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Characterization of Newly Created Mitochondrial Genes Affecting Drosophila melanogaster Sperm Phenotypes through Genetic Manipulation

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Characterization of Newly Created Mitochondrial Genes Affecting *Drosophila melanogaster* Sperm Phenotypes through Genetic Manipulation

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Honors Capstone Project in Biology

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Abstract

Reproductive traits, including phenotypes related to sperm form and function, frequently evolve rapidly under the influence of strong sexual selection. Understanding the genetic basis of this process is of paramount importance to evolutionary biologists and has important implications on our understanding of reproductive outcomes and fertility. The creation of new genes is suspected to contribute to this process as new genes are often expressed in the testis. However, little is currently known about how, and if, these genes acquire novel functions in spermatogenesis. Many novel genes are retrogenes. Retrogenes are formed through reverse transcription of mRNA. A set of retrogenes showing testes specific expression and putative roles in mitochondrial function was selected for genetic manipulation and phenotypic testing. Mitochondrial genes were selected because of their mechanistic role in spermatogenesis and tail elongation. Additionally, there has been a pattern previously identified for prevalent creation of testis specific mitochondrial genes during *Drosophila* evolution. The RNAi (ribonucleic acid interference) knockdown method, using a bipartite system, was used to construct knockdowns of the genes of interest (GOI). This involved crossing a female line containing a Vasa-Gal4 driver with a male line containing the UAS (upstream activation sequence)-GOI construct. The effect of knocking down the GOI was analyzed via three methods: fertility assays, sperm length measurements, and confocal microscopy. Fertility assays were done to determine whether the GOI plays a role in male fertility. Experimental and control males were mated to females and the number of eggs laid and hatched were counted over a four-day period. Four of the RNAi lines exhibited a significant reduction in fertility relative to controls, suggesting a fertility affect for these retrogenes. Sperm length measurements were significantly different from controls for 2 RNAi lines, supporting a role for these mitochondrial retrogenes in sperm tail elongation. Longer sperm length has been correlated with greater fertilization success in *Drosophila*. Finally, seminal vesicles were stained using DAPI and confocal images were taken to compare sperm number. Of particular note, for one gene (CG31003) the knockdown males produced very little or no sperm, implying an important role in sperm production. This work demonstrates that novel mitochondrial genes effect spermatogenesis, sperm traits and fertility. Although more analyses need to be done, this substantiates the importance of new genes in reproduction.

Executive Summary

Sexual selection is the preference of certain characteristics one individual exhibits over another individual of the same species. There are two types of sexual selection, before mating or after mating selection. Both types of selection lead to flashy and diverse traits such as peacock tail feathers or highly diverse sperm.

The diversity of sperm is a result of its rapid evolution. *Drosophila* have evolved an extreme characteristic of giant sperm. Sperm can evolve rapidly by the gain of loss of genes. The gain of genes is often through the acquisition of new genes. One way to create these new genes in by reverse transcription, mRNA is transcribed back into DNA rather than being translated into a protein. The new genes are known as retrogenes and usually acquire mitochondrial function in the testes. Mitochondria are believed to help with the elongation of the sperm tail in *Drosophila*.

Drosophila is being used as the model organism for this study because of its easy maintenance and genetic manipulability. It has been studied for over a century so the genome is well known and provides a strong basis for analysis. Additionally, there are many genetic tools available for genetic manipulation. One of these tools is the GAL4 system. The GAL4 system in conjunction with RNAi can silence specific genes by preventing the translation of the targeted gene.

The goal of this study is to look at the phenotypic characteristics of eleven retrogene knockdown lines. Three assays will be performed to assess sperm length, sperm production, and fertility. The length of the knockdown lines sperm was generally shorter than the control length with two lines being statistically significant. Confocal microscopy imaging was used with DAPI staining (stains DNA) to view the amount of sperm present in the seminal vesicles of the

knockdown flies. The seminal vesicles were examined because that is where mature sperm is stored until ejaculation. Only one line was seen to have a decreased amount of sperm. Finally, fertility assays were performed by mating a virgin wildtype female with a knockdown male. The number of eggs laid and unhatched were then counted over four days. Four lines were found to have statistically significant lower ratios of unhatched to laid eggs ratio than the control.

Additional analyses need to be done to assess more phenotypic characteristic of the knockdown of retrogenes. Some of these analyses may include competitive mating assays, sperm motility, and qPCR. The supplemental analyses will allow more phenotypes to be examined to gain a better overall picture of retrogene roles. However, the results of this study show that novel retrogenes may play a critical role in reproduction.

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Introduction

Sexual selection is the preference of certain characteristics one individual exhibits over another individual of the same species. It occurs in two different ways, pre-copulatory and post-copulatory. Pre-copulatory is what is originally thought of as sexual selection and has had a great amount of research done on it. It can occur through female choice or male-male competition (Shuker, 2014). Post-copulatory selection is much less studied but equally as important. It occurs through female cryptic choice and sperm competition. Sexual selection leads to the production of diverse and flashy traits (Birkhead, 2014).

Both pre-copulatory and post-copulatory selection have consequences on phenotypic characteristics. These characteristics sometimes hinder the organism in some way, but because they help contribute to the overall reproductive fitness of the individual, the characteristics remain. An example of a pre-copulatory consequence is the tail plumage of the male peacock. Their purpose is to attract the attention of females. However, these tail feathers take a great deal of energy to produce and can attract predators, but still the characteristic remains. The same is found for the consequences of post-copulatory selection. It leads to the creation of diverse sperm, sometimes with extreme phenotypes. These traits typically cost the individuals a great deal of energy to produce but have evolved because of their contribution to the overall fitness (Hosken, 2011)

Sperm are some of the most rapidly evolving cells. They can evolve to have different sperm tail lengths, number of flagella, size of the sperm head, and the quantity of sperm produced. In *Drosophila*, sperm length varies and it has been shown that longer sperm have a competitive advantage over shorter sperm in fertilization (Pitnick, 200). Across *Drosophila* species there are many different types of sperm adaptation. These adaptations often reflect the

female seminal receptacle in that they co-evolve (Miller, 2002). The intensity of the post-copulatory competition causes the evolution of these traits to be extremely rapid (Pitnick, 2009).

One cause of the rapid evolution of spermatozoa is by the loss and gain of genes. The gain of genes is usually due to the creation on new genes. When new genes are created, they can either be lost, retain parental function, or gain a new function. Retro transposition is the copying back of mRNA into DNA that can be transposed into a chromosome. This is a common mechanism for the creation of new genes. A large proportion of the time, these retrogenes exhibit mitochondrial function and are often present in spermatogenesis (Rettie, 2015).

Spermatogenesis is the production of spermatozoa by mitosis and meiosis originating in the spermatogonial stem cells. Giant mitochondria have been discovered to play a critical role in sperm tail lengthening and are a limiting factor to the extent of spermatid elongation. During spermatogenesis, giant mitochondria fuse together to form a nebenkern. This nebenkern, along with cytoplasmic microtubules create the structural platform necessary for the elongation of the axoneme (Fig.1) (Noguchi, 2012).

Drosophila have been used as model organisms for over a century. They have been used as a model to study genetics and inheritance, embryonic development, learning, behavior, and aging. *Drosophila* are easy to keep and work with. They have a short life cycle and produce many offspring allowing for genetic manipulation to be done in a reasonable amount of time. Since they have been used for so many years, a well sequenced complete genome is available, providing a solid basis for comparison. Additionally, there is a surplus of molecular tools available that have been developed to allow for the genetic manipulation (Jennings, 2011). One of the tools available is the GAL4 system.

The GAL4 system can be used as a powerful knockdown tool, especially when utilizing ribonucleic acid interference (RNAi). RNAi is a biological process in which particular RNA molecules interfere with the expression of a specific gene by inhibiting the translation of that gene. The GAL4 system is bipartite in that it requires two components. The first component is a Gal4 driver, a transcriptional activator, linked to a tissue specific enhancer. For this study, Vasa was chosen as this enhancer because it is testes specific, meaning that the silencing will only occur in the testes and no other tissues of the organism. The second component is a transgene that is controlled by an upstream activation sequence (UAS) associated with an RNAi sequence. Each gene being analyzed has a specific RNAi construct associated with it. When the Vasa-Gal4 containing female and the UAS containing male are mated, the Vasa-Gal4 driver activates the UAS that causes the transcription on the RNAi sequence. This sequence then inhibits the translation of the gene being analyzed, silencing that gene of interest (GOI). Once the GOI is silenced, it is possible to examine the phenotypic effects of the knockdown (Fig. 2). It is important to note that this is a knockdown technique, not a knockout technique in that the gene is silenced and not removed (Elliott, 2008). Powerful reverse genetic tools such as the GAL4 system, allows us to examine *Drosophila* as a model organism in relation to other species such as humans.

Infertility is a global public health issue (Infertility, 2017). About 12% of males experience some form of infertility causing nearly 1 in 8 couples trouble when trying to conceive (Collura, 2014). Often this infertility has a hidden cause. In many cases the sperm structure and quantity appear to be normal yet an unknown issue is still present. Almost 70% of the *Drosophila* genome has homologs with the human genome, allowing for the research done with the model organism to be highly associated with diseases in humans (Gramates, 2017).

Reproductive biology research is essential in trying to understand why fertility is such a massive issue and can help with basic developmental understanding.

Eleven retrogenes have been selected as part of this study for phenotypic testing. These retrogenes were selected due to their male and testes specificity, their function in mitochondria, and the possibility of their role in spermatogenesis. Their individual functions can be found in Table 1. One of the genes selected is gasket (CG31003). This gene is known to play a critical role in male gamete development (Gramates, 2017). It will serve as an internal control throughout the study to assess that the RNAi knockdown is working properly and as a comparison for the other knockdown phenotypes. Mitochondria are known to play a crucial role in sperm elongation so sperm length of the knockdowns will be assessed. As retrogenes are believed to play a role in spermatogenesis, the quantity of sperm is going to be examined through confocal microscopy. Finally, because the quantity and length of sperm affect the fitness of the individual, fertility assays are going to be conducted. The aim of these assays is gain a better understanding of the role of retrogenes in sperm phenotypes.

Methods

Stocks and Crosses

Flies were maintained in stock bottles at room temperature. A standard cornmeal, yeast, and molasses based media were kept in the bottles and vials as food. All flies with the UAS-RNAi constructs and flies with the Vasa-GAL4 driver were ordered from the Vienna *Drosophila* Resource Center (10,11,12). The constructs for all lines were made from a line with the w [1118] or KK lines. The lines differ in where they originated. These base lines were used as the controls

for each corresponding line. Vasa was chosen as the driver, opposed to Bam, because it shows expression throughout the entire testes.

For each mating, virgin Vasa-GAL4 females were collected and allowed to age for five days. UAS-RNAi young males (newly hatched but not necessarily virgin) were collected and allowed to age for five days. Two five day old males and two five day old females were put in each vial and allowed to mate overnight. The following day, the adults were removed and the vials were kept in a Percival Scientific incubator set at 28ºC until flies were hatched (usually 8-10 days). These first generation (F1) males (now having the GOI knocked-down) were collected, allowed to age for five days in the Shel Lab LIFLY incubator at 24.5ºC, and used for analyses.

Sperm Length Analysis

Five day old F1 males for each GOI and the control were dissected in a 1x phosphate-buffered saline (PBS) solution using a Leica M125 microscope. Sperm was collected from the seminal vesicles and mounted on a microscope slide. Each seminal vesicle was dissected separately and each slide only had the sperm of one individual. Fifteen-twenty individuals were mounted from each line.

Once the sperm was mounted on the slide, it was fixed using a 3:1 acetic acid: methanol solution for one minute. The slides were washed in 1x PBS for three one minute washes and were fully dried in a VWR154 incubator set at 54ºC. After the slides were dried, a mounting solution of 9:1 glycerol: PBS was used to place the slide cover and the slides were sealed with nail polish and allowed to air dry. The sealed slides were kept in a refrigerator to ensure they lasted for several weeks.

The slides were examined on a dark field Olympus B202 microscope at 40x and pictures were taken of individual sperm. ImageJ (a computer program) was calibrated to the correct

length by magnification level and was used to measure the individual sperm tails. Caution was taken to only measure fully intact (no broken) and distinguishable sperm tails. A goal of ten separate sperm tails were measured from each individual's slide, but some individuals had fewer measured due to clumping issues.

Confocal Microscopy of Seminal Vesicles

F1 RNAi males were dissected in a 1x PBS solution using a Leica M125 microscope for their full seminal vesicles. About six seminal vesicles were placed on each slide. Three slides for each GOI line were produced. The seminal vesicles were mounted using a ProLong Diamond Antifade with 4'6-diamidino-2-phenylindole (DAPI) mounting solution, sealed with nail polish and allowed to dry. The sealed slides were kept in a refrigerator so they could last for several weeks. A Zeiss LSM 710 Confocal Microscope was used to take images of the seminal vesicles at different angles to analyze the amount of sperm present within a seminal vesicle. A blue backlight was used to see the DAPI staining of the sperm.

Fertility Assays

Virgin LH_M (wildtype) females, F1 RNAi males and control males were collected and allowed to age for five days in the Shel Lab LIFLY incubator at 24.5^{o}C. One five-day old female and one-five-day old male were put in each vial (labeled Day 1) and allowed to mate overnight. About fifteen vials were set up for each RNAi line and controls. The next day the males were discarded, and the females were moved to a new vial (labeled Day 2). The number of eggs each female laid on Day 1 was counted using a Leica EZ4 microscope. The following day, the females were moved to a new vial (labeled Day 3), the number of eggs laid on Day 2 were counted, and the number of unhatched Day 1 eggs were counted. This pattern continued until laid and

unhatched eggs were counted for four days of vials (Figure 3). The females and vials were then discarded.

Statistical Analyses

A Shapiro-Wilke test was used to test for normality for both sperm length measurements and fertility assay results. The majority of the data was discovered to be not normal. Significance for sperm length was examined using a Mann Whitney test with a Bonferonni correction method since multiple statistical tests were run on the same data. A model comparing laid to unhatched with the different days taken into consideration was used for fertility significance testing. All of the statistical tests were done in R Studio (R Studio Team, 2015). The results for each line for both the sperm length and the fertility assays were adjusted to the control for easier visualization. This was done by subtracting the average control values from each of the experimental values.

Results

This study sought to understand the phenotypic effect of the knockdown of retrogenes through RNAi genetic manipulation. Three studies were used to test a variety of phenotypes. These included sperm tail length, the production of sperm in a quantitative manner, and single mating fertility assays.

Sperm Length Analysis

Sperm length measurements were taken for six of the genes; CG11913, CG4701, CG17856, CG5718, CG7514, and CG10749. The average, standard error within a GOI data set, and standard error between the GOI data sets were calculated for sperm length (Table 2). Average sperm length adjusted to the average control length by subtracting the average control sperm tail length from each of the experimental individual sperm measured and can be seen in

Figure 4. Positive values are longer than the control while negative values are shorter than the control.

The average of the control length is 1.69mm. The genes CG7514 (average=1.71mm, p=0.00156 and CG5718 (average= 1.63mm, p=0.0006) were found to be statistically significant. CG7514 is known to play a role in malate antiporter activity. This is used to create NADH which can then enter the electron transport pathway. CG5718 is believed to be have electron carrier activity in complex II of the electron transport chain.

Confocal Microscopy of Seminal Vesicles

After spermatogenesis, mature sperm are stored in the seminal vesicles until ejaculation. Confocal images were taken to qualitatively examine the number of sperm present. A decreased amount of sperm, would indicate the retrogene plays an imperative role in sperm production. Imaging of CG4701, CG17856, CG11913, CG7514, CG5718, CG10749, and CG31003 (Figure 5.A) were compared to the control (Figure 5.B). The pictures were taken at 40x and were focused on the sperm not the seminal vesicles themselves. The analysis of the amount of sperm present was done by the eye but only CG31003 appeared to have significantly less, almost no, sperm in the seminal vesicle. The DAPI staining allowed for visualization of the rod like sperm heads verses the round somatic cells.

Fertility Assays

Fertility assays were done for all eleven of the retrogenes. The average total eggs, average unhatched eggs, average hatched eggs, percentage of eggs unhatched, and percentage of eggs hatched were calculate (Table 3). The total number of laid eggs was adjusted to the average of the control (W1118=169.08, KK=73.30) by subtracting the average control from each of the experimental lines. Positive values are greater than the control and negative values are smaller

than the control (Figure 6.A). The number of hatched eggs was adjusted the same way (W1118=145.95, KK=68.63) (Figure 6B). Finally, the ratio of hatched to laid eggs was adjusted (W1118=0.863, KK=0.936) (Figure 6C) and tested for significance.

Four genes were found to be statistically significant. They are CG14508 (average ratio=0.04, $p= 5.46 \times 10^{-5}$), CG31003 (average ratio=0.18, $p= 2.45 \times 10^{-5}$), CG11913 (average ratio= 0.26, $p= 1.74 \times 10^{-5}$), and CG4701 (average ratio=0.79, $p= 9.67 \times 10^{-6}$). CG14508 is believed to be an electron transporter in complex III. CG31003 participates in ATP binding. CG11913 facilitates NAD and quinone binding in complex I of the electron transport chain. CG11913 facilitates NAD and quinone binding in complex I of the electron transport chain. All four significant genes play a role in either ATP binding or ATP production.

Discussion

Three assays were done to examine the phenotypic effects of the knockdown of retrogenes. The first assay was sperm length measurements because of retrogenes exhibited mitochondrial function and the putative role that mitochondria play in sperm elongation. The second assay was the use of confocal imaging to qualitatively examine the amount of sperm present in the seminal vesicles. Finally, fertility assays were performed to determine if retrogenes contribute to the fertility success.

In general, knockdown lines showed shorter sperm length than the control. Two lines were found to be statistically significant. The first being CG7514, which is known to play a role in the transport of ions into and out of the mitochondria. This gene was the opposite of what we would expect in that the knockdown was longer than the control. This could mean that for this retrogene, the expense of energy for the longer sperm was not worth the extra length. The second

significant gene is CG5718. CG5718 exhibits electron carrier activity and is found in mitochondrial complex II. This gene exhibits what is expected in that the knockdown gene is shorter than the control. This suggests that the retrogene's function aids the elongation of the sperm tail. For the genes that were not significant, the retrogenes may still play a role in mitochondria, just in ways that do not affect tail elongation.

In the confocal images, only gasket displayed significantly reduced sperm production. This is to be expected since it is known that gasket plays a critical role in male gamete production. The amount of sperm produced was assessed visually in a semi-quantitative manner, so even though there were not a visible reduction in the number of sperm in the other lines, there may have been less that was not discernable to the eye. Additionally, the amount of sperm may not have been significantly different but it is possible that the sperm exhibited structural defects not visible in the images.

Four genes were found to be statistically significant in the fertility assays. The first being CG14508 which is believed to be an electron transporter, facilitating the binding of heme and is found in the mitochondrial complex III. The second gene is gasket, which again was expected because of its known functions including ATP binding. CG11913 was found to be significant and helps facilitate electron flow in the electron transport chain and is found in the mitochondrial complex I. The final significant gene is CG4701, which plays a role in ATP binding. All four of the significant genes either play a role in ATP production or facilitate ATP binding suggesting that the energy created by these retrogenes may be important for fertility. In the genes that were not significant, the retrogenes may lead to increased competitiveness but not increased fertility.

One limitation to the study was that during the sperm length measurement process, there was trouble mounting some of the sperm, specifically in CG4706. This difficulty could have

been due to laboratory complications or it could indicate a phenotype. Maybe the knockdown of CG4706 produces sperm of abnormal structure that does not allow for it to stick to the microscope slides as normal sperm does. Another limitation is that the fertility assays were not all done at once. Differences in temperature, humidity, and the food can all effect the mating of the flies and thus could have influenced the egg numbers. Finally, we were pressured for time as each analysis lasted for a long duration.

To continue the study, more tests need to be run. While RNAi is a powerful knockdown tool and gasket was utilized as an internal control, it would be beneficial to test the efficacy of the knockdown using quantitative polymerase chain reaction (qPCR). qPCR is used to detect a specific DNA sequence and determine the number of that sequence relative to a control. To help address that limitation that this study is in a lab and not in nature competition assay should be done in addition to the single mating fertility assays already performed. In nature, female *Drosophila* mate with more than one male. Finally, there are several other phenotypes that can be tested besides the ones done in this study. One of particular interest is the motility of the sperm because it can lead to highly competitive advantages. Because of this, motility assays should be performed.

The knockdown of retrogenes was shown to effect sperm tail length, sperm production, and fertility. This demonstrated that novel mitochondrial genes do affect the phenotypic characteristics of sperm. Although more analyses need to be done, this study substantiates the importance of new genes in reproduction.

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Figure Legends

Figure 1: **Shematic of Spermatid Elongation-** Schematic showing the process of sperm tail elongation in *Drosohila*. Giant mitochondria fuse together to form a nebenkern. The nebenkern along with microtubules provide a structural platform for the elongation of the axoneme.
(Noguchi, 2011)

Figure 2: RNAi using the **GAL4 System-** Schematic showing the mechanism of the GAL4 system. An enhancer-GAL4 complex containing driver is mated to a UAS-RNAi construct containing responder. The RNAi is activated and silences specific retrogenes in the F1 generation.
(Elliot, 2008)

Figure 3: **Fertility Assays-** Depiction of the methods used in the fertility assays. A male and female were allowed to mate overnight in vial 1. Females are then transferred to a new vial, eggs laid from the first vial are counted and the unhatched eggs are counted the next day. This process is repeated over four days.

Figure 4: **Sperm Length Analysis of RNAi Knockdown Flies-** Sperm length measurements of the knockdown lines were corrected to the control measurements. This was done by subtracting the average control length from the length of each experimental sperm measured. Positive values indicate that the experimental length was longer than the control while negative values indicate that the experimental length was shorter than the control. The statistically significant genes of interest are denoted by an astric.

Figure 5: **Confocal Microscopy Images-** Only gasket was seen to have a decreased amount of sperm present in the seminal vesicles. The images of the control and gasket are compared. A) In gasket, only the round somatic cells are seen. B) In the control, sperm heads can be identified as the rod-shaped cells.

Figure 6: **Fertility Analysis Graphs-** Fertility analysis of the knockdown lines was corrected to the control measurements. This was done by subtracting the average control from each experimental female. Positive values indicate that the ratio is greater than the control while negative values indicate that the experimental ratio is less than the control. A) Total Laid Eggs Adjusted. B) Total Hatched Eggs Adjusted. C) Ratio (Hatched: Laid) Adjusted. Statistically significant genes of interest are denoted by an astric.

Figures

Figure 1- Schematic of Sperm Elongation

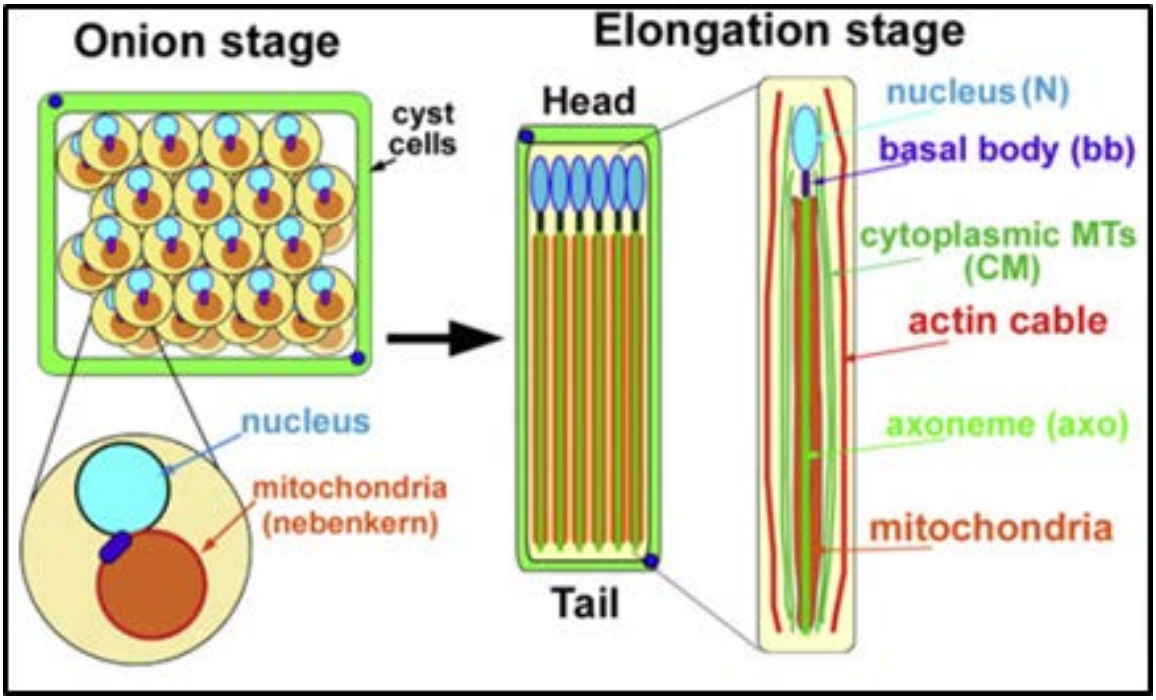


Figure 2- RNAi using the GAL4 System

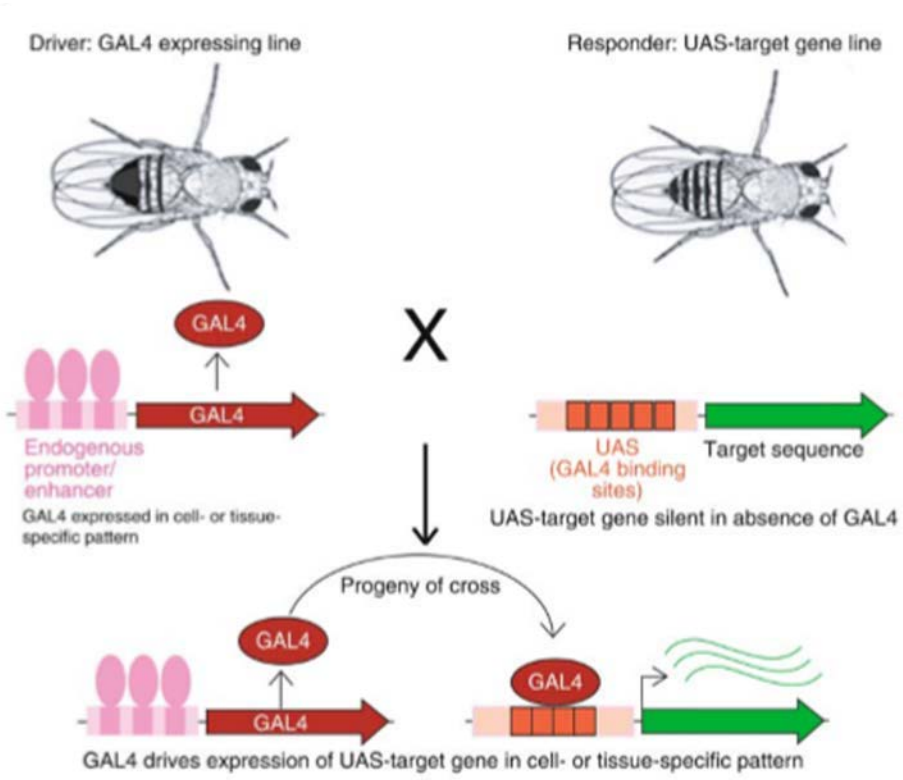
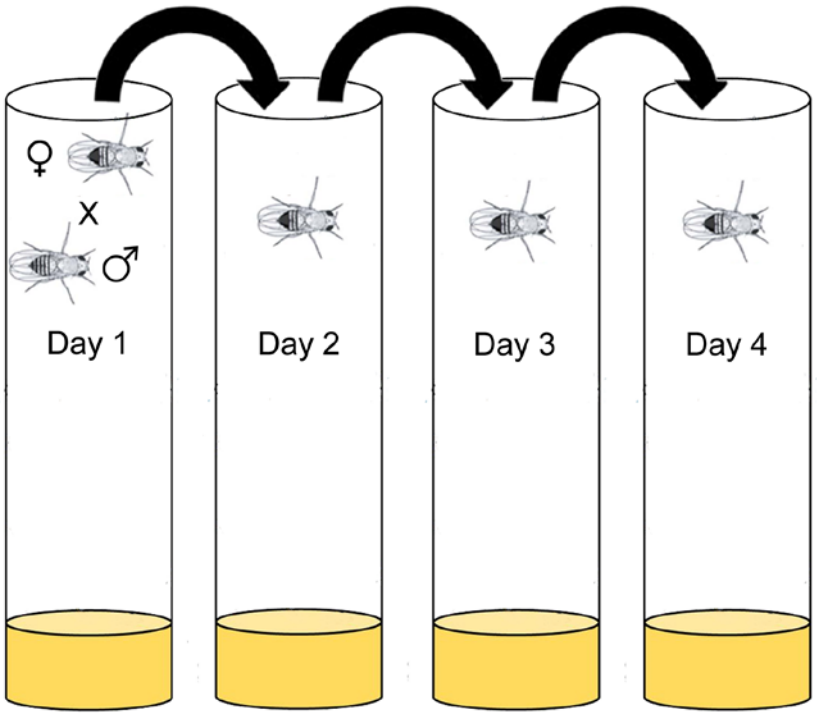


Figure 3- Fertility Assays



Day 1	Mate Flies				
Day 2*	Count Laid	Move Female			
Day 3	Count Unhatched	Count Laid	Move Female		
Day 4		Count Unhatched	Count Laid	Move Female	
Day 5			Count Unhatched	Count Laid	Discard Female
Day 6				Count Unhatched	

*Discard Males on Day 2

Figure 4- Sperm Length Analysis of RNAi Knockdown Flies

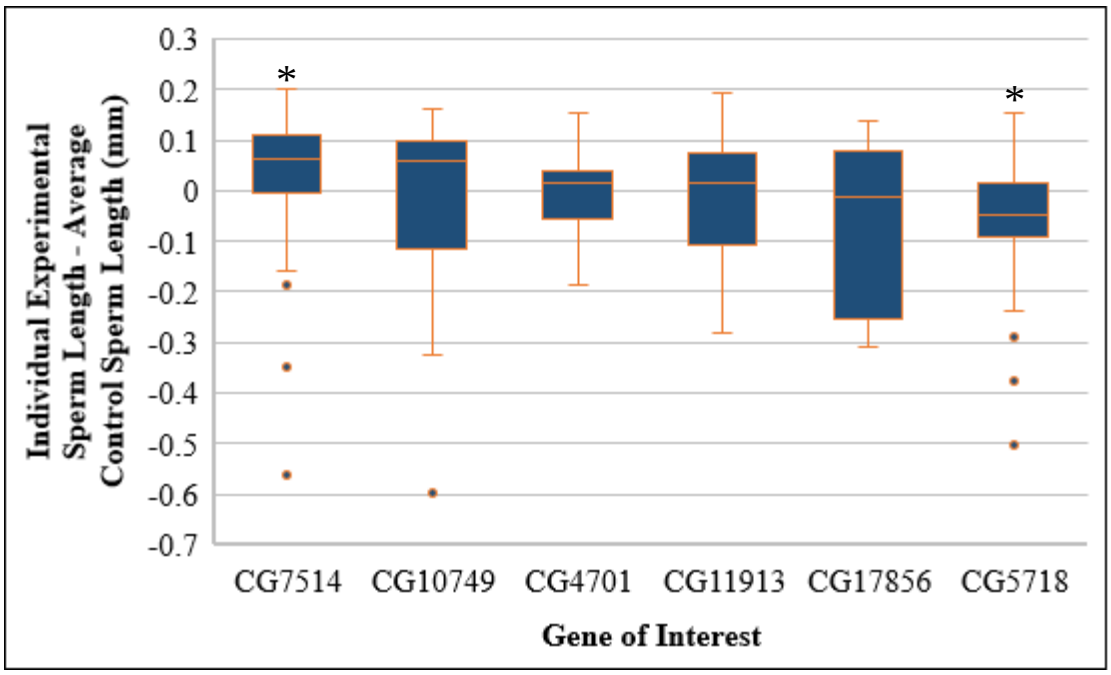


Figure 5- Confocal Microscopy Images

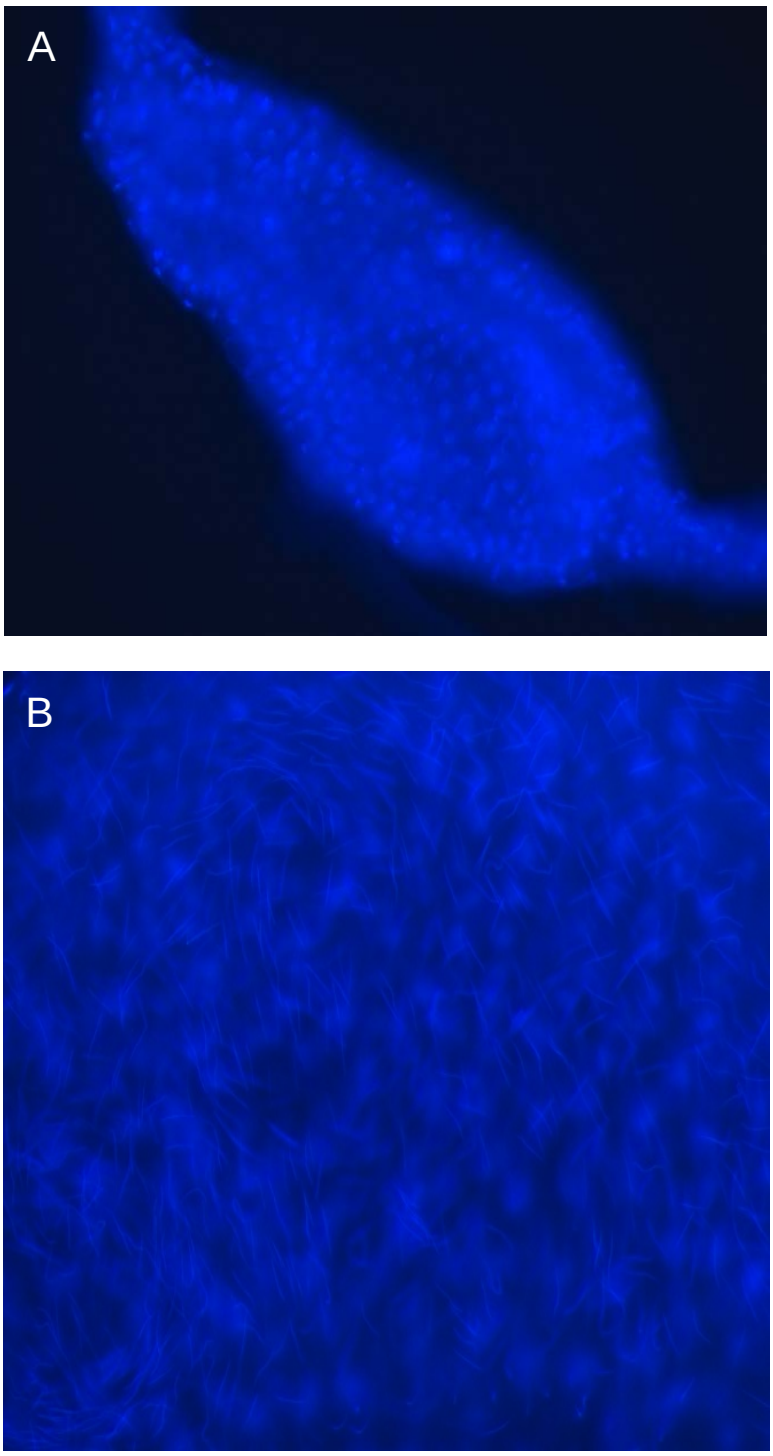
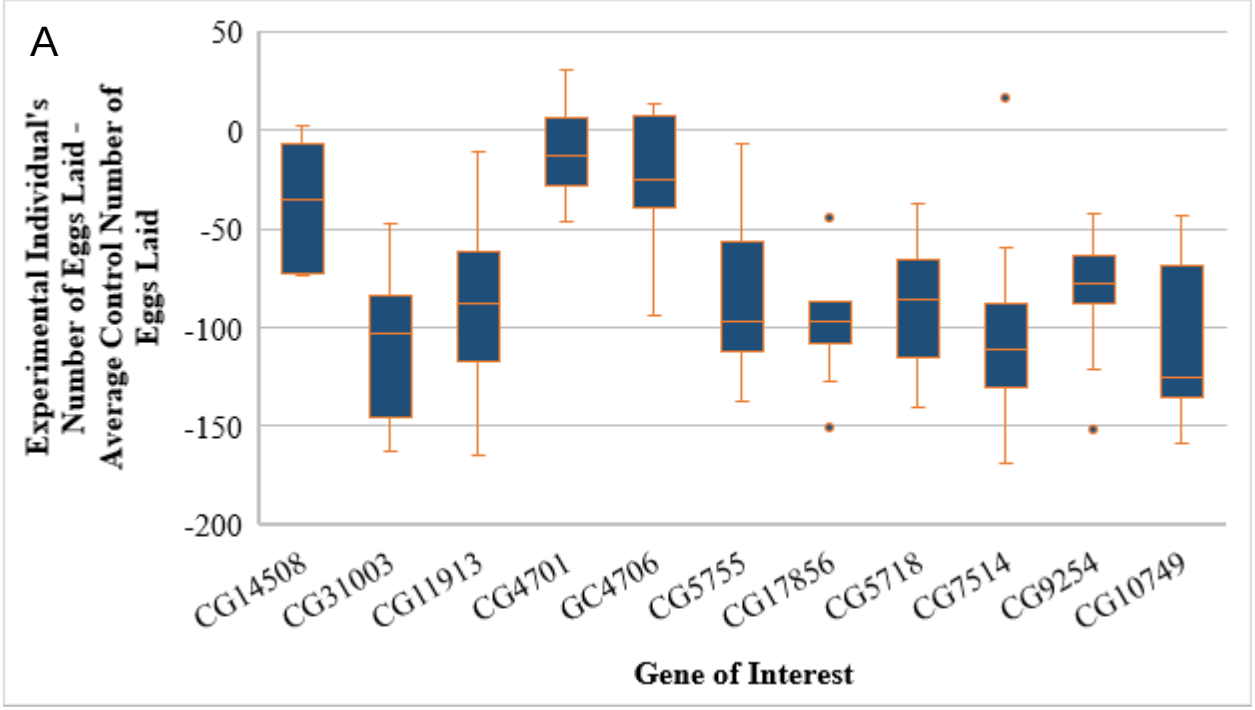
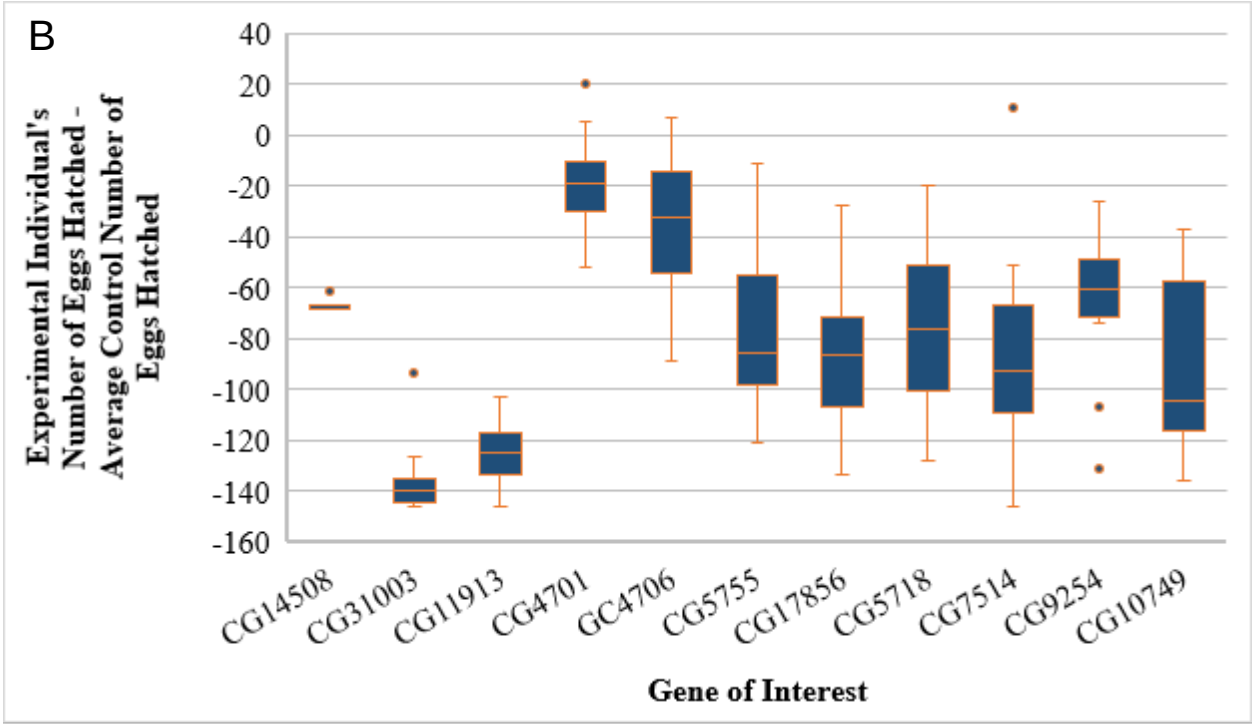
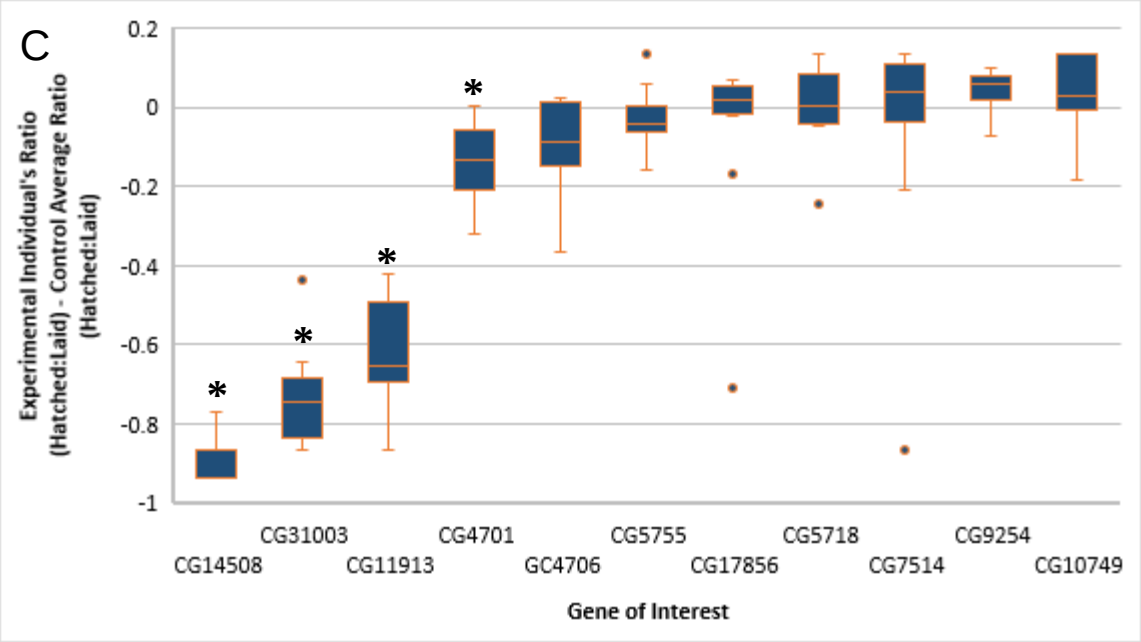


Figure 6- Fertility Analysis Graphs






C
Experimental Individual's Ratio (Hatched:Laid) - Control Average Ratio (Hatched:Laid)
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
*
*
*
*
CG14508
CG31003
CG11913
CG4701
GC4706
CG5755
CG17856
CG5718
CG7514
CG9254
CG10749
Gene of Interest

Tables

Table 1- Molecular functions of selected retrogenes

GOI	**Function**
CG31003	ATP binding; protein kinase activity; male gamete generation
CG11913	NAD binding; mitochondrial electron transport chain; found in mitochondrial complex I
CG14508	Electron transporter, heme binding, found in mitochondrial complex III
CG10749	L-malate dehydrogenase activity, carbohydrate metabolic process
CG5718	Electron carrier activity, found in mitochondrial complex II
CG7514	Oxoglutarate: malate antiporter activity; mitochondrial transport
CG5755	Mitochondrial substrate/solute carrier
CG4701	ATP Binding
CG17856	Ubiquinol-cytochrome-c reductase activity, mitochondrial complex III
CG9254	Sodium: phosphate symporter activity, anion transport

Table 2- Sperm Length Assays

Gene of Interest	Average Sperm Length	Standard Error Between GOI	Standard Error Within GOI	Significance value (p)
W1118	1.69	0.02	0.08	-
kk control	1.72	0.01	0.02	-
CG11913	1.67	0.02	0.09	0.71
CG4701	1.71	0.01	0.02	0.99
CG17856	1.62	0.04	0.05	0.16
CG5718	1.63	0.01	0.09	0.0006
CG7514	1.71	0.01	0.09	0.002
CG10749	1.67	0.02	0.13	0.28

Table 3- Fertility Assays Data

Gene of Interest	**Average Total Eggs**	**Average Unhatched Eggs**	**Average Hatched Eggs**	**% Eggs Hatched**	**Significance value (p)**
W1118	169.08	23.13	145.95	86.32	
KK control	73.30	4.66	68.63	93.64	
CG14508	33.15	31.85	1.31	3.95	5.5 x10E-5
CG31003	60.31	49.69	10.62	17.60	2.5 x10E-5
CG11913	78.79	58.07	20.72	26.29	1.7 x10E-5
CG4706	146.72	33.79	112.92	76.97	0.013
CG4701	62.14	12.79	49.36	79.43	9.7 x10E-6
CG17856	74.50	13.83	60.67	81.43	0.84
CG5755	83.69	14.15	69.54	83.09	0.097
CG5718	146.77	8.87	71.23	48.53	0.98
CG7514	66.00	6.86	59.14	89.61	0.32
CG9254	88.40	8.73	79.67	90.12	0.27
CG10749	58.07	6.57	51.50	88.68	0.27