOTAL	Male	Female	TOTAL
15	30	45	8	7	15
18	22	40	0	0	0
23	36	59	21	27	48
Mean		48.0	Mean		21.0
Standard deviation		9.8	Standard deviation		10.6
Standard error		5.7	Standard error		7.5

The experimental cross revealed a mean of 48.0 flies with a standard deviation of 9.8. The control cross showed a mean of 21.0 flies with a standard deviation of 10.6. A two tailed t-test was done and a P-value of 0.152 was obtained. This value is greater than 0.05, which means that the results are not significant.

3.3.2 Experimental and Control Cross 2

The results from the experimental cross of male progenies from the F1 generation (female K8 crossed with male Gal4 at 25ºC gave F1 generation) with female w^{1118} flies have been presented below. The results from the control cross of male progenies from the F1 generation (female K8 crossed with male w^{1118} at 25ºC gave F1 generation) with female w^{1118} flies were also included.

Table 3.11: Male Fertility Test ♀ x ♂ at 25ºC.

Experimental Cross			Control Cross		
♂ [♀K8 x ♂Gal4](25°C) x ♀ w^{1118} (25°C)			♂[♂K8 x ♀ w^{1118}] (25°C) X ♀ w^{1118} (25°C)		
Number of offspring			Number of offspring		
Male	Female	TOTAL	Male	Female	TOTAL
0	0	0	11	20	31
22	21	43	16	14	30
0	0	0	26	21	47
Mean		14.3	Mean		36.0
Standard deviation		24.8	Standard deviation		9.5
Standard error		14.3	Standard error		5.5

The experimental cross revealed a mean of 14.3 flies with a standard deviation of 24.8. The control cross showed a mean of 36.0 flies with a standard deviation of 9.5. A two tailed t-test was done and a value of 0.231 was obtained. This value is greater than 0.05, which means that the results are not significant.

3.3.3 Experimental and Control Cross 3

The results from the experimental cross of female progenies from the F1 generation (male K8 crossed with female Gal4 at 25ºC gave F1 generation) with male w^{1118} flies have been presented below. The results from the control cross of female progenies from the F1 generation (male K8 crossed with female w^{1118} at 25ºC gave F1 generation) with male w^{1118} flies were also included.

Table 3.12: Female Fertility Test Reciprocal Cross (i.e., ♂ x ♀) at 25ºC

Experimental Cross			Control Cross		
♀[♂K8 x ♀Gal4] (25°C) X ♂ w^{1118} (25°C)			♀ [♂K8 x ♀ w^{1118}] (25°C) X ♀ w^{1118} (25°C)		
Number of offspring			Number of offspring		
Male	Female	TOTAL	Male	Female	TOTAL
20	27	47	23	22	25
10	25	35	11	20	41
15	27	42	14	20	21
Mean		41.3	Mean		36.7
Standard deviation		6.0	Standard deviation		7.4
Standard error		3.5	Standard error		4.3

The experimental cross revealed a mean of 125.7 flies with a standard deviation of 20.6. The control cross showed a mean of 29.0 flies with a standard deviation of 10.6. A two tailed t-test was done and a value of 0.154 was obtained. This value is greater than 0.05, which means that the results are not significant.

3.3.4 Experimental and Control Cross 4

The results from the experimental cross of male progenies from the F1 generation (male K8 crossed with female Gal4 at 25ºC gave F1 generation) with female w^{1118} flies have been presented below. The results from the control cross of male progenies from the F1 generation (male K8 crossed with female w^{1118} at 25ºC gave F1 generation) with male w^{1118} flies were also included.

Table 3.13: Male Fertility Test Reciprocal Cross (i.e., ♂ x ♀) at 25ºC

Experimental Cross			Control Cross		
♂[♂K8 x ♀Gal4] (25°C) X ♀ w^{1118} (25°C)			♂[♂K8 x ♀ w^{1118}] (25°C) X ♀ w^{1118} (25°C)		
Number of offspring			Number of offspring		
Male	Female	TOTAL	Male	Female	TOTAL
0	0	0	26	8	34
0	0	0	15	19	34
12	9	0	13	18	31
Mean		7.0	Mean		33.0
Standard deviation		12.1	Standard deviation		1.7
Standard error		8.6	Standard error		1.2

The experimental cross revealed a mean of 0.0 flies with a standard deviation of 0.0. The control cross showed a mean of 35.0 flies with a standard deviation of 6.5. A two tailed t-test was done and a value of 0.00271 was obtained. This value is much lower than 0.05, which means that male flies show significantly reduced fertility in comparison to the controls as a result of the knockdowns.

3.3.5 Summary of Results

The results were combined in a bar graph represented below. The experimental group was placed next to its corresponding control group. For the 25°C temperature, the data showed a significant decrease in fertility for experimental males in comparison to control males for experimental cross 2 (♂ [♀K8 x ♂Gal4](25°C) x ♀ w^{1118} (25°C)) and control cross 2 (♂ [♀K8 x ♂ w^{1118}](25°C) x ♀ w^{1118} (25°C)). For the 27°C temperature, significant decreased in fertility was also observed for experimental cross 4 (♂[♂K8 x ♀Gal4] (25°C) X ♀ w^{1118} (25°C)) and control cross 4 (♂[♂K8 x ♀ w^{1118}] (27°C) X ♀ w^{1118} (25°C)). The data showed complete sterility for experimental males in comparison to

control males for experimental cross 2 (♂ [♀K8 x ♂Gal4](27°C) x ♀ w^{1118} (25°C)) and control cross 2 (♂ [♀K8 x ♂ w^{1118}](27°C) x ♀ w^{1118} (25°C)). Complete sterility was also observed for experimental cross 4 (♂[♂K8 x ♀Gal4] (27°C) X ♀ w^{1118} (25°C)) and control cross 4 (♂[♂K8 x ♀ w^{1118}] (27°C) X ♀ w^{1118} (25°C)).

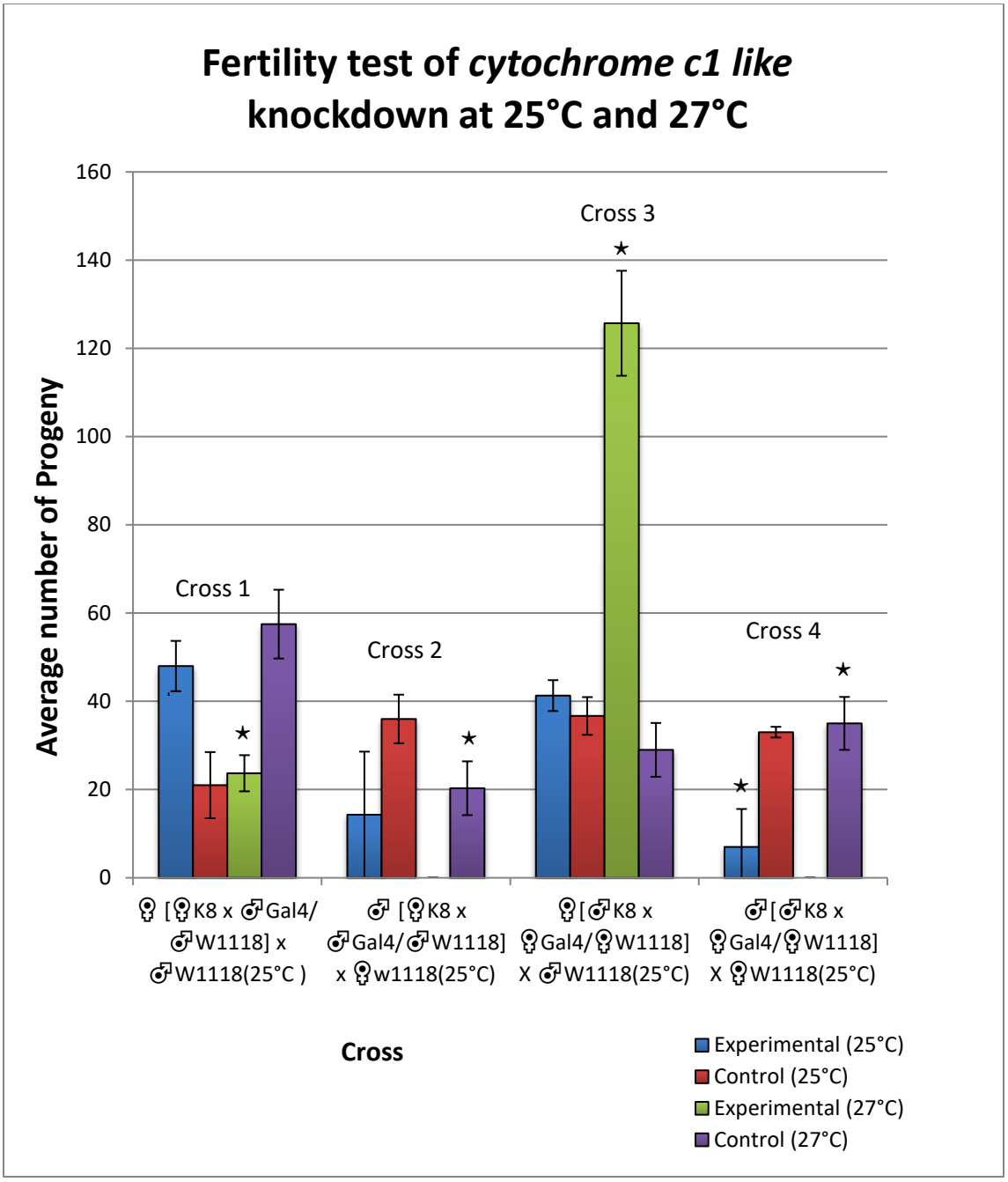

Figure 3.6: Bar Graph of the Number of Progeny vs Identity of Cross for *Cytochrome c Distal* Knockdown at 25°C and 27°C (KK Library). Stars Above Bars Indicate Significant Result

CHAPTER 4

DISCUSSION

In this project, I studied the function of two testis-specific duplicated genes on the fertility of *D. melanogaster.* The knockdown was achieved through a single controlled cross. In this cross, flies were produced that carried the transgene expressing the yeast Gal 4 protein (driver), and the transgene producing the hairpin and small interference RNAs under control of the Upstream Activation Sequence (UAS) of yeast in the genome that Gal 4 targets. The progeny of these flies were obtained and crossed with a standard laboratory strain at 25ºC and 27ºC, and the number of progeny were counted. We expected that the males would show a significant decrease in fertility to a point of complete sterility. However, we did not expect any noteworthy results from the female crosses, as the gene duplicates are highly expressed in males. Therefore, the results from the female crosses were not analyzed.

It is worth mentioning that for one of the genes (*cytochrome c distal*), we had data relating to the effects of knockouts. So in this project, we checked if the RNAi results recapitulated previous observations of decreased fertility in males.

According to the results, most crosses for both genes resulted in a significant decrease in male fertility as observed from the lack of overlaps of the error bars in the bar graphs, and most importantly, the two tailed t-tests. Some of the crosses that were at a temperature of 27ºC even revealed the effect of complete sterility in the male specimens. The only exception to this was experimental cross 2 (♂ [♀G7 x ♂Gal4] (27°C) x ♀W1118

(25°C)) and control cross 2 (♂ [♀G7 x ♂W1118 (27°C) x ♀W1118 (25°C)) of the *cytochrome c distal* knockdown at 27°C for the GD library. Other students in the lab repeated this cross and a significant reduction in fertility was observed every time the cross was done. The other observations were also in agreement with the assumption that the two genes of interest have detrimental effects on fertility by reducing the number of offspring produced by the males. As a result, the null hypothesis was rejected and it was concluded that knockdown of either of the two genes, *cytochrome c1 like* and *cytochrome c distal,* caused a decrease in fertility to the point of complete sterility. The sterility was observed at higher temperatures due to the higher efficiency of Gal4. Similar results were obtained by other students working on the KK library of *cytochrome c1 like*.

The possible errors that could have taken place during the experiment involved progeny counting. When counting a large number of flies, there is a chance of underestimation or overestimation due to unavoidable human errors. This is assumed to be a random effect that increases the standard error and that is why a t-test must be done to account for this. Since the t-tests revealed significant differences between the experimental and control crosses, the standard errors will be present despite the measures taken to narrow down the error. Another possible error could be in the timing of the fly collection. During experimentation, virgins were collected every eight hours as this was the time required to gain sexual maturity. It could be possible that for some of the vials used, virgins were collected a few minutes past eight hours, which could mean that some of the females were not virgins at the time of collection. This would then affect the experimental results, as the progeny counted would represent an inaccurate value of the cross. Furthermore, identifying a male fruit fly from a female is a task that requires a skilled eye. Even then, it is probable

to take a male for a female or vice versa. To avoid these issues, the vials were checked thoroughly in order to see if a female virgin vial was contaminated with a male fly. If a male was discovered in the female vial, the set of females was discarded. Nonetheless, this possible error does not affect the results or conclusions significantly as the project is only interested in the total number of offspring.

The experiment could be improved by conducting more replicates of crosses to add a greater degree of power to detect even small fertility effects (Glover and Mitchell, 2008). If more than three replicates were performed, then the anomalous result for experimental cross 2 (♂ [♀G7 x ♂Gal4] (27°C) x ♀W1118 (25°C)) and control cross 2 (♂ [♀G7 x ♂W1118 (27°C) x ♀W1118 (25°C)) of the *cytochrome c distal* knockdown at 27°C for the GD library could be avoided. This was because the experimental cross had a standard error of 10.5, where the mean was 10.7. This showed the data had a large deviation from the mean and was not reliable. So, more replicates could have added reliability to the data by causing a decrease in the value of the standard error. Apart from that, the method of knocking out of the genes with advanced technology like CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats-Cas9) that can be used to directly cut the gene out and replace it by a reporter gene (Deltcheva et al., 2011) could be used to confirm the result of the knockdown. While knockdowns decrease the function of a gene, knockouts completely remove the gene from the genome, making the results more reliable (Deltcheva, et al. 2011). This is an initiative that is a part of our upcoming study in order to add validity to the knockdown results. This method of knockout will also help us to narrow down the function of the genes. Students working in the cytology of the knockdown males testis in the lab have observed that the genes that are related to energy production in the

mitochondria of the sperm play an important role in sperm maturation. Cytological results observed by other students in the lab clearly support this hypothesis as they observe defects in sperm individualization. Individualization is the last step in sperm production and the excess cytoplasm is removed after head and tail, including mitochondria, are elongated. Structural problems in the mitochondria or energy problems might explain this phenotype and needs to be explored.

The results of this project could potentially provide further advancements to a process called the 'Trojan female technique' (Gemmell et. al., 2013), a method used for controlling invasive pests. This method involves finding N-mt mutations that reduce male fertility and then incorporates these mutations in female carriers before releasing them in the fields. The male progeny from the female carriers eventually reduce the reproduction rate of the whole population (Gemmell et. al., 2013). Since this research involves finding the genes that have an effect on male sterility, we believe, it can potentially help the pest control industry to produce carrier females at a faster rate. Lastly, species that are pests can be studied for the presence of these testis-specific genes that affect male fertility and tests could be performed to observe if this system works.

REFERENCES

AJ, Tabone CJ, Crosby MA, Emmert DB, Falls K, Goodman JL, Hu Y, Ponting L, Schroeder AJ, Strelets VB, Thurmond J, Zhou P and the FlyBase Consortium. (2017) FlyBase at 25: looking to the future. Nucleic Acids Res. 45(D1): D663-D671.

Arama, E., Agapite, J. and Steller, H., 2003. Caspase activity and a specific cytochrome C are required for sperm differentiation in Drosophila. *Developmental cell*, *4*(5), pp.687-697.

Ben-David, Geulah, Eli Miller, and Josefa Steinhauer. "Drosophila spermatid individualization is sensitive to temperature and fatty acid metabolism." *Spermatogenesis* 5.1 (2015): e1006089.

Castrillon, Diego H., et al. "Toward a molecular genetic analysis of spermatogenesis in Drosophila melanogaster: characterization of male-sterile mutants generated by single P element mutagenesis." *Genetics* 135.2 (1993): 489-505.

Deltcheva, E., Chylinski, K., Sharma, C.M., Gonzales, K., Chao, Y., Pirzada, Z.A., Eckert, M.R., Vogel, J. and Charpentier, E., 2011. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature*, *471*(7340), pp.602.

Dietzl, Georg, et al. "A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila." *Nature* 448.7150 (2007): 151.Dorus, Steve, et al. "Genomic and functional evolution of the Drosophila melanogaster sperm proteome." *Nature genetics* 38.12 (2006): 1440.

Duffy, Joseph B. "GAL4 system in Drosophila: a fly geneticist's Swiss army knife." *genesis* 34.1-2 (2002): 1-15.

Eslamieh, M., Williford, A., and Betrán, E., 2017. Few Nuclear-Encoded Mitochondrial Gene Duplicates Contribute to Male Germline-Specific Functions in Humans. *Genome Biology and Evolution*, *9*(10), pp.2782-2790.

Gallach, M. and Betrán, E., 2011. Intralocus sexual conflict resolved through gene duplication. *Trends in Ecology & Evolution*, *26*(5), pp.222-228.

Gray, Michael W. "Mosaic nature of the mitochondrial proteome: Implications for the origin and evolution of mitochondria." *Proceedings of the National Academy of Sciences* 112.33 (2015): 10133-10138.

Gramates LS, Marygold SJ, dos Santos G, Urbano J-M, Antonazzo G, Matthews BB, Rey Gaudet, P., Livstone, M., Thomas, P. (2010-). Gene Ontology annotation inferences using phylogenetic trees. GO Reference Genome Project. http://www.geneontology.org/cgi-bin/references.cgi#GO_REF0000033

Gemmell, Neil J., et al. "The Trojan female technique: a novel, effective and humane approach for pest population control." *Proc. R. Soc. B* 280.1773 (2013): 20132549.

Giles, R.E., Blanc, H., Cann, H.M. and Wallace, D.C., 1980. Maternal inheritance of human mitochondrial DNA. *Proceedings of the National academy of Sciences*, *77*(11), pp.6715-6719.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A. and Charpentier, E., 2012. A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. *Science*, *337*(6096), pp.816-821.

Kakidani, Hitoshi, and Mark Ptashne. "GAL4 activates gene expression in mammalian cells." *Cell* 52.2 (1988): 161-167.

Martin, W.F., Garg, S. and Zimorski, V., 2015. Endosymbiotic theories for eukaryote origin. *Phil. Trans. R. Soc. B*, *370*(1678), p.20140330.

Mendes, C.S., Arama, E., Brown, S., Scherr, H., Srivastava, M., Bergmann, A., Steller, H. and Mollereau, B., 2006. Cytochrome c-d regulates developmental apoptosis in the Drosophila retina. *EMBO reports*, *7*(9), pp.933-939.

Ohno, Susumu, Ulrich Wolf, and Niels B. Atkin. "Evolution from fish to mammals by gene duplication." *Hereditas* 59.1 (1968): 169-187.

Sokal, R. R., and F. J. Rohlf. "Biometry: the principles and practice of statistics in biological sciences." *WH Free Company, New York, USA* (1995).

Tait, Stephen WG, and Douglas R. Green. "Mitochondria and cell signaling." *J Cell Sci* 125.4 (2012): 807-815.

Wang, Yonghua, Lixia Wang, and Zhaohui Wang. "Transgenic analyses of TGIF family proteins in Drosophila imply their role in cell growth." *Journal of Genetics and Genomics* 35.8 (2008): 457-465.

Wasbrough, Elizabeth R., et al. "The Drosophila melanogaster sperm proteome-II (DmSP-II)." *Journal of proteomics* 73.11 (2010): 2171-2185.

BIOGRAPHICAL INFORMATION

Imran was born and raised in Bangladesh. He came to the United States to attend UT Arlington as an undergraduate student in 2015 and pursued a major in Biology along with a minor in Chemistry. During his freshman year, Imran joined Dr. Liping Tang's lab to help develop cancer traps for melanoma cells and learned about the importance that genes play in the development of cancer. A few semesters later, while taking an introductory genetics class, he developed an interest in exploring the field of evolutionary genomics through hands-on research. He joined Dr. Esther Betrán's lab in the summer of 2017. The first project he worked on involved exploring the correlation between sperm tail lengths of organisms and the number of mitochondria present in the tails. Then in the fall of 2017, he used RNA interference technology to knockdown (reduce function) testis-specific gene duplicates in fruit flies in order to study effects on fertility. He presented the results of this study as his Honors thesis. In the upcoming semesters, he plans to keep helping in the Betran lab with the knockout (completely remove) of the same genes to confirm the results of the knockdown.

Imran aspires to be a physician and believes his research work will help him gain a crucial understanding of the innate mechanisms that drive the physiological processes in human beings. He is confident that his research experience will help him provide better care to his patients because of the knowledge he is gaining from studying primary scientific papers.