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WHAT GENE(S) SPECIFY EGG SIZE VARIATION AMONG *DROSOPHILA* SPECIES?

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WHAT GENE(S) SPECIFY EGG SIZE VARIATION AMONG *DROSOPHILA* SPECIES?

A THESIS APPROVED FOR
SCHOOL OF BIOLOGICAL SCIENCES

BY THE COMMITTEE CONSISTING OF

Dr. John P. Masly, Chair

Dr. Gavin Woodruff

Dr. Ashlee Rowe

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DEDICATION

I dedicate this thesis to my amazing son, Leon Enderson. You are my greatest inspiration. Thank you for your patience, strength and love throughout this journey. I pray you always see how kind, capable and deeply loved you are.

Acknowledgement

I am grateful to God, for His grace upon me in my life. Thank you, dear Endy Enderson, for your loving support, encouragement and prayers to keep me going. Thank you for your patience and for taking care of Leon and keeping the house going while I was at the lab every day of the week and on most nights when I stayed at the lab till late. To my amazing advisor, Dr. John P. Masly, I wouldn't be here if you had not taken me as your graduate student. Thank you for being extremely patient with me, for mentoring me into a great researcher, for pushing me to be better each day and follow what I truly love, and for constantly supporting me throughout my graduate studies here at the University of Oklahoma. Thank you to my committee members, Dr. Ashley Rowe and Dr. Gavin Woodruff, as well as Dr. Christina Bourne. Your advice at every step of my progress was unwavering.

I would like to thank my lab friends, Mehrnaz and Azom for being there for me whenever I needed you, and to the entire Masly's lab undergraduate research assistants for all your help in the lab. At the entire School of Biological Sciences, you provided me with resources that made my journey smoother. Finally, thank you to my beloved family and friends. My sweet mom, Mrs. Olypher Lumula, for always listening to my fly stories and laughing with me, and my dad Mr. John Lumula, for your prayers and advice whenever I needed it. All this wouldn't have been possible without each one of you.

I would like to thank Bloomington *Drosophila* Stock Center (NIH P40-OD018537).

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ABSTRACT

There exists great variation in egg morphology among *Drosophila* species. However, little is known about the mechanism(s) that specify egg shape and size of *Drosophila*. Some studies suggest that pre-embryonic gene expression might influence egg size variability. *Drosophila sechellia* is known to produce much larger eggs, and *Drosophila sechellia* is also known to produce fewer eggs compared to its sister species *D. mauritiana, D. melanogaster*, and *D. simulans*. This egg size difference presents an opportunity to leverage the arsenal of genetic tools available among these species to understand the molecular bases of egg size variation. Here, we study a collection of interspecific genetic introgression that have a mostly *D. sechellia* genomic background with a small segment of *D. mauritiana* genomic material. We collected, measured, and analyzed egg length data and discovered that *3Q1(A)* was one of the three introgression lines had an effect on egg length size. *3Q1(A),* produces much smaller eggs compared to *D. sechellia* pure species. To identify the gene(s) that causes these differences in egg length, we sequenced the genome of *3Q1(A)* and compared it to the genomes of the parental *D. sechellia and D. mauritiana* lines used to create the introgression. We have identified the limits of the *D. mauritiana* genetic material within the *3Q1(A)* introgression region and we have performed functional genetic tests of these candidates using resources available in *D. melanogaster* and identified genes that have an effect on egg length. We anticipate that our results will provide direction for future developmental studies of oogenesis among these species.

BACKGROUND AND SIGNIFICANCE

Life history evolution has been extensively studied using *Drosophila melanogaster* as a model organism (Azevedo et al. 1997). The size of an egg in insects, including other life history traits like female fecundity, survival, and longevity, are used as proxies of fitness (Barghi et al. 2023). Embryonic viability, hatching rate, and embryonic development (Azevedo *et al*. 1997), are some of the life history traits affected by egg size in *Drosophila melanogaster* (Markow et al, 2008). Egg size is influenced by factors such as temperature (Avelar, 1993; Azevedo et al. 1997; Imai, 1932), larval overcrowding (Venkitachalam et al. 2022), diet, as well morphological features of anterior-posterior patterning (Huang et al. 2020; Lott et al. 2007). These morphological differences are believed to be shaped by evolution since *Drosophila mauritiana, D. simulans* and *D. sechellia* diverged around 0.24-0.5 MYA (million years ago) from each other and roughly 5 MYA from *D. melanogaster* (David et al. 2007; Lachaise et al. 1986; Tamura et al. 2004). A recent study suggests that during speciation, introgression might be common in genus *Drosophila* (Suvorov et al. 2022). In sister species, not only is there a variation in the number of eggs laid by the female, but there is also a distinct variation in the egg sizes as well that suggests that egg production is closely related to female fitness (Bouletreau-Merle et al*,* 1982) and may be a pleiotropic effect of genes involved in other adaptations (Jones, 2004). Apart from having much larger eggs, *D. sechellia* is known to retain fertilized eggs for a long period of time in their reproductive tracts (Markow et al. 2009). This characteristic is notable even when *D. sechellia* is raised in its native host *Morinda citrifolia* (Legal et al. 1994) also known as noni fruit, *D. sechellia. D. sechellia* is endemic to an island living

and has evolved to consume this fruit that is toxic to other *Drosophila* species. It appears that evolution has shaped species egg differences (Kambysellis and Heed, 1971; Montague, 1984). This observation indicates that there is a genetic basis to this variability (Markow et al, 2009). In evolutionary population biology studies, egg size, and more specifically egg length, is rarely studied. This is due to the low throughput of the manual measurement of egg size (Barghi *et al.* 2023).

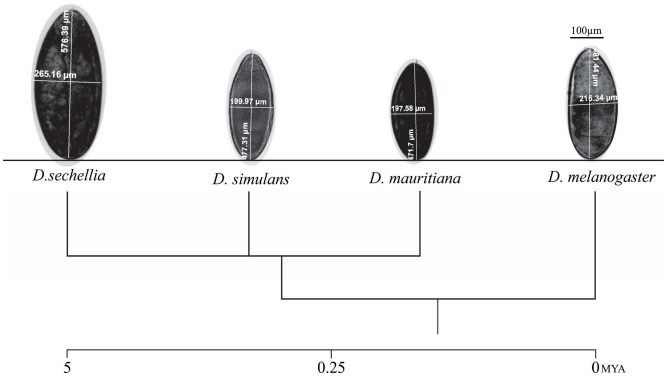


Figure 1: Phylogenetic Tree of the four sister species. The Tree represent the divergent relationship between *D. mauritiana*, *D. simulans*, *D. sechellia* and, *D. melanogaster*. Scale bar represent the number of divergence in millions of years (MYA) (David et al. 2007; Lachaise et al.1986; and Tamura et al.2004).

In developmental studies, *Drosophila* ovaries are easily studied as they provide a cellular basis for understanding the genetic regulation of oogenesis (Spradling et al. 2022; King, 1970; Mahowald & Kambysellis, 1980; Lin & Spradling 1993; Hudson & Cooley, 2014; Hughes et al. 2018). *Drosophila* has two sets of ovaries, each containing varying numbers of ovarioles that resemble a string of 6 or 7 developing egg chambers sequentially. In each egg chamber, there contain 16 germ-line cells, one oocyte and 15 nurse cells that are all surrounded by somatic follicle cells (Lin & Spradling, 1993). The development of the ovariole undergoes through 14 stages. Fully developed oocytes are produced after the egg chambers undergo drastic changes in their size and structure (Lebo & McCall, 2021). Oocyte maturation is observed during stages 12-14 (Aviles-Pagan & Orr-Weaver, 2018). To study cell cycle differentiation and regulation, the egg chamber follicle cell epithelium is a model system that is well established (Spradling, 1993;

Deng et al. 2001; Sun & Deng, 2005; Sun & Deng, 2007; Jia et al. 2015; Lopez-Schier, 2001; Jia et al. 2015; Ge et al. 2015; Jouandin et al. 2014; McConnell et al. 2012; Mitra et al. 2012; Lammens et al. 2008; Mata et al. 2000; Maines et al. 2004; Brun et al. 2006; Conder et al. 2007; Jones et al. 2017). At the tip of each ovariole is the germarium (Lin & Spradling, 1993). This is where *Drosophila* oogenesis begins. Studies shows that in *Drosophila* species, the anterior and posterior axis are defined during oogenesis. During this time, *bicoid* and *oskar* mRNA are localized and directed by the microtubule cytoskeleton to the anterior and posterior poles when the oocyte undergoes polarization (Martin et al. 2003). Not only do egg length differ among Drosophila species, ovariole numbers also differ among Drosophila species. This is an inverse relationship indicating that the smaller the eggs, the more the ovarioles and the larger the eggs, the fewer the ovarioles.

To identify the gene(s) that specifies egg size variation in *Drosophila*, it is important to take advantage of the *GAL4/UAS* system. The *GAL4/UAS* system is a tool used to target tissue specific gene expression in *Drosophila.* One of the main uses of this system is to be able to screen for any mutations that are affecting any process of interest by cellular marking (Duffy, 2002). Our interest is in oocyte development in the female germline. This system uses two types of transgenes which are, a *GAL4,* which acts as a driver, and the other, a *GAL4*-responsinve UAS (Rorth, 1998). The GAL4 drivers are crossed to a responder line that contains a RNAi gene (Brand & Perrimon, 1993; Duffy, 2002). For instance, to study the effect of a gene of interest in the female germline, it is appropriate to use a responder that is expressed in the female germline during oogenesis (Duffy, 2002). A *GAL4* driver that is known to give high-level expression in the female germline is *nanos-GAL4* (Van Doren *et al.* 1998). In this case, a *nanos-GAL4*, which is a female germline-specific

GAL4, would be crossed to a specific responder line, *RNAi*. The progenies from this cross would be tested and analyzed for the specific effect.

MATERIALS AND METHODS

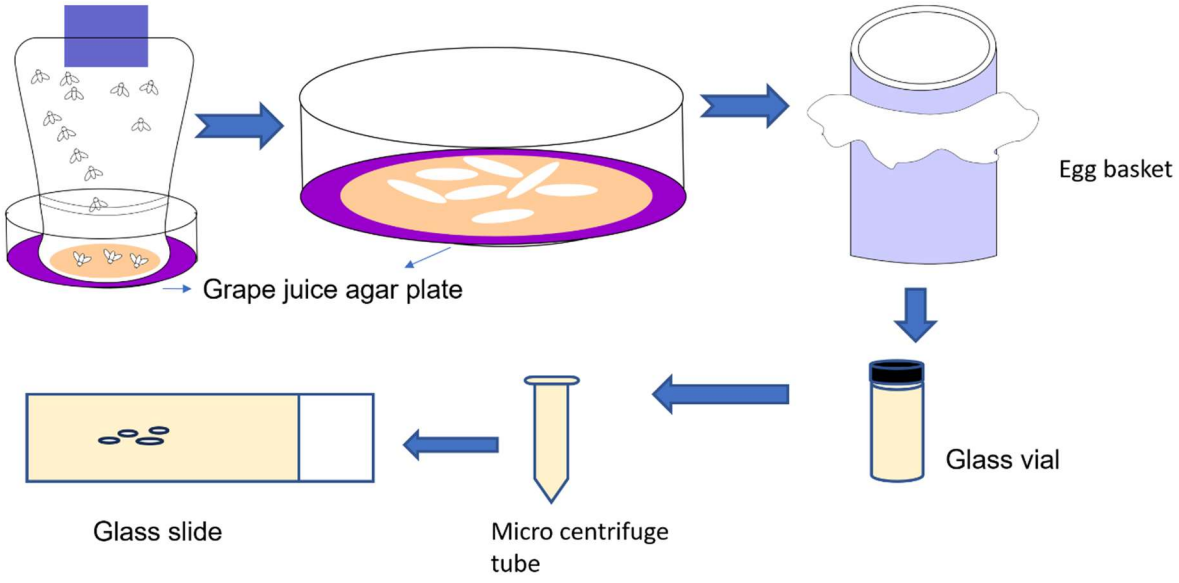
Drosophila stocks-Introgression lines

All stocks were maintained on standard cornmeal-yeast-molasses medium at room temperature (23-25C°) on a 12h light:dark cycle. The introgression lines were constructed previously using *D. sechellia w* and a collection of *D. mauritiana P-element* insertion stocks that each bear a visible marker *w+* (Masly and Presgraves 2007). The introgression lines used for this study are: *2Q2(B);50CD, 2X2;G30 EF, 3J1(B);780B, 3Q1(A);35GF, 3Q1(B), 4C2(A);Q1(A), I1(B):99D, Neneh2(A), Neneh2(B), Q1(A), Rylah1(A), Yar1(A).*

Egg collection, fixation and imaging

For this experiment, we used light carbon dioxide anesthesia to collect virgin male and female individuals. We aged these individuals for 3-4 days and placed them in mating chambers or vials of freshly made fly food for 24 hours. 50-100 females from each of the introgression lines were placed on a grape juice media plate supplemented with yeast paste for 12-17 hours at 25°C. We prepared the grape juice media using a grape juice plate protocol (Wood 2017). To prepare the grape juice media, we made and autoclaved a solution termed as solution “A”, containing 15g of Bacto-Agar and 350 mL of distilled water for 1hr. We also made a second solution termed as solution “B”, containing 10mL 70% ethanol and 0.25g Tegosept. After solution “A” cooled down to 50°C, we mixed it with solution “B” and added it to 150mL of grape juice concentrate. We made 50 small plates each containing 10mL of the grape juice media.

Eggs were collected, dechorionized, and fixed using methanol following the *Drosophila* Protocols (Sullivan et al 2000) Chapter 9: Protocol 9.1-9.8 respectively. We used the following supplies and equipment for the egg collection. A 6-ounce plastic fly "collection" bottle with a fitted cotton-filled breathing hole, grape juice agar plate containing a dollop of yeast paste in the middle, an egg collection basket with synthetic mesh (120-mm 206 821 7345; Research Nets Inc., fine paint brush, glass vial (5ml), and masking tape.



We placed 50-100 female flies in the 6-ounce plastic collection bottle that is fitted with the cotton-filled breathable home and covered it with the grape juice agar plate containing a dollop of yeast paste in the middle. The grape juice agar plate was secured to the egg collection bottle using masking tape and placed in the incubator for 12-17hours at 25°C.

Using a fine paint brush and distilled water, we washed the eggs from the grape juice agar plate and placed them in the egg basket. We used a squirt bottle to carefully clean the eggs. We rinsed the eggs using a bleach solution that is 50% bleach (Clorox) and 50% distilled water. After rinsing the eggs, we used the fine paint brush carefully to remove the eggs from the mesh and placed all the eggs in a 5ml glass vial. We fixed the eggs using methanol by pipetting 1ml of methanol into the vial and shaking it vigorously for 15 seconds and let them stand for 1 minute to allow the devitellinized eggs to sink to the bottom of the vial. We discarded the eggs that did not sink to the bottom. Using a Pasteur pipette, we transferred the fixed eggs into a 1.5ml microcentrifuge tube. To rehydrate the eggs, we prepared a PBTA solution using 50ml 1x PBS, 5g 1% BSA, 250-ml 0.05% Triton X-100, 0.1g 0.02% Sodium Azide and adjusted the volume to 500ml by adding 250ml of distilled water.

We gently added 250 ml of the PBTA solution, and 250 ml methanol into the 1.5ml microcentrifuge tube and swirled the tube for 5 seconds. We used as much PBTA and methanol as possible.

Finally, we added 1ml of PBTA solution into the microcentrifuge tube and allowed the eggs to sink to the bottom. We placed the microcentrifuge tube on the shaker for 15 mins. Afterwards, the PBTA solution was removed from the microcentrifuge tube. Using a fine paint brush, eggs were mounted on glass slides with glycerol and cover slips.

A compound microscope equipped with a Zeiss AxioCam digital imaging system was used to capture the images at a resolution of x100 magnification.

We performed the Bonferroni corrections with multiple comparisons test using the R studio statistical program and displayed our results in a box and whisker plot.

Body measurement

To measure the body size of individual females, we dissected one frontal tibia from 50 females from each genotype. Tibia length serves as a proxy for body size (Shingleton et al 2009). Prior to dissecting the frontal tibias from these females, carbon dioxide anesthesia was used. Flies were placed on 1x PBS for dissection in a well of a depression slide and mounted on glass slides with glycerol and cover slips. A compound microscope equipped with a Zeiss AxioCam digital imaging system was used to capture the images at a resolution of x100 magnification.

Dissection of Ovaries

Ovaries from these same flies whose tibia was dissected were retrieved to count the number of ovarioles on each ovary. We followed a protocol from Mohit et al 2007 (A protocol for culturing *Drosophila melanogaster* stage 9 egg chambers for live imaging). Using a pair of forceps, we pinched a bit of the abdominal cuticle and pulled out the female reproductive tract with the ovaries attached to it. Female fruit flies have two functioning ovaries. We used a micro dissecting needle to separate the ovarioles from each other. We pulled the ovarioles carefully from the germarium and counted the number of ovarioles on each ovary. We repeated this process on 50 females from each of the introgression lines.

Egg volume calculations

To calculate the volume of each egg, we used the formula $(1/6)pW^2L$ (Markow et al 2009).

Whole genome sequencing

Using NEBnext Ultra ll FS DNA library kit and NEBnext Multiplex oligos (New England Biolabs, Inc.), we constructed Illumina sequencing libraries. Library sequence pair-end lengths were between 120 to 130 bp, sequenced on a Novaseq 6000 at a minimum of 15X coverage. We used FastQC (Andrews, 2010) to check to the quality of the libraries.

We used various bioinformatic tools as stated below:

1) We used Geneious prime software (Chong, Yoong Min, et al. 2020) to align the library created to *D. sechellia* genomes and *D. mauritiana* chromosomes and created a consensus introgression line chromosome for the *3Q1(A). 3Q1(A)* is found on the left hand second chromosome. Additionally, we mapped the *p*-element flanking region to the chromosome as a potential mid-point for the introgression. We subsequently used a second alignment software known as IGV (Integrative Genome Viwer) (James et a. 2011) to double check our alignment.

2) After identifying potential gaps of 3Q1(A), we used Gepard software (Krumsiek et al. 2007) to create a dot plot to compare and visualize the gaps in the genome and find the breakpoint between *D. sechellia* and *D. mauritiana* genetic material as in results (Fig. 7). This gap/region is 629,455bp long.

3) We used FlyBase (Jenkins et al. 2022, Ozturk-Colak et al. 2024) a free database for genetic studies, and identified 73 total genes in this region. 43 of these genes are protein coding genes whilst the other 30 genes are non-coding genes.

4) We used the comparative genome viewer (Rangwala et al. 2024) to compare the genes found within this region between *D. sechellia, D. mauritiana, D. simulans, and D. melanogaster.*

5) We used FlyAtlas to check the expression of each individual gene in the ovary (Krause et al. 2020).

Primer designs

We designed primers using NCBI's primer design tool (Altschul et al. 1990) using 500bp from each side of the breakpoint as follows:

Oligo Name	Sequence 5'-3'
ALSF	CGACTGGCAGTTACCCCTTA
ALSR	TAACTTCTCGGTCGGCAGTC
CLSF	TCGAACCCATTTAAACGCGG
CLSR	CATCGGTGGTCAGAAAAGGC
ARSF	GCTATGGTCCCCTACTGGTC
ARSR	TGGTTCCCCTATAGGCCAAC

We used the 1% agarose gel and imaged our results using the BIO-RAD Molecular Imager® Gel Doc™ XR+ with Image Lab™ Software.

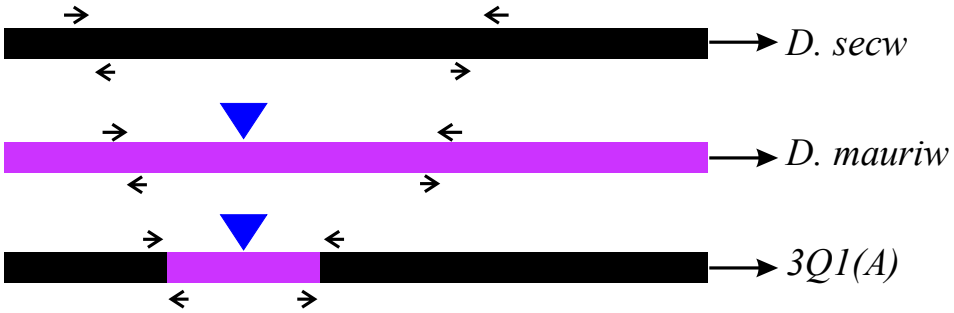


Figure 2: Genomic sequences of the three species, *D.sechelliaw, D. mauritianaw* and *3Q1(A)*. Blue triangle shows the location of the P-element instertion site. The magenta rectangle shows where the *3Q1(A)* introgression material was found in the *D. mauritiana* genome. Black arrows indicate where the primers were designed to amplify to identify the *3Q1(A)* genetic material.

Genetics stocks were obtained from Bloomington *Drosophila* stock center (NIH P40ODO018537) see Table 2 in Appendix.

We used the following 4 *Gal4* driver lines: *nanos/Tm3,Sb on the 3rd chromosome, Nanos-Gal4/nanos-Gal4* on the X-chromosome, *otu-Gal4/otu-Gal4* on the X-chromosome and *ovo Gal4/ovo-Gal4* - on the X-chromosome. An X-chromosome balancer, *FM7, Kr>GFP* graciously provided by Dr Wan Hee, and was used to maintain specific genetic combinations. We crossed virgin female *Gal4* from each genotype to male with *FM7, Kr >GFP*. Progenies from these crosses were screened and the females containing both the *Gal4* and *FM7, Kr >GFP* were backcrossed to the adult males from the *FM7, Kr >GFP* parental stocks. Virgin progenies were collected and

screened for both the *Gal4* and *FM7, Kr>GFP*. Females were then crossed to male *UAS-Drc2/Drc2* on the 2nd chromosome. Progenies from these crosses were screened to identify the right genotype and phenotype to create the stocks. The process was repeated for each of the 4 *Gal4s*.

The following stocks were made from the crosses above:

1) *UAS-Dcr2/+; nanos-Gal4/Tms,Sb*
2) *nanos-Gal4/FM7, Kr >GFP;Dcr2/+;+/+*
3) *otu-Gal4/FM7, Kr >GFP;Dcr2/+;+/+*
4) *ovo-Gal4/FM7, Kr >GFP;Dcr2/+;+/+*

RNAi knockdowns were performed by crossing virgin females from the *Gal4* stocks above to males from each 16 RNAi stocks. Progenies from these crosses were used for the oviposition to collect eggs. Tibia and ovaries were dissected from the same progenies following protocols mentioned previously above.

AIMS

1: Characterization of egg length sizes, egg volume, body size and ovariole numbers

We tested the hypothesis that major effect genes specify the differences in egg length by using the introgression approach to identify those genes. We measured the eggs of the 12 introgression lines and analyzed the data to identify if there are variations in egg length among these species. We compare each individual introgression line to pure species *D. sechellia.* Subsequently, we compared the egg length sizes to body size and overall egg volume. We investigate whether ovariole number correlates with egg number and size. Ovarioles ultimately give rise to egg size.

Not only do the eggs differ in overall size, ovariole numbers differ as well among Drosophila species. The smaller the eggs, the more the ovarioles and the bigger the eggs the fewer the ovarioles.

2: Identifying the regions that cause egg length variations among *Drosophila* species.

We identified genomic regions that affect egg morphology by identifying the genes that are present in the *3Q1(A)* introgression. Using the *Drosophila melanogaster* knock-out flies we took advantage of the *GAL4/UAS* system to identify the role and effect of these candidate genes on morphology.

RESULTS

1. There is a significant difference in egg size between *Drosophila* and its sister species

Drosophila sechellia is known to have bigger eggs than sister species as seen in (Fig.3).

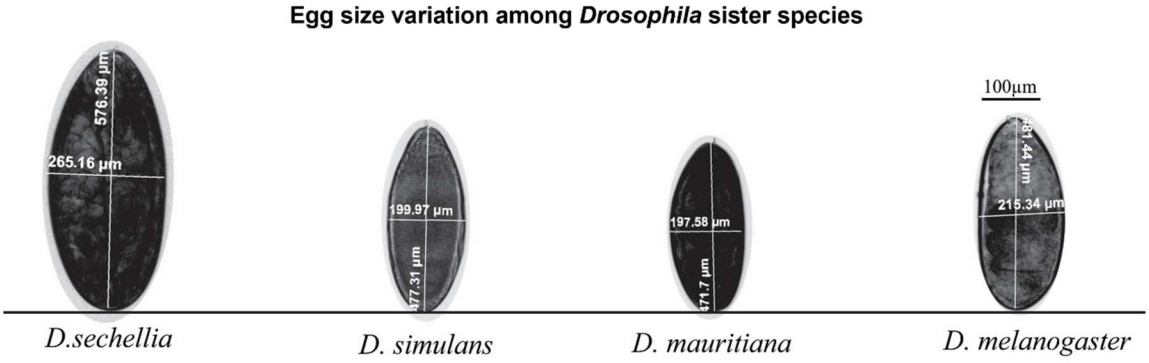


Figure 3: Egg size variation among the four species of the *Drosophila melanogaster* complex. Vertical lines show egg length, and horizontal lines show egg width measurements.

2: There are significant differences in egg length among these 12 introgression lines. We collected 50 eggs from each of the 12-introgression lines. We used *D. sechellia w* as a control and compared the results for each of the 12 genotypes to *D. sechellia.*). The result, as shown (Fig. 4), indicate that there is a significant difference between three genotypes *3Q1(A),* and *Rylah1* being smaller than *D. sechellia* and *Yar1* being larger than *D. sechellia.*

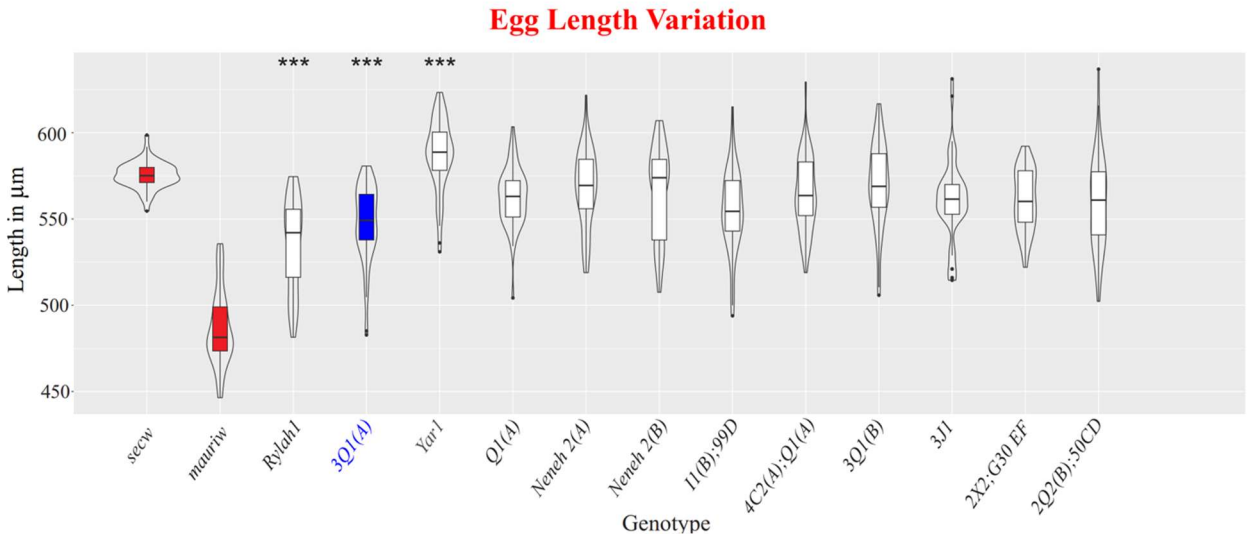


Figure 4: Variation in egg length among the *D. sechellia-D. mauritiana* introgression lines. Each genotype was statistically compared to *D. sechellia* (red) using a t-test. Significance threshold of $p < 0.05$ was used for calculation by performing the Bonferroni correction test with multiple comparisons, using R studio statistical program. Three of the genotypes: *3Q1(A), Rylah1* and *Yar1*, had statistically significant *p <values. *< 0.05, **< 0.001, ***< 0.0001*. Sample size was 50 eggs per genotype.

We focused on this *3Q1(A)* introgression line for this research study. The *3Q1(A)* introgression line is found on the second chromosome arm (Fig. 5) (Masly et al. 2011). Previous studies that analyzed *Drosophila* chromosomes found that the second chromosome has the most egg production genes (Jones, 2004).

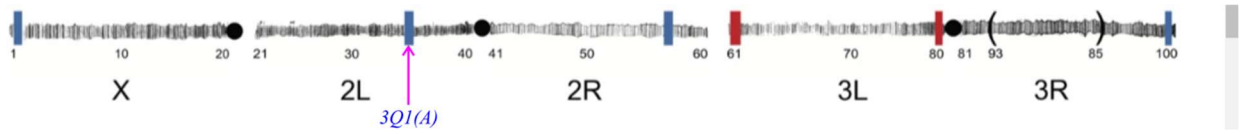


Figure 5: Genomic distribution of the *D. mauritiana* introgressions across the five major chromosome arms. Introgression line 3Q1(A), is found on the second chromosome arm (2L) (Figure adapted from Masly, *et al*. 2011)

3: *Drosophila* body size does not correlate with the egg length size.

We asked ourselves whether *Drosophila* body size correlated with egg length by using tibia length. Tibia length serves as a good proxy for overall body size (Shingleton et al. 2009). We dissected one frontal tibia each from 50 female flies for each of the 12 genotypes. We also performed the same dissection procedure on *D. sechellia* and *D. mauritiana.* We compared the results of each of the 12 genotypes to *D. sechellia* as seen in (Fig. 6). When applying for egg length size, we corrected for body size using the tibia length by dividing the egg length by the tibia length size. Summary of these results are shown in (Table 1)(Appendix). This had no effect on the overall results of our comparisons. Our results led us to conclude that *Drosophila* species body size and egg length size are independent of each other. The volume of the eggs was calculated using the formula $(1/6)\pi W^2 L$ (Markow et al. 2009), summary of the results are in the appendix, (Table 1). Although calculations for egg width are prone to error, they are necessary to find the volume of the egg. Egg volume has also been used as a proxy for maternal investment in each offspring (Church et al. 2019). In *Drosophila* species, egg volume is dependent on diet.

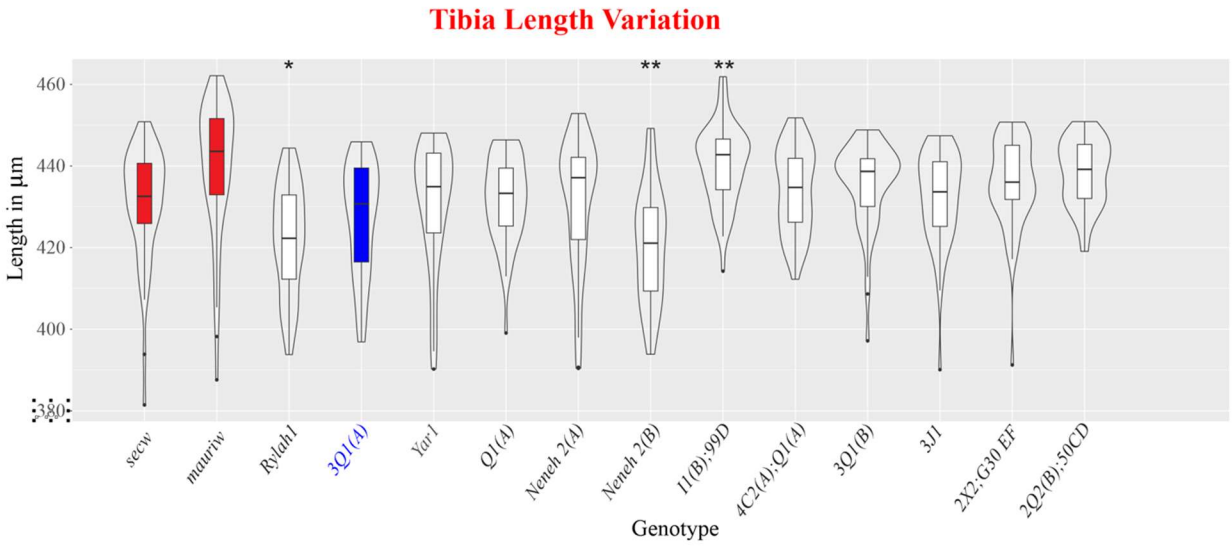


Figure 6: Variation in tibia length among the *D. sechellia-D. mauritiana* introgression lines. Each genotype was statistically compared to *D. sechellia* (red) using a t-test. Significance threshold of $p < 0.05$ was used for calculation by performing the bonferroni correction test with multiple comparisons, using R studio statistical program. This compared each introgression line to the control *secw*. Three of the genotypes, *Rylah1, Neneh2(B),* and *I1(B)*, had statistically significant *p <values.* There were no significant differences between *3Q1(A)* (highlighted in blue) and *D. sechellia*. *< *0.05, **< 0.001, ***< 0.0001*. Sample size was 50 eggs per each genotype.

4: There is a correlation between ovariole number and egg size

We dissected 50 females from each genotype and analyzed their ovaries by counting the number of ovarioles per ovary. Studies have shown that there is a correlation between egg size and ovariole number. (Church *et al*. 2019). A mentioned previously, ovarioles ultimately give rise to egg size. Not only do the eggs differ in overall size, ovariole numbers differ as well among *Drosophila* species. The smaller the eggs, the more the ovarioles and the bigger the eggs the fewer the ovarioles. As shown is (Table 1), there were no significant differences in ovariole numbers among the introgression lines compared to *D. sechellia* . This might be due to the fact that all introgression lines have the same background.

5: Genome sequencing and identifying candidate genes. We performed a genomic analysis to compare the genomic sequences of *3Q1(A), D. mauritiana* and *D. sechellia*. This was done by

using bioinformatic tools through prior knowledge in terminal and basic shell script. This enabled us to map the genomic sequences of *3Q1(A)* to *D. sechellia* and likewise to *D. mauritiana*. We were able compare and view these sequences using a free genomic viewer software known as Geneious prime (Chong, Yoong Min, et al. 2020) and subsequently compared our alignments using Integrative Genomic Viewer (IGV) (James et a. 2011). Two distinct breakpoints were identified on either side of the *3Q1(A)* introgression region and visualized using a dot plot (add citation) as seen in (Fig. 7B). We designed primers to perform a polymerase chain reaction (PCR) to detect genetic materials from *D. mauritiana*, *3Q1(A),* and *D. sechellia.* We performed a gel electrophoresis test to identify any amplification of the genetic materials according to their sizes and seen in (Fig. 7C). There was a clear distinction between the three genotypes. This region is 629,455 base pairs long.We identified 72 genes in the *3Q1(A)* introgression region by using resources available on *D. melanogaster* on Flybase.org (Jenkins et al. 2022, Ozturk-Colak et al. 2024), a free database for *Drosophila* genetics. Only 43 of these genes are protein coding genes and 30 are long noncoding RNAs (lncRNAs). The lncRNAs are nucleotides that lack protein coding potential, and their precise function is unclear (Maeda et al. 2018). This makes it challenging to study their effect on morphological structure. On the other hand, protein coding genes can be tested using the *GAL4/UAS* system. We divided the protein coding genes to those that have already been characterized and named, and those that have not been named nor characterized yet, (*CG*) genes also known as computer generated genes.

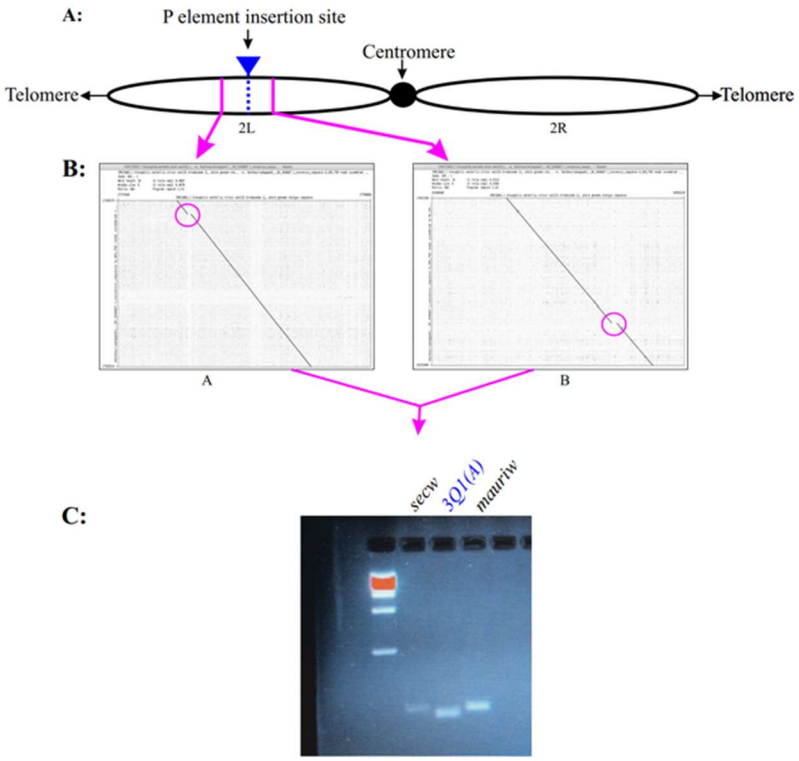


Figure 7: A) Image depicting the *Drosophila* chromosome 2 arm. B) Dot plot used to visualize the left breakpoint (A) and the right breakpoint (B). C) Gel electrophoresis image showing the amplification of the *3Q1(A)* genetic material.

6: Designing a GAL4/UAS system to perform the RNAi knockdown experiment. The GAL4/UAS system is a tool used to target gene expression Drosophila. One of the biggest challenges in using this system is that it was believed to be less efficient in the female germline. However, *nanos-GAL4* is known to give high levels of expression in the female germline. To compare this germline expression, we will also use other germline specific *GAL4s*; *ovo-GAL4(BL62770#)*, and *otu-GAL4(BL#58424)*. Since most of the RNAi stocks are currently not available in the United States, we tested only those that were currently available from Bloomington *Drosophila* stock center (NIH P40ODO018537). This experiment enabled us to visually see any morphological differences on the eggs and be able to ascertain the role of the gene that has the effect.

7: 12 out 16 genes have an effect on egg length

Sixteen RNAis genes were knocked down by *nanos-Gal4/FM7,Kr>GFP;UAS-Dcr2/+;+/+*, *otu-Gal4/FM7,Kr>GFP;UAS-Dcr2/+;+/+*,ovo-*Gal4/FM7,Kr>GFP;UAS-Dcr2/+;+/+*, and *UAS-*

Dcr2/+;*nanosGal4/TM, Sb*. We only knocked down the 16 genes because they were the only ones available in the United States through Bloomington *Drosophila* Stock center. Out of the 16 genes knocked down, 12 genes showed moderate to significant effect on egg length. For each RNAi gene, we generated a control, and an experimental line indicated as C and E on each gene's name. Based on normality and homogeneity of variance, we compared control to experimental for each RNAi line with each other by performing a t-test. The p-values were adjusted using the Bonferroni correction test. These results proved that the 4 Gal4s used in this study were effective in gene knockdown and that the second chromosome indeed carries genes that influence *Drosophila* egg size.

8: *ovo-Gal4/FM7, Kr>GFP* X RNAi gene knockdown

When we knocked down each of the 16 genes to *ovo-Gal4/FM7, Kr>GFP*, 8 genes, showed a significant effect on the egg length, *t-test p value >0.05*, as seen in (Fig. 8). For each pair of genes, *C* indicated the control and is colored gray, while the *E* indicated the experimental, with no color. These genes are as follows: *btv, cadN, cadN2, CG5783, CG5790, CG31802, JMJD4* and *ninaD.*

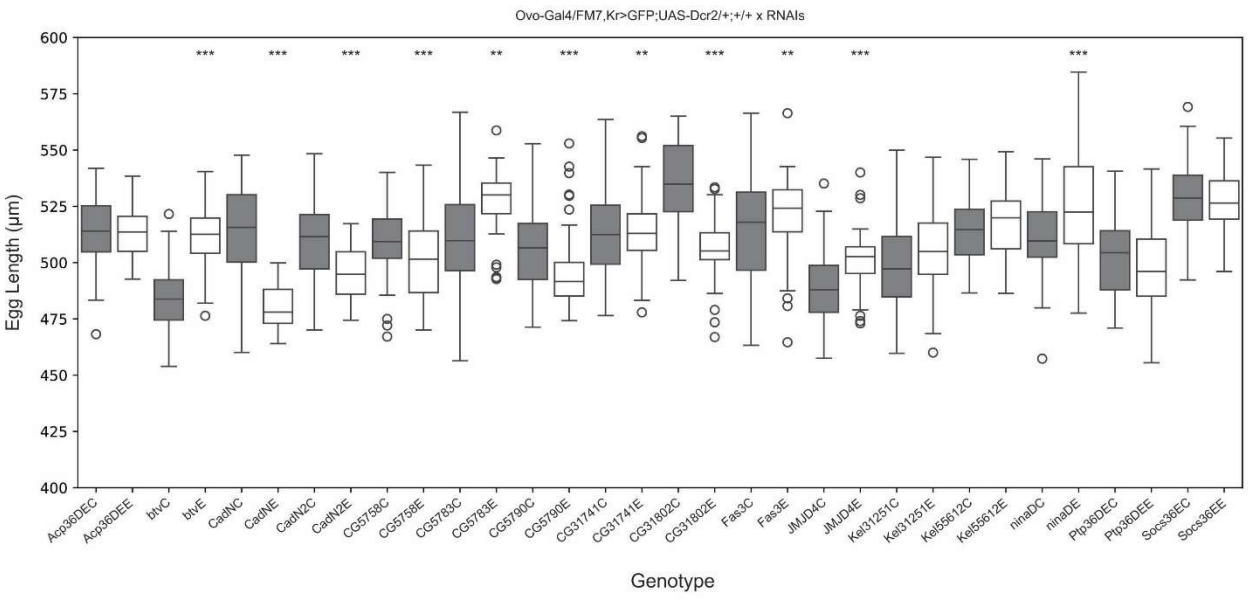
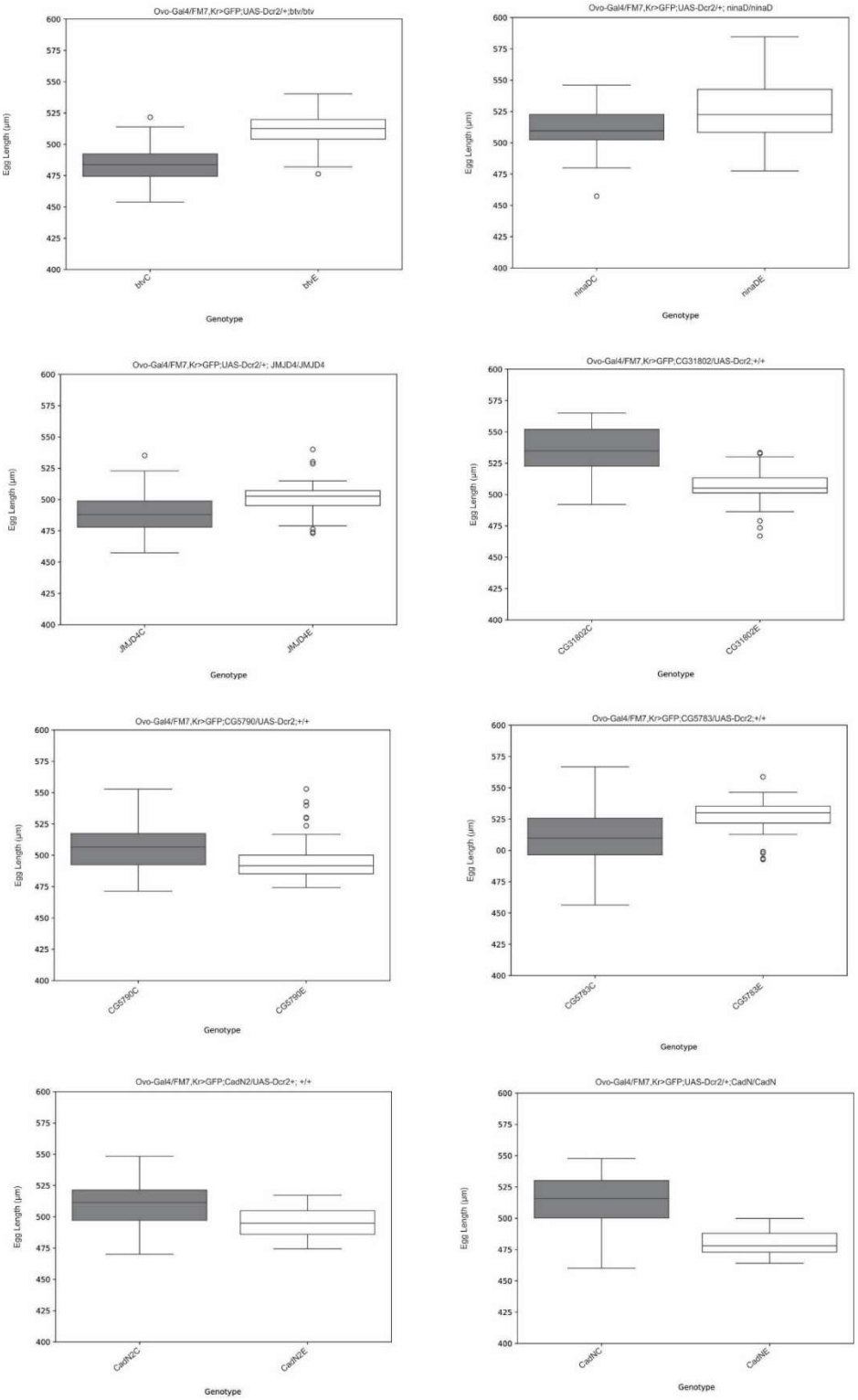


Figure 8: Egg length comparisons between each RNAi genotype. Controls are marked with a C and colored in gray, while the experimental are marked with an E with no color. A *t-test* was performed and a Bonferroni correction test with multiple comparisons, using R studio statistical program with a significance threshold of >0.05 using r studio. Each control RNAi was compared its experimental. *< 0.05, **< 0.001, ***< 0.0001. Sample size was 50 eggs per each RNAi pair (C and E).

Panel 1 shows the 8 genes with significant effect on egg length. We checked the expression of each gene in the female germline by using FlyAtlas2 (Krause et al.2020), a database containing quantified RNA-Seq transcripts of genes and their expression in individual tissues of *Drosophila melanogaster*. The results of each gene were displayed with an estimated abundance value calculated in the specific tissue. Since our interest is in the female *Drosophila* reproductive tract, we were able to identify the genes that are expressed in the ovary to better ascertain whether they are ideal candidates of egg length effect. The abundance of each gene in the adult female ovary was as follows from the highest; *JMJD4 6.1, cadN 0.8, CG5783 0.4* and *btv 0.1* whilst *cadN2, CG5790, CG31802*, and *ninaD*, each had a *0.0* abundance value. Although these genes show a low expression in the female germ line, more tests must be performed to fully understand how they affect the overall length of the egg.

Panel 1: Comparisons of individual genes. Control and experimental are marked as C and E respectively. These are the 8 genes that showed significant effect in egg length when knocked down by *ovo-Gal4/FM7,Kr >GFP*



9: *otu-Gal4/FM7, Kr>GFP* X RNAi gene knockdown

When we knocked down each of the 16 genes to *otu-Gal4/FM7, Kr>GFP*, 8 genes, showed a significant effect on the egg length as seen in (Fig. 9). For each pair of genes, *C* indicated the control and is colored in gray, while the *E* indicated the experimental, with color white. These genes are as follows: *btv, cadN, cadN2, CG5783, CG5790, CG31802, JMJD4* and *kel (BL# 31251)* *p >0.05.*

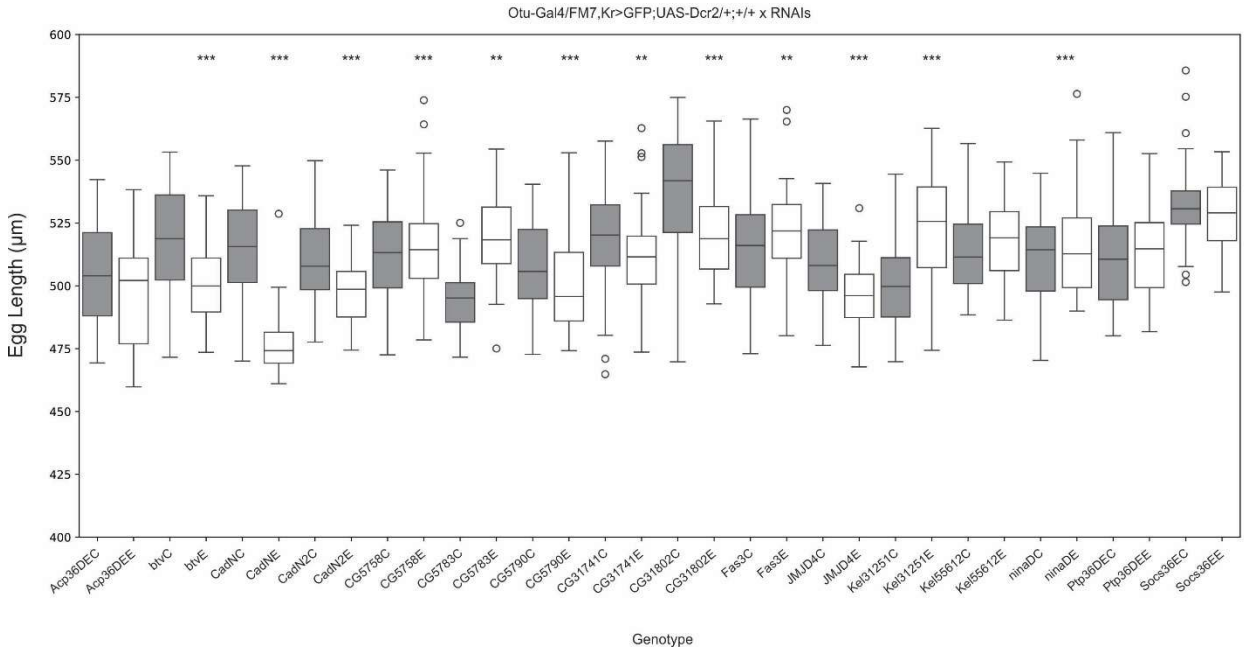
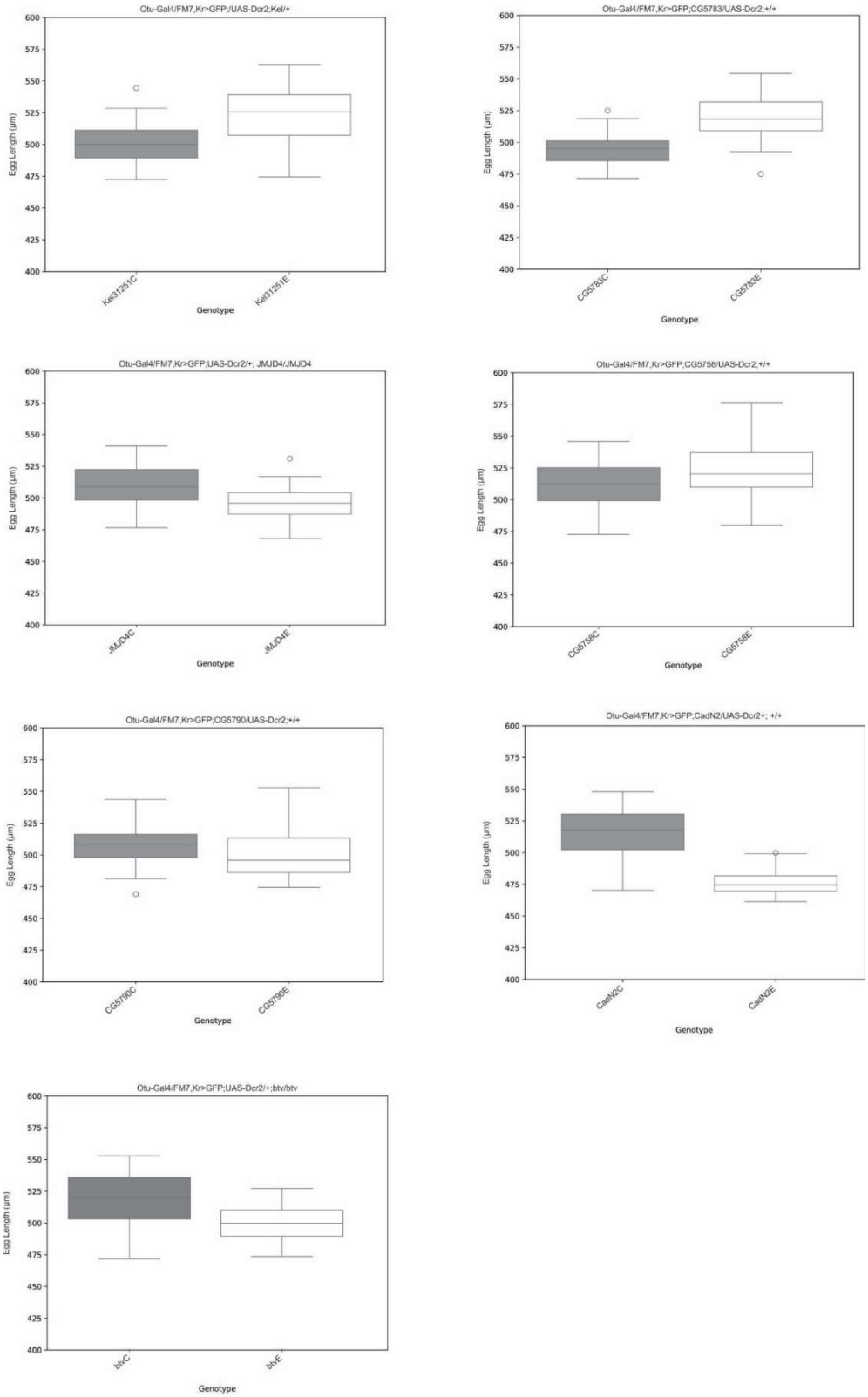


Figure 9*:* Egg length comparisons between each RNAi genotype. Controls are marked with a C and colored in gray, while the experimental are marked with an E with no color. A *t-test* was performed with a significance threshold of >0.05 using r studio. . *< 0.05, **< 0.001, ***< 0.0001. Sample size was 50 eggs per each RNAi pair (C and E).

Panel 2 shows the 7 genes with significant effect on egg length. We checked the expression of each gene in the female germline by using FlyAtlas2 (Krause et al.2020), a database containing quantified RNA-Seq transcripts of genes and their expression in individual tissues of *Drosophila* melanogaster. The results of each gene were displayed with an estimated abundance value calculated in the specific tissue. Since our interest is in the female *Drosophila* reproductive tract, we were able to identify the genes that are expressed in the ovary to better ascertain whether they

are ideal candidates of egg length effect. The abundance of each gene in the adult female ovary was as follows from the highest; *kel (BL#3125) 6.7, JMJD4 6.1, cadN 0.8, CG5783 0.4* and *btv 0.1* whilst *cadN2, CG31802,* and *CG5790* had a *0.0* abundance value. Although these genes show a low expression in the female germ line, more tests must be performed to fully understand how they affect the overall length of the egg.



Panel 2: Comparisons of individual genes. Control and experimental are marked as C and E respectively. These are the 7 genes that showed significant effect in egg length when knocked down by *otu-Gal4/FM7,Kr >GF*

10: *nanos-Gal4/FM7, Kr>GFP* X RNAi gene knockdown

When we knocked down each of the 16 genes to *nanos-Gal4/FM7, Kr>GFP*, 9 genes, showed a significant effect on the egg length as seen in (Figure 10). For each pair of genes, *C* indicated the control and is colored in gray, while the *E* indicated the experimental, with color white. These genes are as follows: *btv, cadN, cadN2, CG5758, CG5783, CG31802, JMJD4, Fas3,* and *Ptp36E, t-test pvalue> 0.05.*

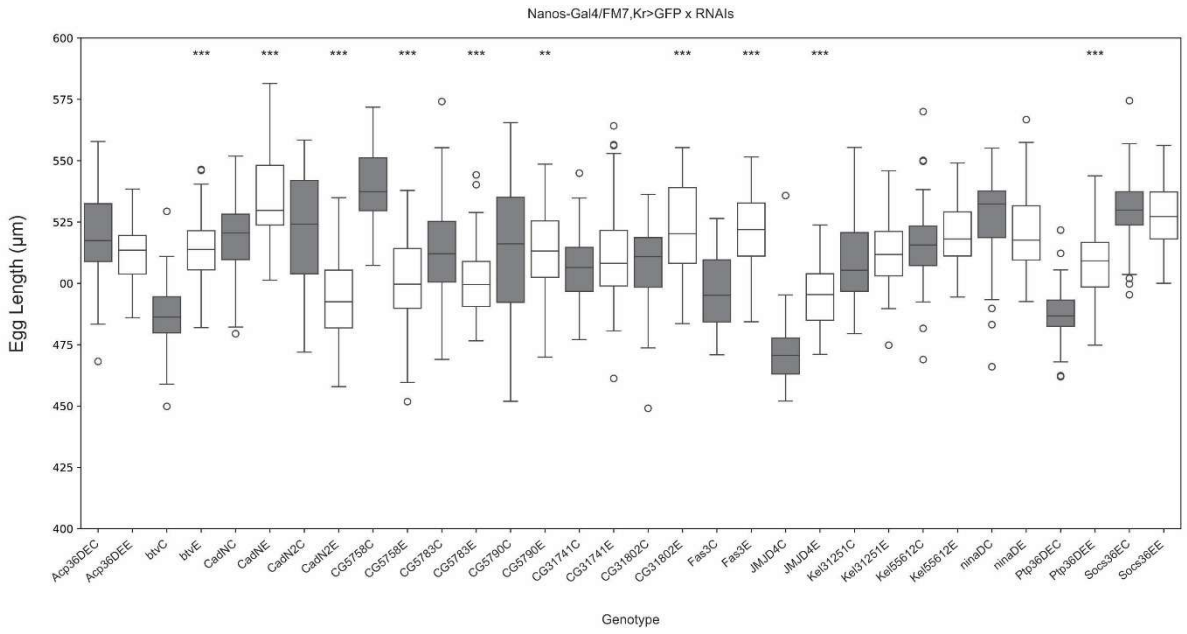
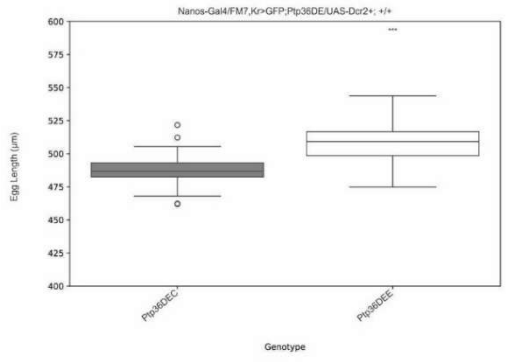


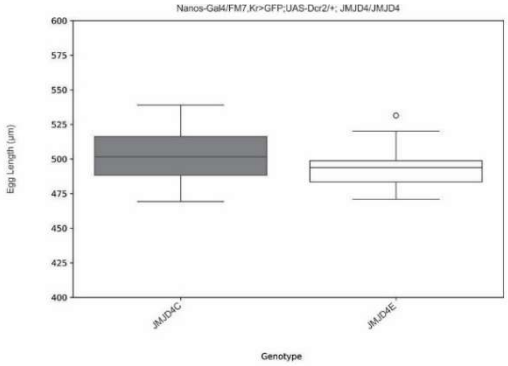
Figure 10: Egg length comparisons between each RNAi genotype. Controls are marked with a C and colored in gray, while the experimental are marked with an E with no color. A *t-test* was performed with a significant threshold of *p>0.05* using r studio. . *< *0.05,* **< *0.001,* ***< *0.0001*. Sample size was 50 eggs per each RNAi pair (C and E).

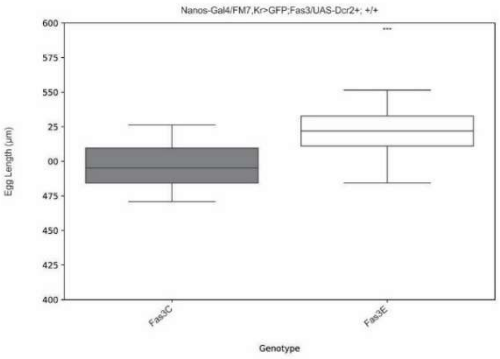
Panel 3 shows the 9 genes with significant effect on egg length. We checked the expression of each gene in the female germline by using FlyAtlas2 (Krause et al.2020), a database containing quantified RNA-Seq transcripts of genes and their expression in individual tissues of *Drosophila* melanogaster. The results of each gene were displayed with an estimated abundance value calculated in the specific tissue. Since our interest is in the female *Drosophila* reproductive tract, we were able to identify the genes that are expressed in the ovary to better ascertain whether they

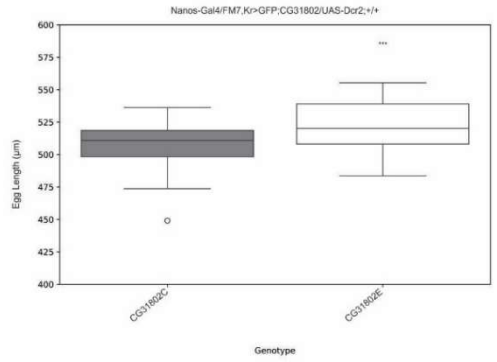
are ideal candidates of egg length effect. The abundance of each gene in the adult female ovary was as follows from the highest; *JMJD4 6.1, Fas3 5.3, cadN 0.8, CG5758 0.6, CG5783 0.4*, *Ptp36E 0.3*, and *btv 0.1* whilst *cadN2, and CG31802* each had a *0.0* abundance value. Although these genes show a low expression in the female germ line, more tests must be performed to fully understand how they affect the overall length of the egg.

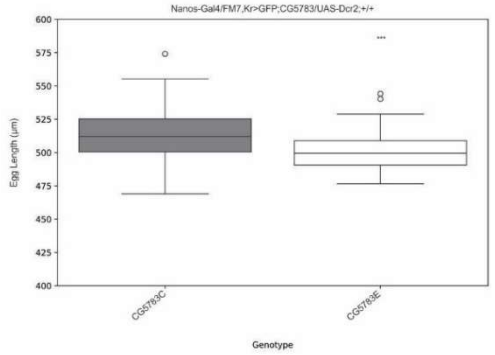
Panel 3: Comparisons of individual genes. Control and experimental are marked as C and E respectively. These are the 9 genes that showed significant effect in egg length when knocked down by *Nanos-Gal4/FM7,Kr >GFP*

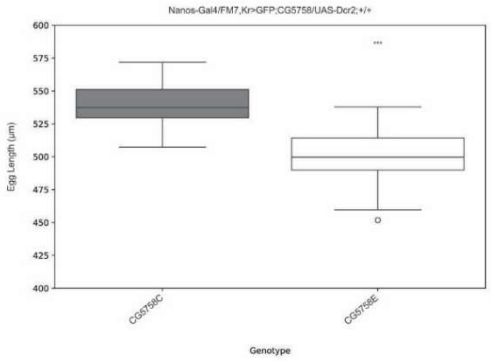


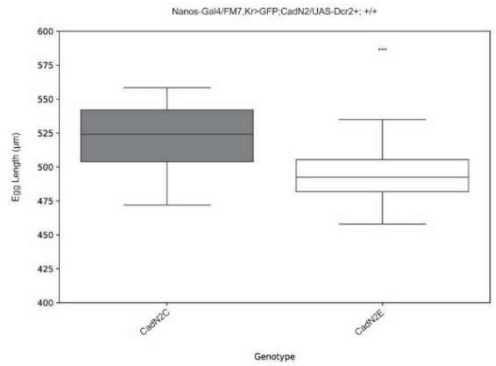


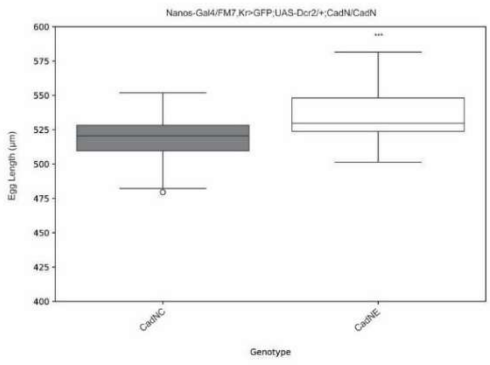


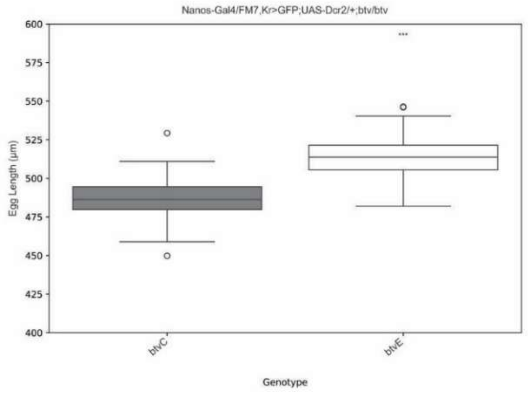








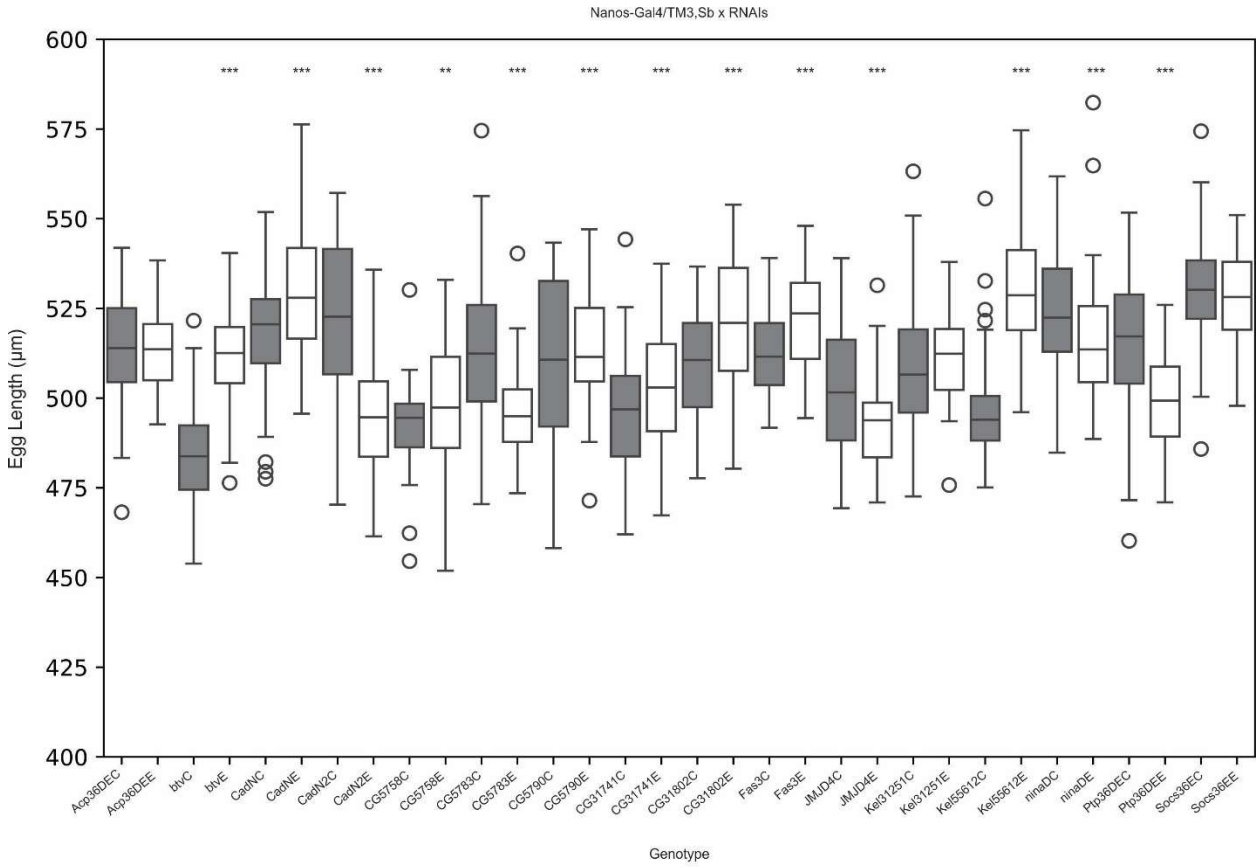




11: *UAS-Dcr2/+; nanos-Gal4/TM3,Sb* X RNAi gene knockdown

When we knocked down each of the 16 genes to *UAS-Dcr2/+; nanos-Gal4/TM3,Sb***,** 11 genes, showed a significant effect on the egg length as seen in (Fig. 11). For each pair of genes, *C* indicated the control and is colored in gray, while the *E* indicated the experimental, with color white. These genes are as follows: *btv, cadN, cadN2, CG5783, CG31741, CG31802, JMJD4, Fas3, Ptp36E, kel (BL#55612),* and *ninaD, t-test pvalue >0.05.*

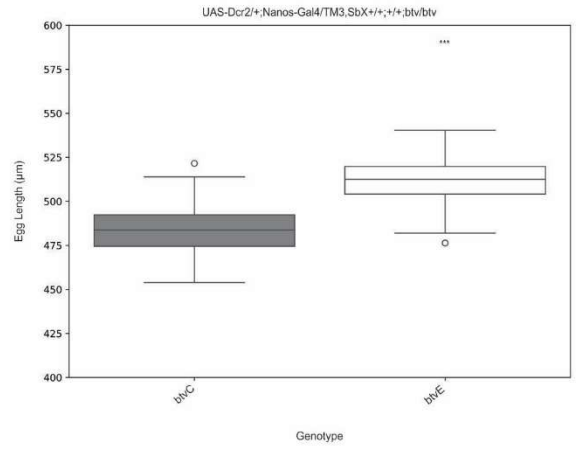
Figure 11: Egg length comparisons between each RNAi genotype. Controls are marked with a C and colored in gray, while the experimental are marked with an E with no color. A *t-test* was performed with a significance threshold of >0.05 using r studio. *< 0.05, **< 0.001, ***< 0.0001. Sample size was 50 eggs per each RNAi pair (C and E).

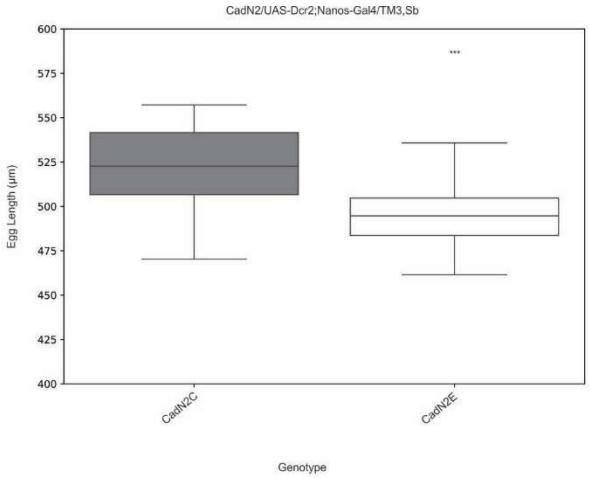


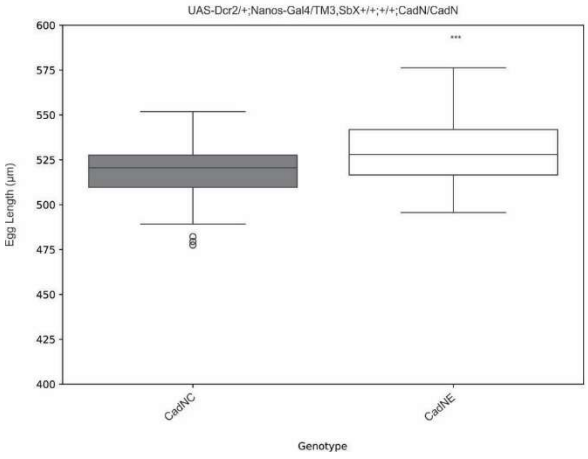
Panel 4 shows the 11 genes with significant effect on egg length. We checked the expression of each gene in the female germline by using FlyAtlas2 (Krause et al.2020), a database containing quantified RNA-Seq transcripts of genes and their expression in individual tissues of *Drosophila* melanogaster. The results of each gene were displayed with an estimated abundance value calculated in the specific tissue. Since our interest is in the female *Drosophila* reproductive tract, we were able to identify the genes that are expressed in the ovary to better ascertain whether they are ideal candidates of egg length effect. The abundance of each gene in the adult female ovary was as follows from the highest; *kel (BL 55612) 6.7, JMJD4 6.1, Fas3 5.3, cadN 0.8, CG5783 0.4*, *Ptp36E 0.3*, and *btv 0.1* whilst *cadN2, CG31802*, *CG31741,* and *ninaD*, each had a *0.0* abundance

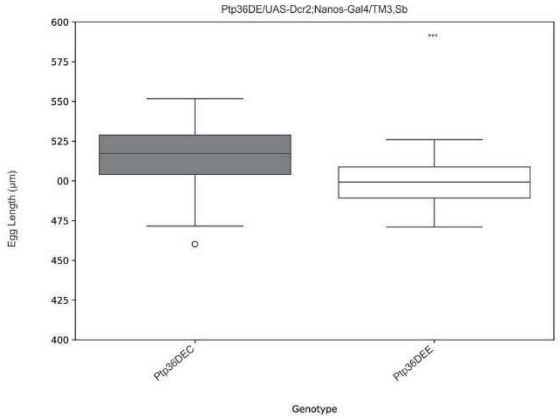
value. Although these genes show a low expression in the female germ line, more tests must be performed to fully understand how they affect the overall length of the egg.

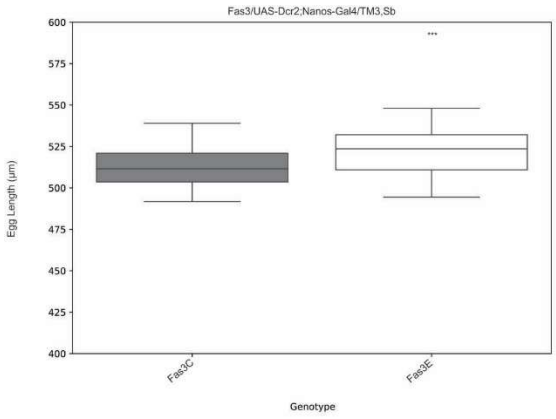
Panel 4: Comparisons of individual genes. Control and experimental are marked as C and E respectively. These are the 11 genes that showed significant effect in egg length when knocked down by *UAS-Dcr2/+;Nanos-Gal4/TM3,Sb*

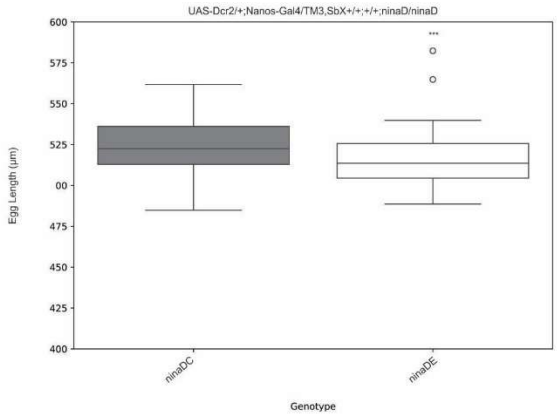


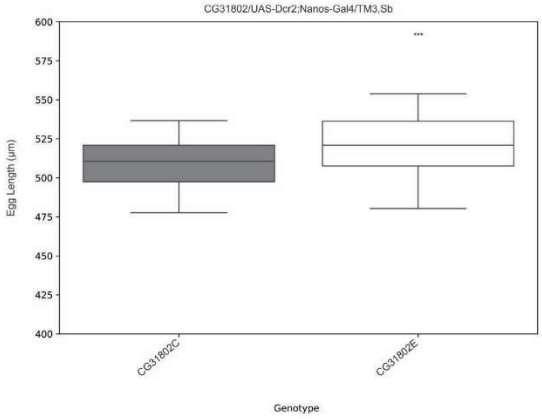


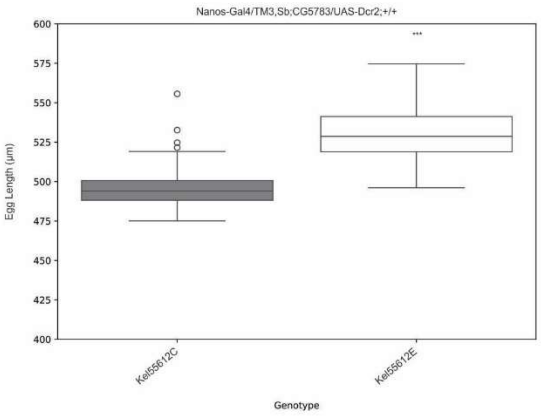


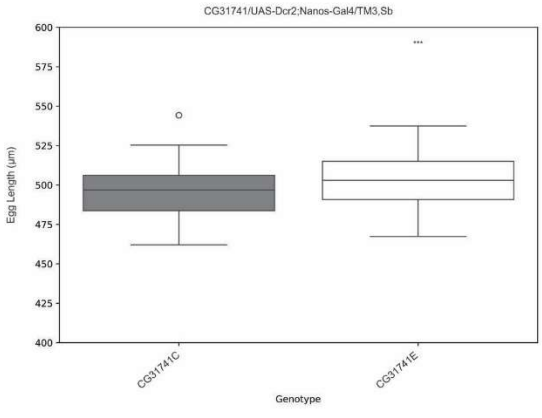

Ptp36DE/UAS-Dcr2;Nanos-Gal4/TM3,Sb
Egg Length (µm)
Ptp36DEC
Ptp36DEE
Genotype

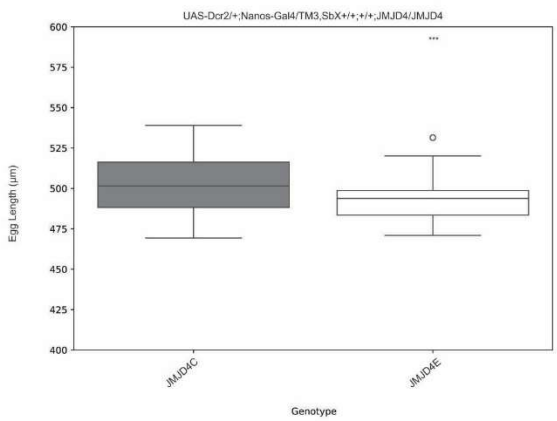

Fas3/UAS-Dcr2;Nanos-Gal4/TM3,Sb
Egg Length (µm)
Fas3C
Fas3E
Genotype

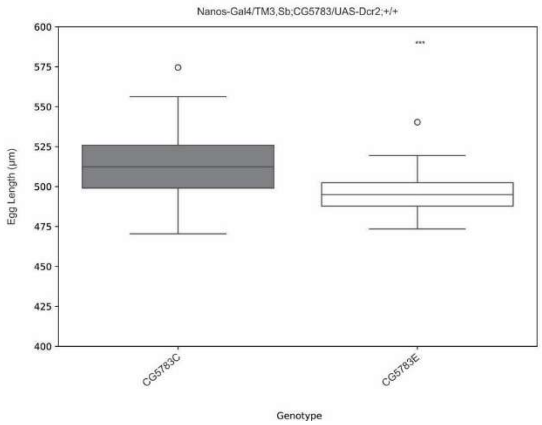

UAS-Dcr2/+;Nanos-Gal4/TM3,SbX+/+;+/+;ninaD/ninaD
Egg Length (µm)
ninaDC
ninaDE
Genotype


CG31802/UAS-Dcr2;Nanos-Gal4/TM3,Sb
Egg Length (µm)
CG31802C
CG31802E
Genotype


Nanos-Gal4/TM3,Sb;CG5783/UAS-Dcr2;+/+
Egg Length (µm)
Kel55612C
Kel55612E
Genotype


CG31741/UAS-Dcr2;Nanos-Gal4/TM3,Sb
Egg Length (µm)
CG31741C
CG31741E
Genotype


UAS-Dcr2/+;Nanos-Gal4/TM3,SbX+/+;+/+;JMJD4/JMJD4
Egg Length (µm)
JMJD4C
JMJD4E
Genotype


Nanos-Gal4/TM3,Sb;CG5783/UAS-Dcr2;+/+
Egg Length (µm)
CG5783C
CG5783E
Genotype

12: Gene knockdown effect by each *Gal4*

From our results, we also noticed that 5 genes showed consistent results affecting the egg length when knocked down by all 4 Gal4s. The genes *btv, cadN, cadN2, CG5783, CG31802, and JMJD4* showed consisted results respectively. When genes were knocked down by the *nanos/TM3,Sb* and *Nanos/FM7, Kr>GFP*, 8 genes showed consistent results as well. However, we also noticed that individual genes showed significant results when knocked down by each individual Gal4. These genes are *kel (BL# 31251)* which only had significant effect in egg length when knocked down by the *otu-Gal4/FM7, Kr>GFP. CG5758* had significant on egg length only when knocked down to *nanos-Gal4/FM7, Kr>GFP*. When knocked down by both *nanos-Gal4/FM7, Kr>GFP* and *nanos/TM3,Sb*, both *Fas3* and *Ptp36E* showed an effect in egg length. *kel (BL#55612*) and *CG31741* showed an effect in egg length when knocked down by *nanos-Gal4/FM7, Kr>GFP* and *nanos/TM3,Sb*. One gene, *ninaD* showed significant effect when knocked by both *ovo-Gal4/FM7, Kr>GF*P and *nanos-Gal4/TM3,Sb* and not when knocked down by *nanos-Gal4/FM7, Kr>GFP* and *otu-Gal4/FM7, Kr>GFP.* The summary of these results is shown in the upset plot (Fig. 12) below.

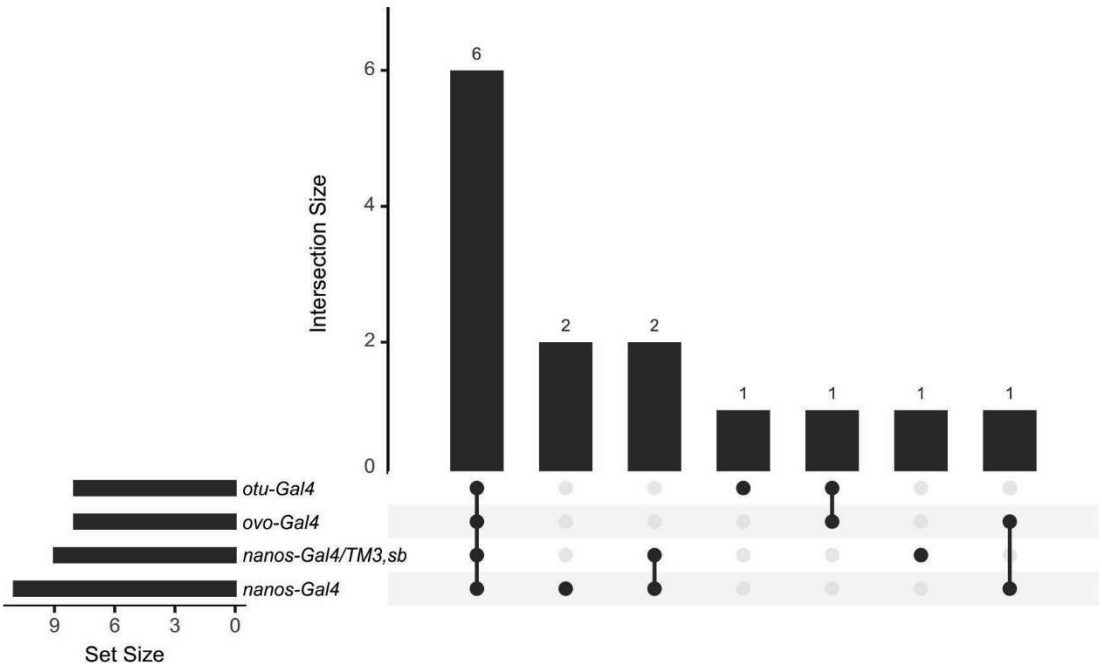


Figure 12: Gene knockdown effect by the different Gal4 driver lines. This upset plot summarizes the genes that had an efect on egg length as per knockdown by specific Gal4 driver line.

13: Gene knockdown affected egg laying behavior in specific experimental lines

Out of the 16 RNAi we crossed, adult females from 5 experimental RNAi's were unable to lay their eggs as normal. Although all the flies were raised on the same standards and for the same 5 days, we decided to extend the egg laying to a total of 10 days and there were still no eggs laid. The 4 RNAis are, *kel (BL# 31251), CG31802, CG5783, Fas3* and *CadN.* We were able to dissect their ovaries to extract the eggs for egg fixation, imaging and analysis. It is important to note that these RNAis did lay eggs when crossed to different Gal4s. For instance, when knocked down by *ovo-Gal4/FM7, Kr >GFP*, *kel (BL#31251), CG31802*, and *CG5783*, these females did not lay any eggs, but when crossed to other three *Gal4s,* the adult females were able to lay their eggs normally. Similarly, when *Fas3* and *cadN* were knocked down by *otu-Gal4/FM7, Kr >GFP*, the adult females did not lay any eggs, but when crossed to the other three *Gal4s* they were able to lay eggs

normally. This indicated that some Gal4s may be more effective in gene knockdown than others in the same tissues. Oogenesis was not disrupted as the eggs were able to develop to maturity. However, the knockdown using the *ovo-Gal4/FM7, Kr >GFP* affected the female's oviposition.

14: The female adult body size does not correlate with egg length

We tested whether the body size correlates to egg length size. Our results indicated that body size did not correlate with egg length. These results led us to conclude that the RNAi's body size and egg length size are independent of each other. We generated a scatterplot to show the correlation Results are displayed in (Fig 13, Fig.14, Fig15, Fig. 16). We performed a welch test to understand the difference between egg length and tibia length. Results are displayed in (Table. 2, Table. 3, Table. 4, Table. 5) in the appendix.

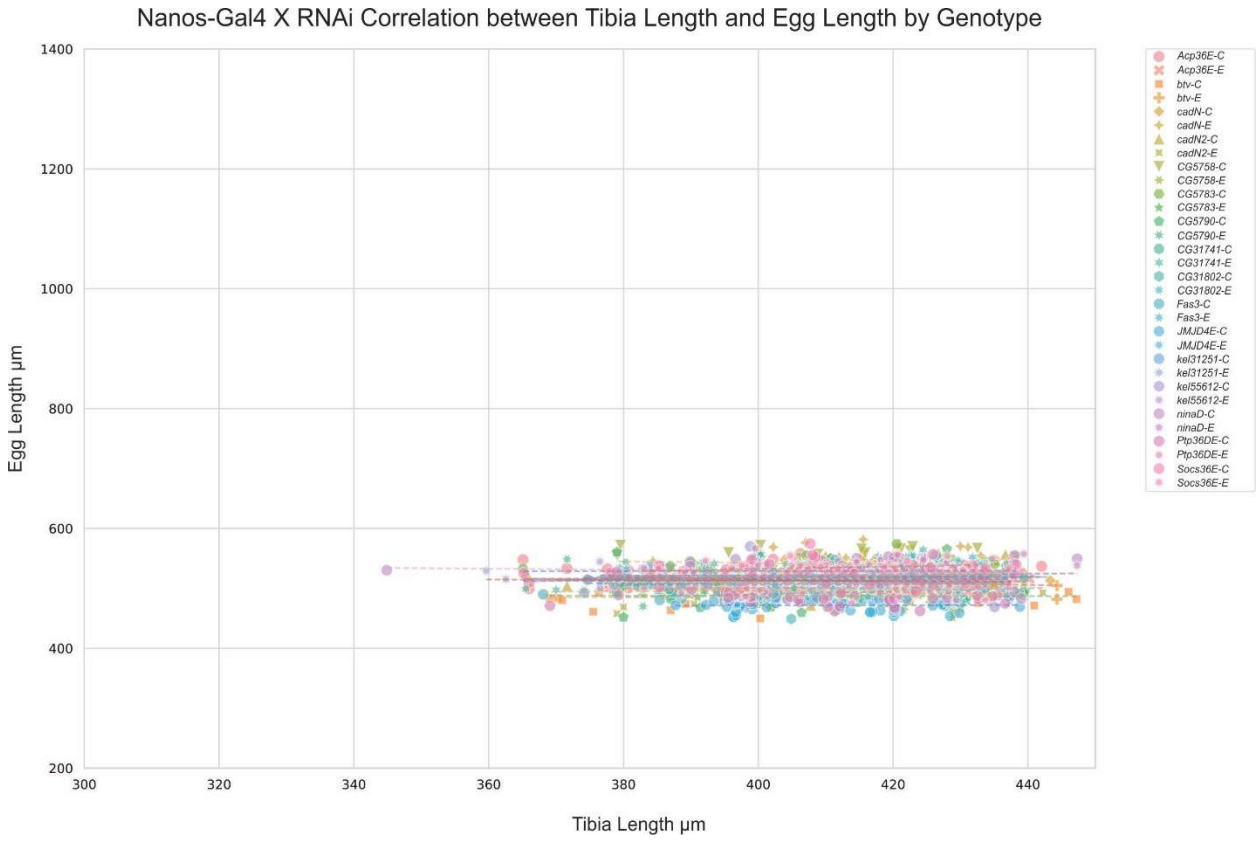


Figure 13: Correlation between Tibia Length and Egg Length when *Nanos-Gal4* was crossed to all RNAis. Scatterplot generated to best visualization. Results indicate that there are no correlations between the two. Sample sizes are 50 eggs per genotype and 50 tibias per genotype.

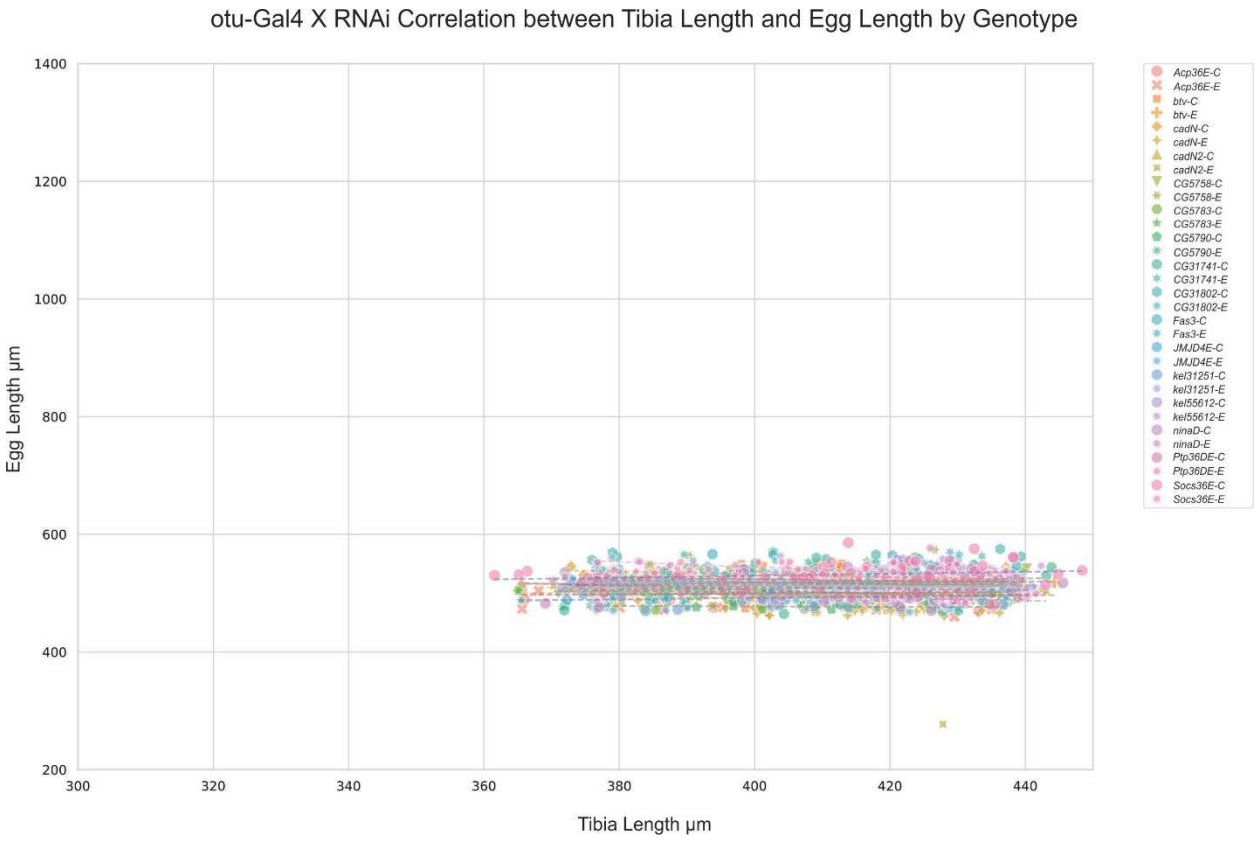


Figure 14: Correlation between Tibia Length and Egg Length when *otu-Gal4* was crossed to all RNAis. Scatterplot generated to best visualization. Results indicate that there are no correlations between the two. Sample sizes are 50 eggs per genotype and 50 tibias per genotype.

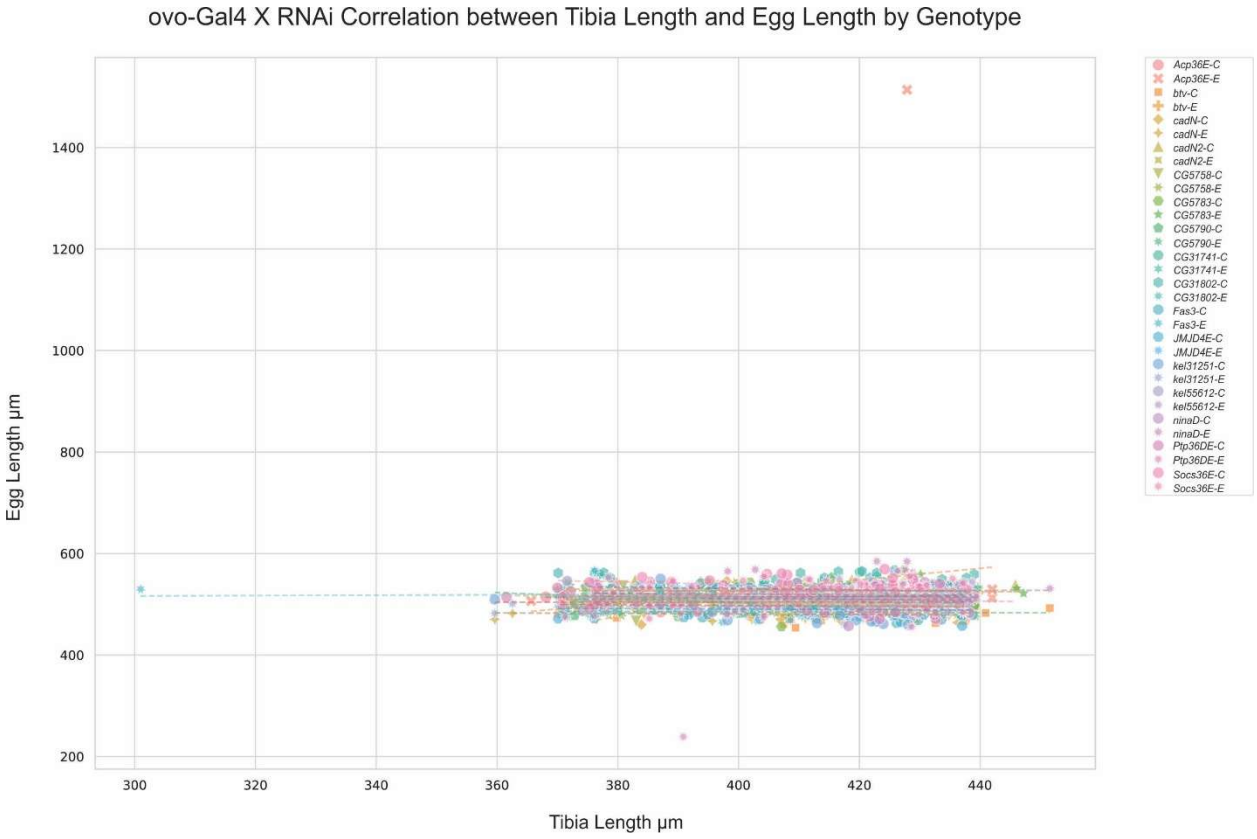


Figure 15: Correlation between Tibia Length and Egg Length when *ovo-Gal4* was crossed to all RNAis. Scatterplot generated to best visualization. Results indicate that there are no correlations between the tibia and egg lengths. Sample sizes are 50 eggs per genotype and 50 tibias per genotype.

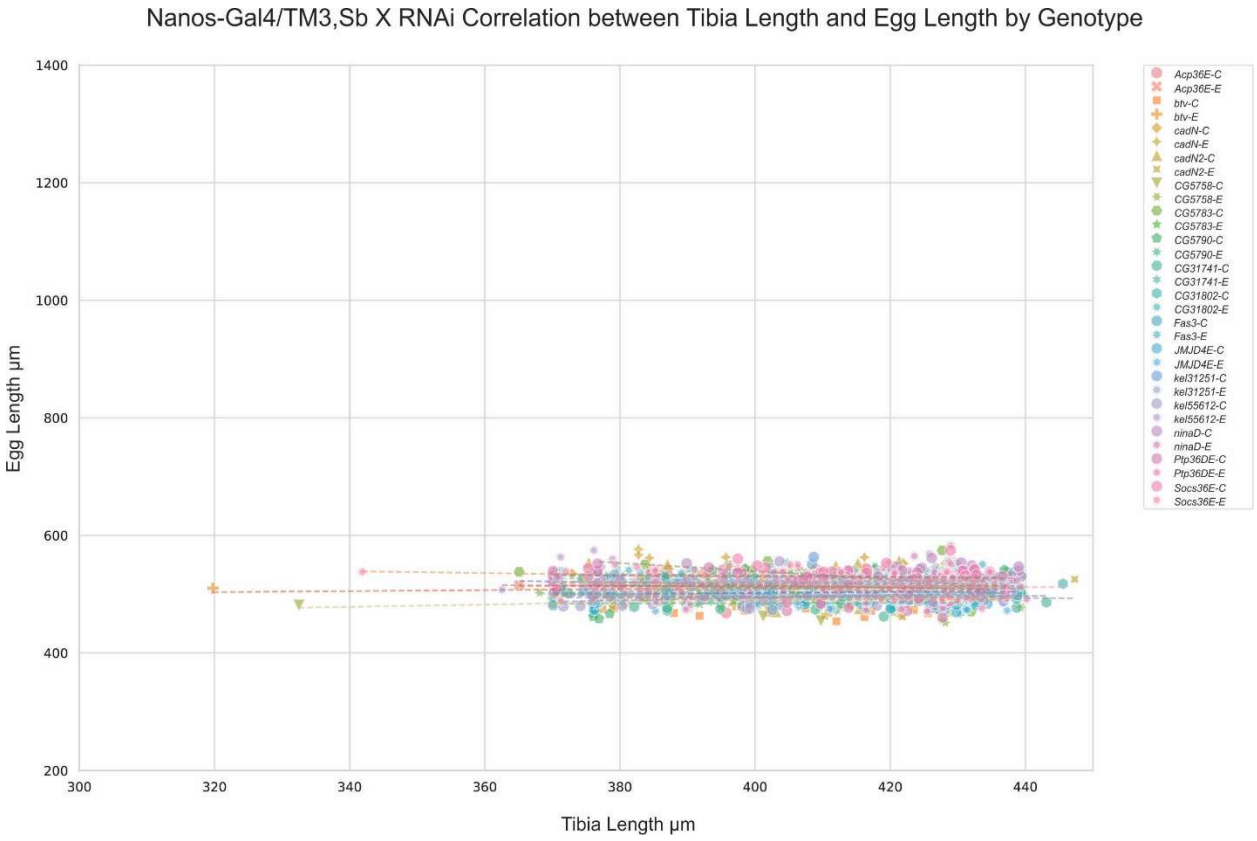


Figure 16: Correlation between Tibia Length and Egg Length when *Nanos-Gal4/TM3,Sb* was crossed to all RNAis. Scatterplot generated to best visualization. Results indicate that there are no correlations between the tibia and egg lengths. Sample sizes are 50 eggs per genotype and 50 tibias per genotype.

We also wanted to know whether there were any differences between tibia sizes between control and experimental RNAi. We performed an F test and our results indicated that there were no significant differences between tibia sizes of female before and after gene knockdown as show in (Fig. 17, Fig. 18, Fig. 19, Fig. 20). We also plotted the results in a box and whisker plot as shown in (Fig. 21, Fig. 22, Fig. 23, Fig. 24) in the appendix.

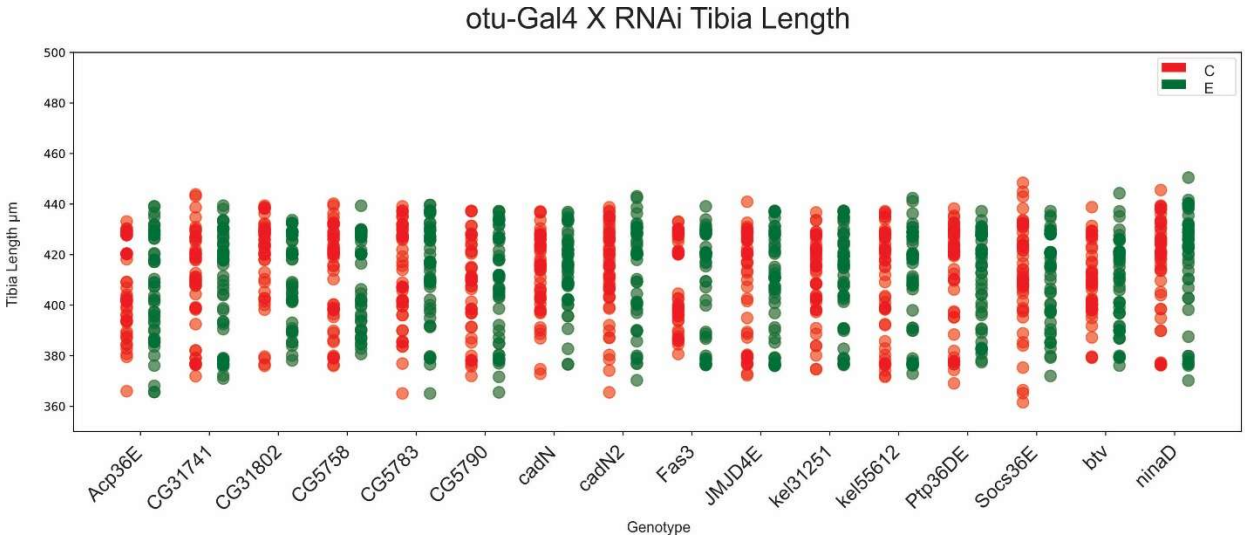


Figure 17: Tibia length. This scatterplot shows the correlation between experimental and control RNAi whose tibias were measured and analyzed. There were no statistical differences between the control vs experimental tibia lengths. F test p-value <0.05.

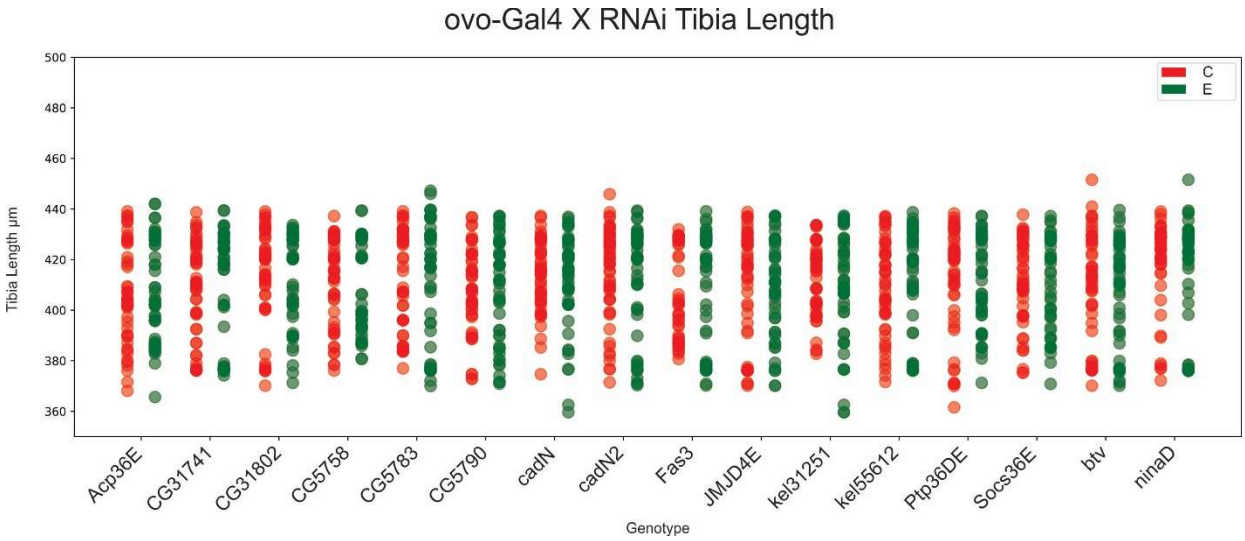


Figure 18: Tibia length. This scatterplot shows the correlation between experimental and control RNAi whose tibias were measured and analyzed. There were no statistical differences between the control vs experimental tibia lengths. F test p-value <0.05

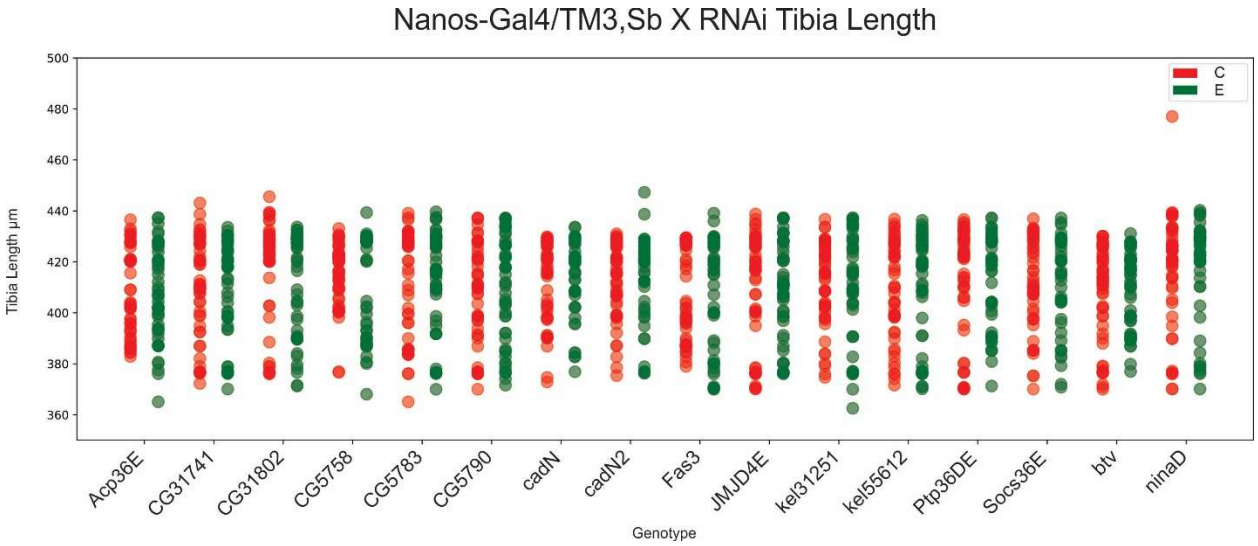


Figure 19: Tibia length. This scatterplot shows the correlation between experimental and control RNAi whose tibias were measured and analyzed. There were no statistical differences between the control vs experimental tibia lengths. F test p-value <0.05

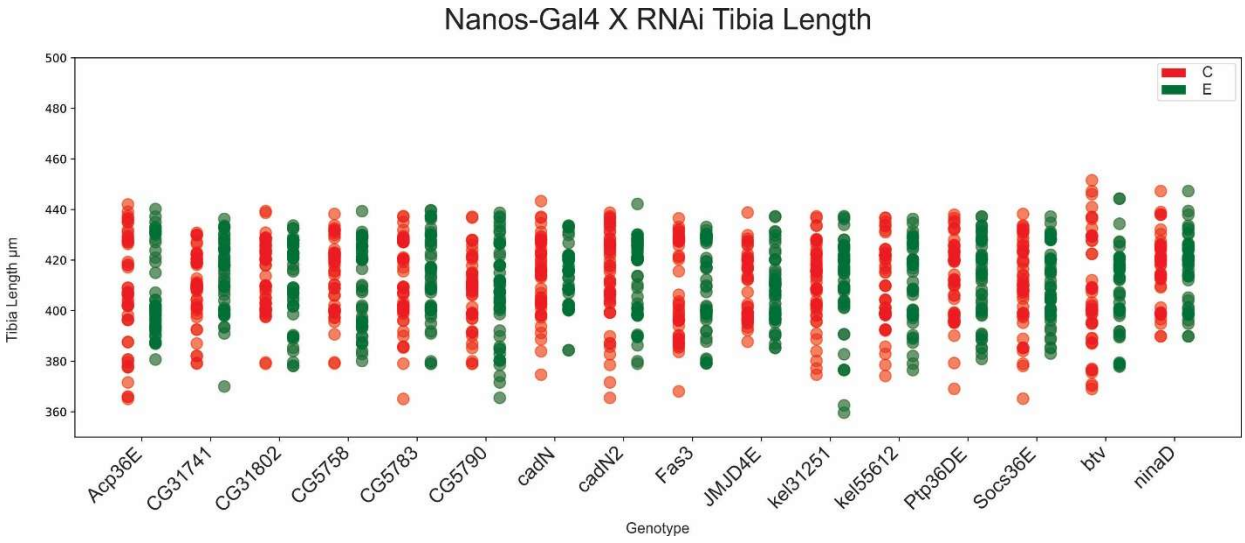


Figure 20: Tibia length. This scatterplot shows the correlation between experimental and control RNAi whose tibias were measured and analyzed. There were no statistical differences between the control vs experimental tibia lengths. F test p-value <0.05.

15. Gene knockdown did not affect the ovariole numbers.

We dissected and counted the number of ovarioles from each ovary for every female in each genotype. There were no differences in ovariole numbers between the control and the experimental adult flies (Table. 2, Table. 3, Table. 4, Table. 5) in the Appendix and the scatterplots below (Fig. 25, Fig. 26, Fig. 27, Fig. 28). To further analyze this, we dissected the RNAi females that were not crossed to any Gal4 and counted their ovarioles. The number of ovarioles remained the same in each genotype. These results concluded that although there is a correlation between egg size and ovariole number, gene knockdown did not affect ovariole numbers, instead it affected only the egg length. This experiment was necessary because study have shown that some genes that affect overall egg size may also affect ovariole number. This was not the case for our results.

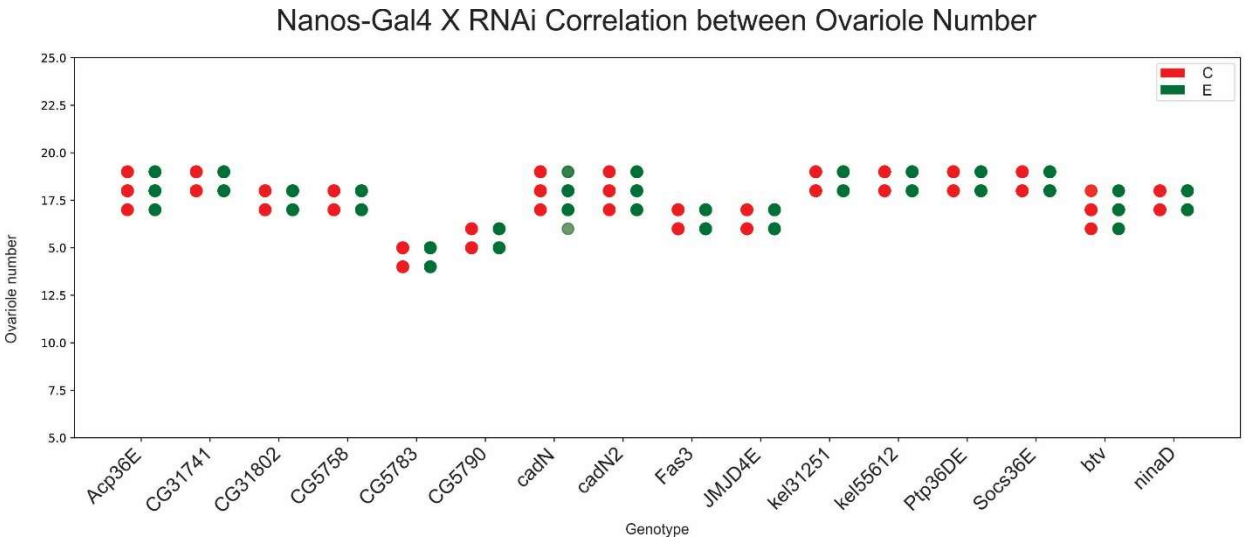


Figure 25: Ovariole number. This scatterplot shows the correlation between experimental and control RNAi whose ovarioles were counted. There were no statistical differences between the two as the number of ovarioles remained the same for each genotype.

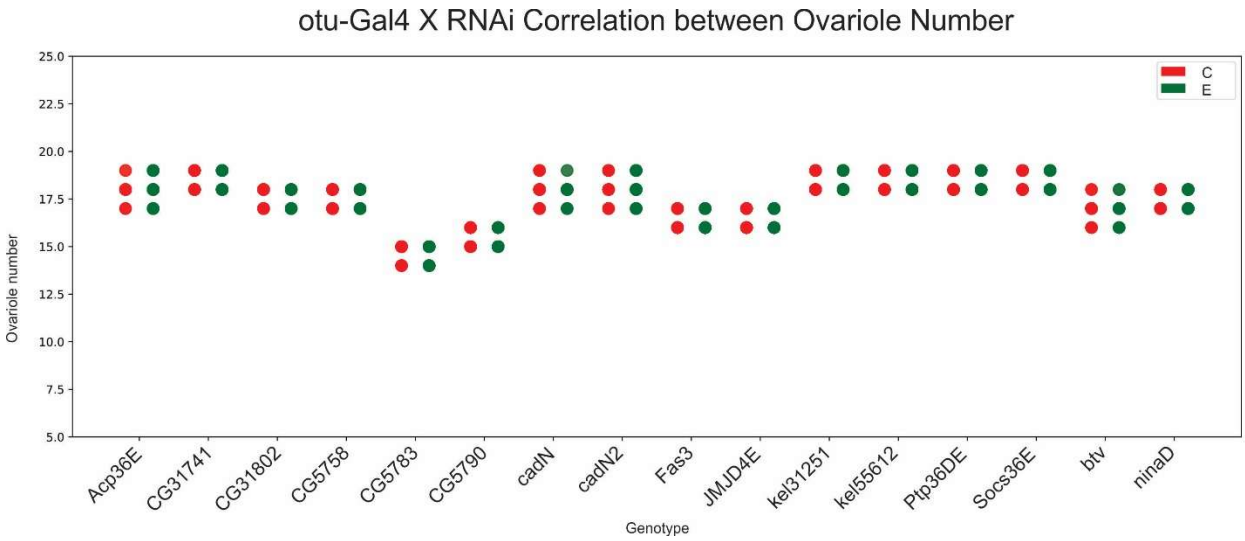


Figure 26: Ovariole number. This scatterplot shows the correlation between experimental and control RNAi whose ovarioles were counted. There were no statistical differences between the two as the number of ovarioles remained the same for each genotype.

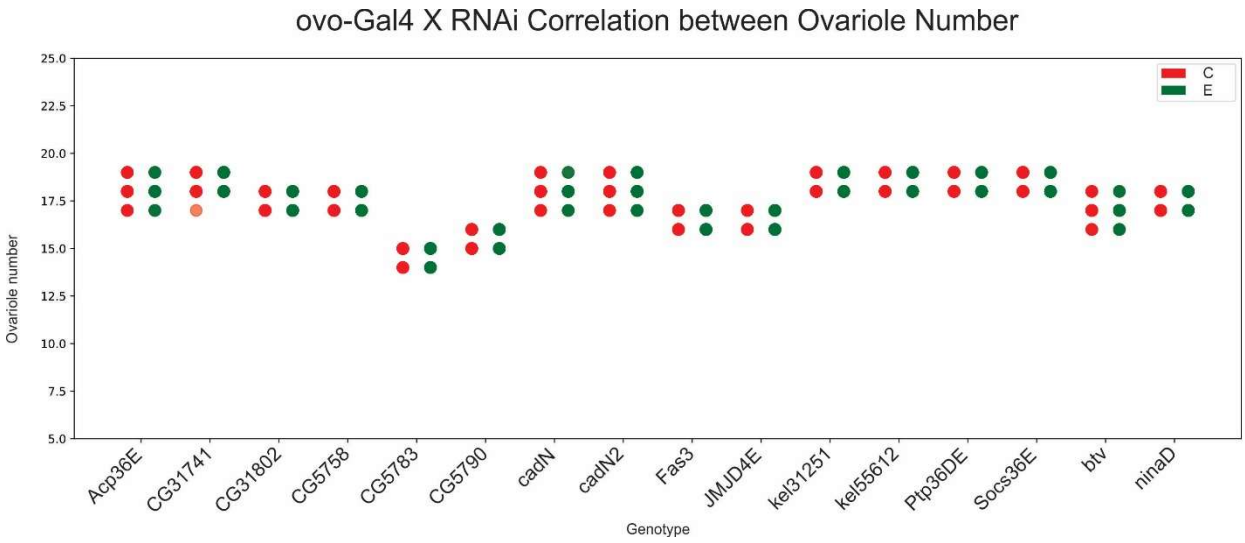


Figure 27: Ovariole number. This scatterplot shows the correlation between experimental and control RNAi whose ovarioles were counted. There were no statistical differences between the two as the number of ovarioles remained the same for each genotype.

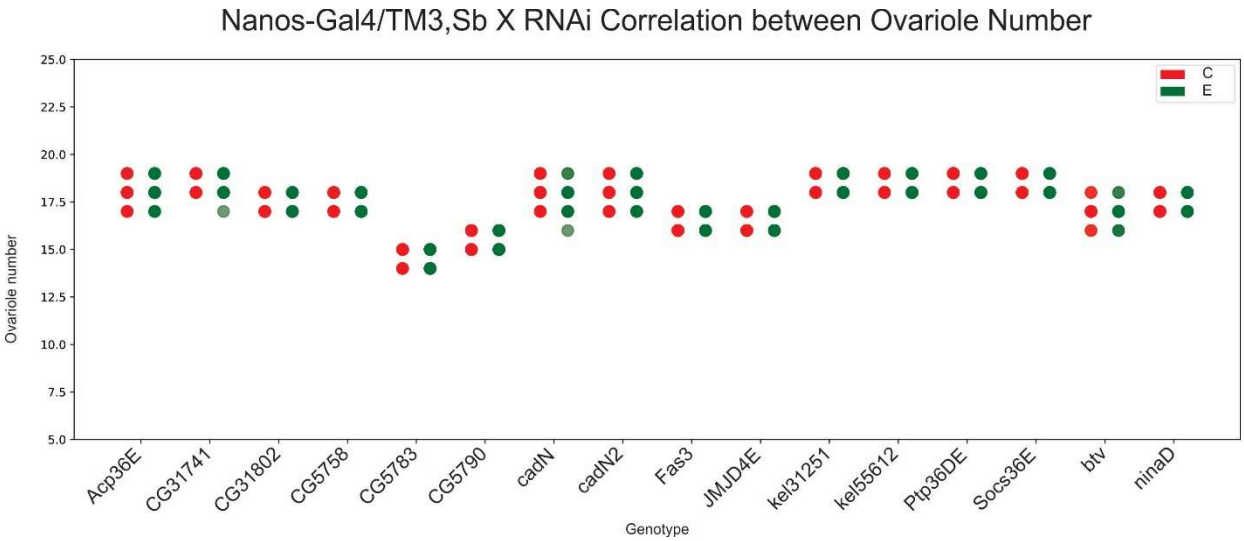


Figure 28: Ovariole number. This scatterplot shows the correlation between experimental and control RNAi whose ovarioles were counted. There were no statistical differences between the two as the number of ovarioles remained the same for each genotype.

DISCUSSION

In this study, we investigated the gene(s) that cause egg length variation among *Drosophila* species by taking advantage of our lab's stock of introgression lines. These introgressions have mostly *D. sechellia* background with a small segment of *D. mauritiana* genomic material (Masly and Presgraves 2007).

Based on our initial results, we aligned the genomic sequences of *3Q1(A)* to those of *D. sechellia* and *D. mauritiana.* We were able to identify the *3Q1(A)* genomic region and identified genes that lie therein. This region is 629,455 base pairs long and contains 72 genes. 43 of theses are protein coding genes and the other 30 genes are lncRNAs (Long non-coding RNAs). Based of availability, we were able to test 16 genes.

We performed genetic crosses using the *Gal4-UAS* system to knock down each gene and measured its effect on the egg length. We have identified 12 genes that have significant effect on egg length and 6 of those genes are promising to be ideal candidates for egg length effect. This is because the 6 genes showed consistent results of influencing egg length when knocked down by all 4 Gal4s

driver lines. Our results provide an opportunity to study the effectiveness of tissue specific Gal4 knockdown by proving that while the 4 Gal4s we used were specific to the female germline, some were more effective than others. This is due to their distinct expression patterns in the female germline. *Ovo-Gal4* is highly expressed in the female germline and is maintained throughout oogenesis. *Otu-Gal4* is expressed in the germline stem cells and has a high expression pattern during the early egg chamber formation and in the germarium. On the other hand, we used two nanos-gal4. One was balanced using the *TM3,sb*, a third chromosome balancer for *Drosophila* genetic research. This balancer carries dominant markers known as stubble that helps in maintaining lethal or deleterious mutations and easily identifying flies that carry the balancer. The flies with this marker had shortened hair "stubble" on their backs and were used as controls while the flies with normal stubble were experimental. The second nanos-gal4 was balanced using FM7, an x chromosome balancer tagged with GFP for sibling control and better comparisons. Flies containing the GFP were used as controls while those without the GFP were the experimental. Nanos-Gal4 is expressed at the tip of the germarium to the mature egg. Using the different Gal4 driver lines provided us with a broader understanding of the expression patterns of each gal4 in the ovary as well as the effectiveness of gene knockdown in the specific tissues in the ovary.

The 12 genes that had an effect on egg length are as follows: *cadN, cadN2, Fas3, btv, CG5783, CG5758, CG5790, CG31741, CG31802, JMJD4, Ptp36E,* and *Kel.* Some of these genes have been known to have an effect in oogenesis while others do not. For instance, *Kel* is a gene well studied and known to be involved in the formation of ovarian ring canals in adult female *Drosophila* (Robinson et al. 1994). Without *kel,* the overall size of egg is affected (Xue and Cooley, 1993).

The CG (Computer Generated) genes are *CG5783, CG5758, CG5790, CG31741, CG31802.* While their direct expression in the female germline is not yet known, we believe that they are all involved in oocyte maturation as they all affected egg length. More tests must be done to expand this.

NinaD is a gene that is localized in the plasma membrane and is essential for the cellular uptake of carotenoids. While its known function is its relation to vision, we believe it is involved in oocyte development as well. Studies have shown that *ninaD* works in conjunction with *CD36 Scavenger* receptor *Bez* (Carrera et al. 2024) to ensure that lipids are properly transferred from the fat body to the ovaries. High amounts of lipids are stored in the female ovaries to support oocyte maturation. Due to this reason, we believe that any gene that disrupts the process of fat body accumulation in the ovaries will have an effect on overall egg size and shape.

Studies have shown that the *btv* gene (*beethoven*) plays a major role in the sensory organ function involved in auditory and vibration detection (Eberl et al. 1997). While its involvement in the egg development is unknown, this gene might have an indirect effect in the pathways that lead to egg development causing an effect on egg length as seen in our results.

Ptp36E is highly expressed in the adult *Drosophila's* head as well as in the female germline (Agrawal and Hardin, 2016; Fitzpatrick et al. 1995). This gene is involved in regulating the germline stem cells as well as ensuring that oocyte develops properly (Fitzpatrick et al. 1995). Although its involvement in egg morphology is unknown, our results indicated that it caused the experimental egg length to be smaller than the controls.

JMJD4 gene is expressed in the female germline and is crucial in the development and maintenance of the female germline stem cells (Holowatyj et al. 2015). *JMJD4* influences histone modifications and gene expression. Studies have also shown that mutations in *JMJD4* gene may

cause abnormal development of oocyte leading to changes in overall egg size (Holowatyj et al. 2015).

Our results also show that three of the genes that showed an effect in egg length were neuronal genes. The genes, *CadN* and *CadN2* are among the N-cadherin genes that are known to be involved in neuronal circuit formation (Kurusu et al. 2012) while *Fas3*, is part of a group of neural adhesion genes that also play a role in neural circuit formation (Vaikakkara et al. 2024). Cadherin genes are transmembrane proteins that mediate cell-cell adhesions. They provide guidance and growth of normal processes. This might explain why they showed an effect in egg length. While these genes showed an effect on egg length, they did not disrupt the process of oogenesis, as the eggs were able to develop to maturity.

We also noticed that these cadherin genes showed an opposite effect when knocked down by the different gal4 driver lines. When knocked down by *ovo-Gal4*, and *otu-Gal4*, both *cadN* and *cadN2* caused the experimental eggs to become bigger than the control. When knocked down by *nanos-Gal4* the *cadN* caused the eggs to be bigger than the control while the *cadN2* caused the egg to be smaller than the control. These differences in egg length might be due these genes being abundant in the ovary.

Studies have shown that cadherin genes also play a role in regulating overall egg size in Drosophila. This is due to their involvement in oogenesis and when knocked down, might cause defects in egg development (Vaikakkara et al. 2024). One of the cadherin genes, Fas3, is hypothesized to be involved in cell shape-change and rearrangements that are necessary for transforming the initially flat epithelium into a tube during oogenesis (Vaikakkara et al. 2024). Fas3 synaptic target mediates cell adhesion, axon outgrowth and does not directly control egg size (Vaikakkara et al. 2024).

Our results provide an opportunity to further studies in reproductive biology to understand the role of each gene during oogenesis. Based on our results from the females who were unable to lay eggs, our analysis proved that the eggs were fully matured which means that oogenesis was not disrupted. While we cannot ascertain that the gene knockdown might have disrupted their neuronal pathway that plays a role in oviposition, we can study this phenomenon. Let us recall that, although sex peptides play a role in female oviposition, they are only effective if the female fly is mated. However, all females in this study were virgins which indicated that there might be other reasons as to why oviposition was disrupted.

These results, together, provide direction for future developmental and reproductive biology in studies on oogenesis among *Drosophila* species.

Appendix

Table 1: Summary of Egg length means, Tibia length means, Egg length and Tibia length correction, and Egg volume and ovariole numbers in the 12 introgression lines as well as *D. mauritiana* and *D. sechellia*. Means are reported ± standard error.

Genotypes	Egg Length (mm)	Tibia Length (mm)	Egg/Tibia (mm)	Egg Volume (mm^3) ($x10^{-3}$)	Ovariole number
2Q2	0.559± 0.0038	0.423± 0.0021	13.21± 0.0111	2.075± 0.002	16
2X2	0.561± 0.0025	0.427± 0.0021	13.13± 0.0087	1.535± 0.001	16
3J1	0.561± 0.0033	0.42± 0.0022	13.35± 0.0107	1.623± 0.002	16
3Q1(A)	0.547± 0.0031	0.411± 0.0024	13.31± 0.011	1.477± 0.001	17
3Q1(B)	0.569± 0.0034	0.424± 0.002	13.42± 0.0104	1.721± 0.002	16
4C2A;Q1A	0.564± 0.003	0.397± 0.0022	14.21± 0.0111	1.358± 0.001	16
I1(B)	0.554± 0.0033	0.431± 0.0018	12.85± 0.0095	1.604± 0.002	17
Mauriw	0.486± 0.0031	0.389± 0.0013	12.49± 0.0091	0.891± 0.001	36
Neneh 2(A)	0.567± 0.0031	0.413± 0.0026	13.72± 0.0116	1.572± 0.002	17
Neneh 2(B)	0.565± 0.0037	0.405± 0.0024	13.95± 0.0126	1.775± 0.002	16
Q1(A)	0.562± 0.0024	0.411± 0.0018	13.67± 0.0086	1.311± 0.001	16
Rylah1	0.535± 0.0037	0.403± 0.0026	13.27± 0.0128	1.203± 0.001	16
Sechw	0.575± 0.011	0.413± 0.0025	13.92± 0.0088	1.777± 0.002	15
Yar1	0.587± 0.029	0.408± 0.0027	14.38± 0.012	1.739± 0.002	15

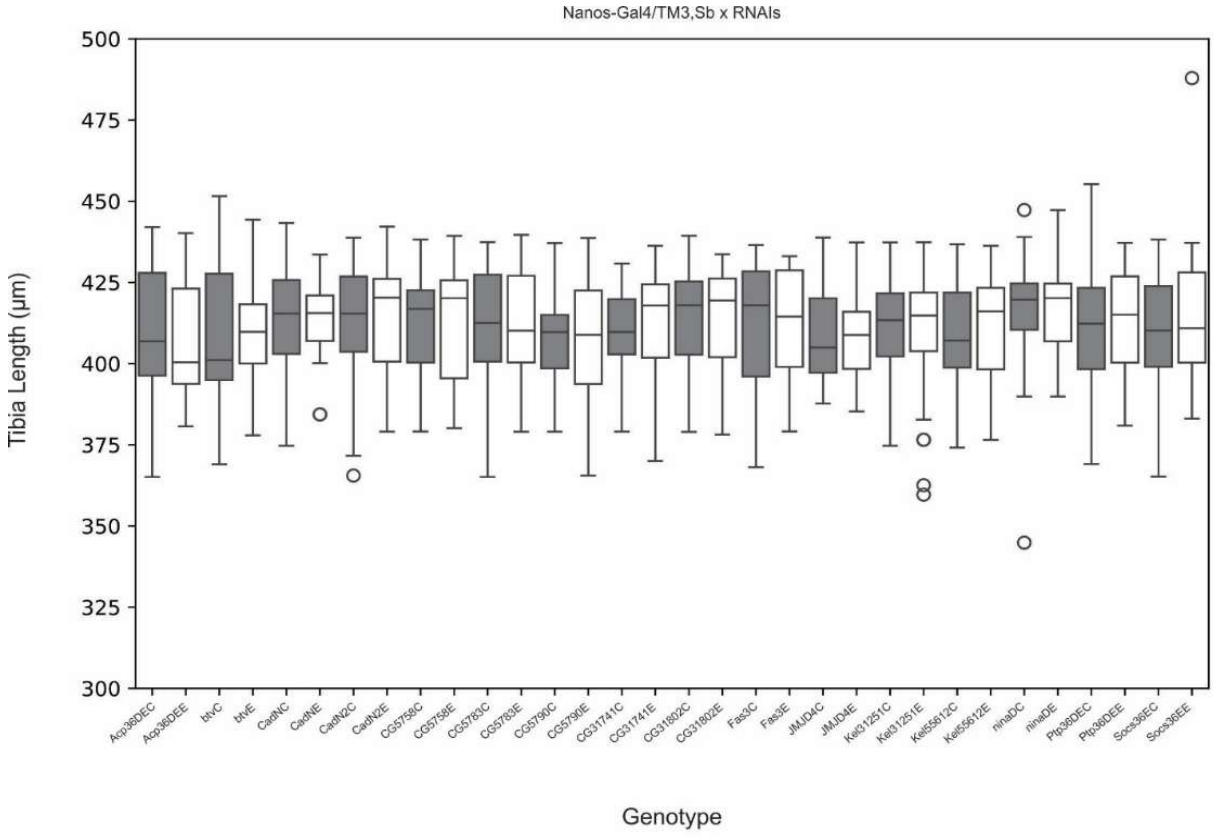


Figure 21: Tibia length comparisons between each RNAi genotype. Controls are marked with a C and colored in gray, while the experimental are marked with an E with no color. A *t-test* was performed with a significance threshold of >0.05 using r studio. *< 0.05, **< 0.001, ***< 0.0001. Sample size was 50 eggs per each RNAi pair (C and E).

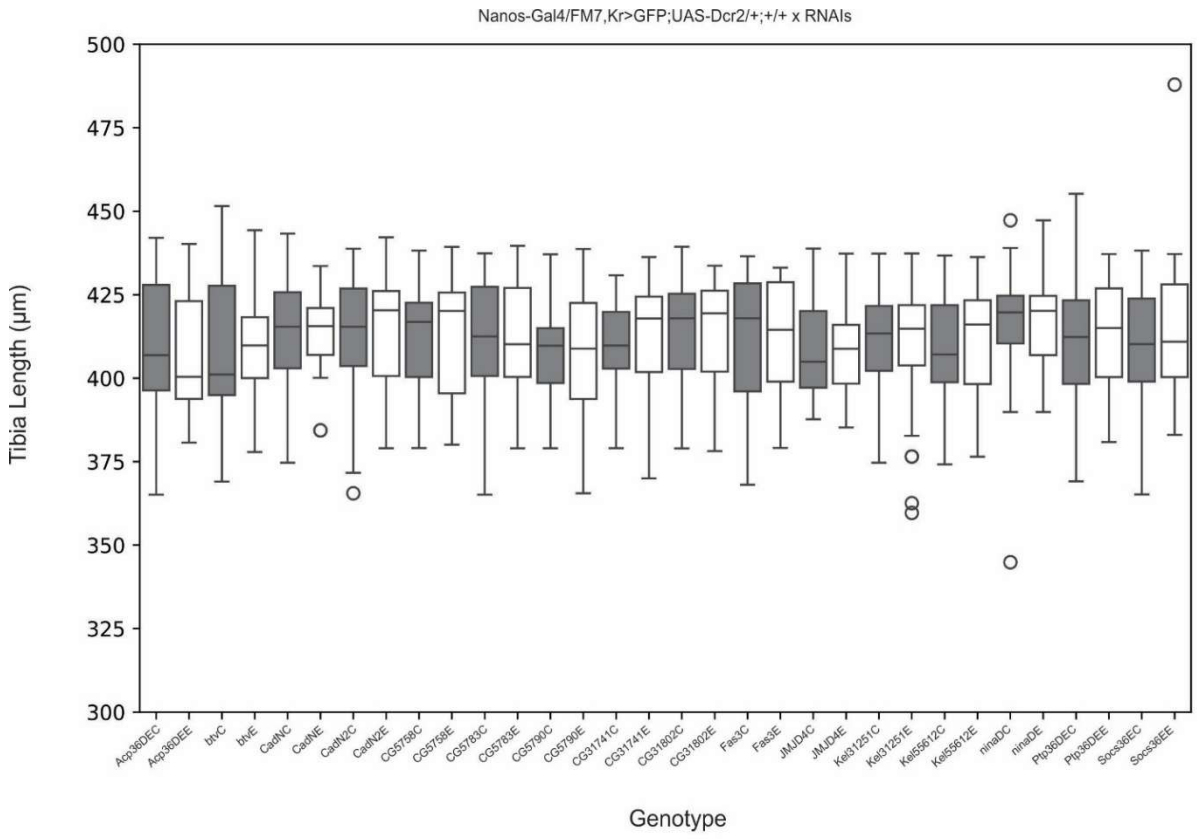


Figure 22: Tibia length comparisons between each RNAi genotype. Controls are marked with a C and colored in gray, while the experimental are marked with an E with no color. A *t-test* was performed with a significance threshold of >0.05 using r studio. *< 0.05, **< 0.001, ***< 0.0001. Sample size was 50 eggs per each RNAi pair (C and E).

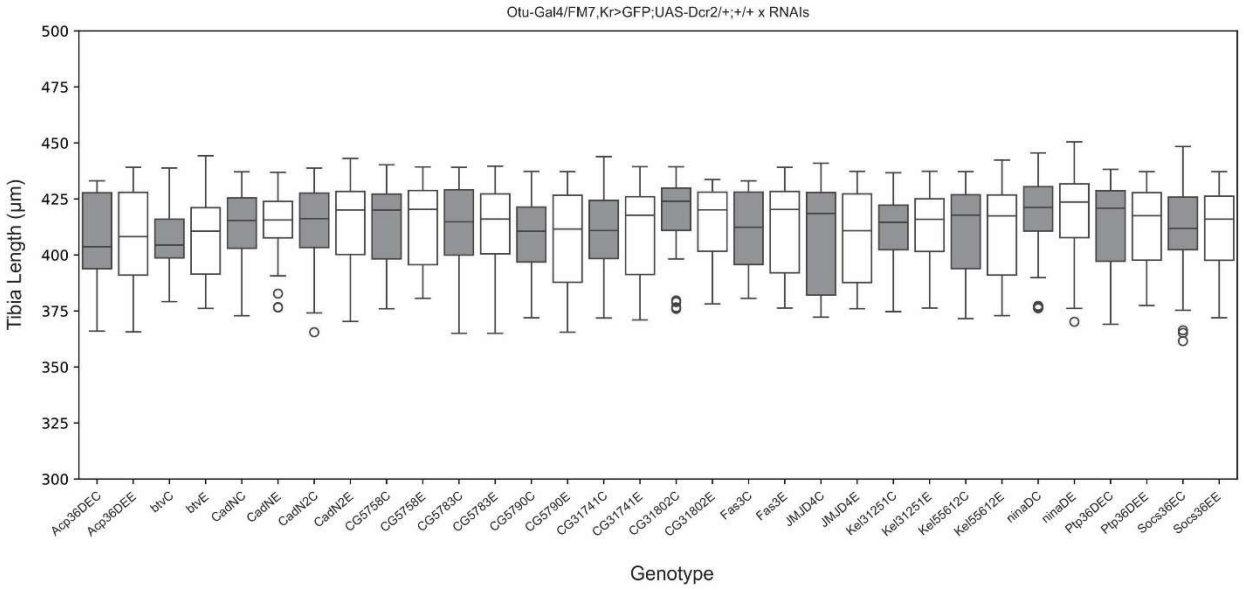


Figure 23: Tibia length comparisons between each RNAi genotype. Controls are marked with a C and colored in gray, while the experimental are marked with an E with no color. A *t-test* was performed with a significance threshold of >0.05 using r studio. *< 0.05, **< 0.001, ***< 0.0001. Sample size was 50 eggs per each RNAi pair (C and E).

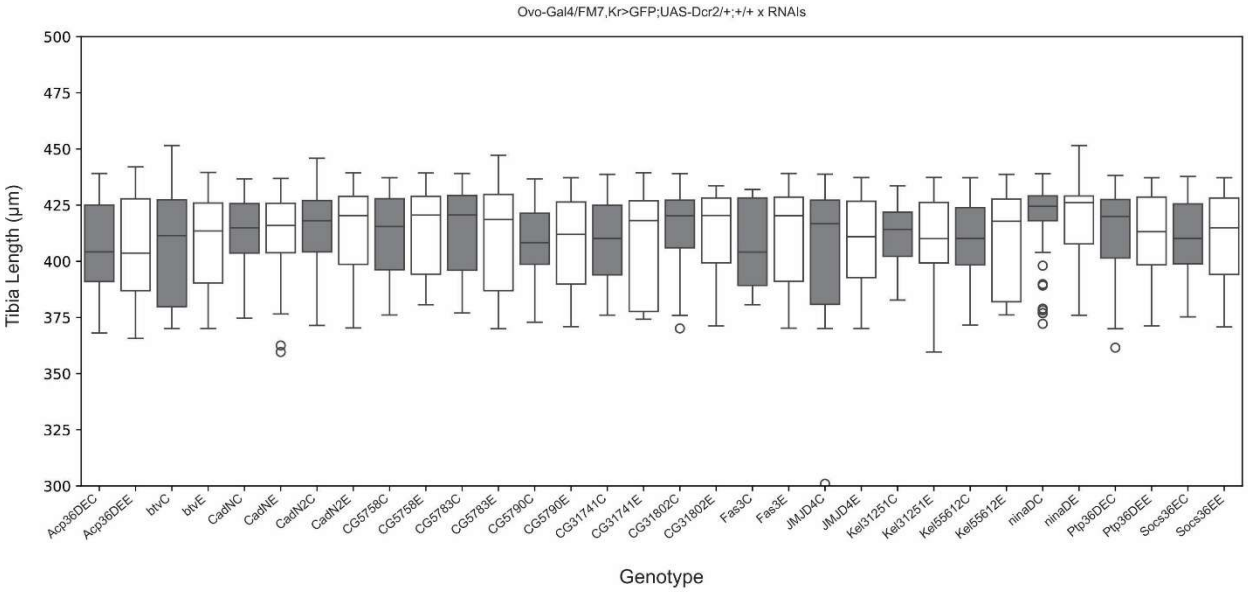


Figure 24: Tibia length comparisons between each RNAi genotype. Controls are marked with a C and colored in gray, while the experimental are marked with an E with no color. A *t-test* was performed with a significance threshold of >0.05 using r studio. *< 0.05, **< 0.001, ***< 0.0001. Sample size was 50 eggs per each RNAi pair (C and E).

nanos – Gal4/FM7, Kr>GFP; Dcr2/+ X all RNAis Summary.

Table 2: Summary of Egg length means, Tibia length means, Egg length and Tibia length correction, and Egg volume and ovariole numbers in the 16 RNAi pairs of control (C) and experimental (E). Means are reported ± standard error.

Genotype	Egg Length (mm)	Tibia Length (mm)	Egg/Tibia (mm)	Egg Volume (mm^3) ($x10^{-3}$)	Ovarioles
Acp36DE-E	0.514±0.0023	0.407±0.0031	12.7±0.001	1.050±0.001	18
Acp36DE-C	0.533±0.0020	0.406±0.0024	12.6±0.001	0.811±0.001	17
btv-C	0.484±0.0019	0.406±0.0031	12.0±0.001	0.963±0.001	18
btv-E	0.510±0.0017	0.408±0.0022	12.6±0.001	1.431±0.001	17
CadN-C	0.518±0.0024	0.413±0.002	12.5±0.001	1.903±0.003	18
CadN-E	0.529±0.0027	0.413±0.0016	12.9±0.001	1.235±0.004	18
CadN2-C	0.521±0.0031	0.413±0.0025	12.7±0.001	1.597±0.000	19
CadN2-E	0.494±0.0023	0.413±0.0021	11.9±0.001	2.166±0.001	18
CG5758-C	0.492±0.0017	0.413±0.0020	12.8±0.002	1.380±0.004	17
CG5758-E	0.486±0.0088	0.411±0.0028	12.0±0.002	3.340±0.003	18
CG5783-C	0.514±0.0029	0.412±0.0023	12.5±0.002	1.092±0.002	14
CG5783-E	0.495±0.0018	0.412±0.0023	12.2±0.001	1.069±0.002	14
CG5790-C	0.509±0.0034	0.407±0.0020	12.4±0.002	0.897±0.003	16
CG5790-E	0.507±0.0067	0.407±0.0027	12.6±0.001	1.119±0.003	15
CG31741-C	0.495±0.0023	0.409±0.0018	12.4±0.001	1.232±0.002	19
CG31741-E	0.503±0.0022	0.414±0.0019	12.3±0.001	0.923±0.003	19
CG31802-C	0.509±0.0020	0.414±0.0019	12.3±0.001	1.128±0.003	18
CG31802-E	0.521±0.0025	0.412±0.0023	12.7±0.001	1.120±0.003	17
Fas3-C	0.512±0.0017	0.411±0.0026	11.9±0.002	1.972±0.003	16
Fas3-E	0.522±0.0018	0.411±0.0024	12.7±0.001	1.440±0.003	17
JMJD4E-C	0.502±0.0026	0.408±0.0018	11.5±0.001	1.378±0.002	17
JMJD4E-E	0.492±0.0017	0.408±0.0019	12.1±0.001	1.813±0.001	18
Kel31251-C	0.507±0.0027	0.410±0.0021	12.4±0.001	1.158±0.003	18
Kel31251-E	0.511±0.0017	0.410±0.0025	12.5±0.001	1.272±0.003	18
Kel55612-C	0.495±0.0021	0.409±0.0022	12.6±0.001	1.225±0.003	18
Kel55612-E	0.531±0.0025	0.410±0.0022	12.7±0.001	1.155±0.002	19
ninaD-C	0.523±0.0024	0.415±0.0023	12.7±0.001	1.218±0.002	18
ninaD-E	0.516±0.0025	0.417±0.0018	12.5±0.001	1.062±0.002	18
Ptp36DE-C	0.514±0.0029	0.412±0.0023	11.8±0.001	1.247±0.001	18
Ptp36DE-E	0.498±0.0019	0.412±0.0022	12.3±0.001	0.190±0.002	18
Socs36E-C	0.529±0.0022	0.410±0.0024	12.9±0.001	1.259±0.002	18
Socs36E-E	0.527±0.0019	0.413±0.0025	12.8±0.001	1.016±0.001	18

UAS-Dcr2/+; nanos - Gal4/TM3.Sb X all RNAis Summary.

Table 3: Summary of Egg length means, Tibia length means, Egg length and Tibia length correction, and Egg volume and ovariole numbers in the 16 RNAi pairs of control (C) and experimental (E). Means are reported ± standard error.

Genotype	Egg Length (mm)	Tibia Length (mm)	Egg/Tibia (mm)	Egg Volume (mm^3) ($x10^{-3}$)	Ovarioles
Acp36DE-E	0.513±+0.0024	0.408±0.0023	12.59±0.001	1.010±0.001	17
Acp36DE-C	0.506±0.0074	0.407±0.0025	12.45±0.002	0.804±0.001	18
btv-C	0.484±0.0019	0.407±0.0026	11.92±0.009	0.922±0.001	18
btv-E	0.510±0.0017	0.406±0.0027	12.58±0.001	1.252±0.001	17
CadN-C	0.518±0.0024	0.413±0.0022	12.57±0.009	0.951±0.001	18
CadN-E	0.529±0.0027	0.414±0.0020	12.80±0.001	1.127±0.001	18
CadN2-C	0.521±0.0031	0.412±0.0020	12.67±0.001	1.607±0.001	18
CadN2-E	0.494±0.0023	0.413±0.0023	11.93±0.009	0.903±0.001	19
CG5758-C	0.492±0.0017	0.413±0.0023	11.92±0.007	0.936±0.001	17
CG5758-E	0.486±0.0088	0.412±0.0028	11.80±0.002	0.878±0.001	18
CG5783-C	0.514±0.0029	0.412±0.0029	12.51±0.001	1.096±0.001	14
CG5783-E	0.495±0.0018	0.412±0.0027	12.03±0.001	1.006±0.001	14
CG5790-C	0.509±0.0034	0.408±0.0027	12.51±0.001	0.908±0.002	16
CG5790-E	0.507±0.0067	0.408±0.0029	12.44±0.001	1.253±0.001	15
CG31741-C	0.495±0.0023	0.409±0.0027	12.13±0.009	0.906±0.003	19
CG31741-E	0.503±0.0022	0.409±0.0027	12.32±0.009	1.122±0.003	19
CG31802-C	0.509±0.0020	0.411±0.0071	13.06±0.008	1.112±0.003	18
CG31802-E	0.521±0.0025	0.412±0.0028	12.66±0.001	1.062±0.002	17
Fas3-C	0.512±0.0017	0.408±0.0025	12.57±0.008	0.931±0.002	16
Fas3-E	0.522±0.0018	0.407±0.0031	12.85±0.001	1.456±0.001	17
JMJD4E-C	0.502±0.0026	0.408±0.0032	12.34±0.001	1.268±0.002	17
JMJD4E-E	0.492±0.0017	0.408±0.0027	12.06±0.009	0.986±0.003	18
Kel31251-C	0.507±0.0027	0.410±0.0023	12.38±0.001	1.160±0.003	18
Kel31251-E	0.511±0.0017	0.410±0.0029	12.51±0.001	1.296±0.002	18
Kel55612-C	0.495±0.0021	0.409±0.0027	12.13±0.008	0.974±0.002	18
Kel55612-E	0.531±0.0025	0.409±0.0030	12.99±0.001	1.451±0.002	19
ninaD-C	0.523±0.0024	0.417±0.0030	12.58±0.001	1.214±0.001	18
ninaD-E	0.516±0.0025	0.417±0.0028	12.40±0.001	1.008±0.001	18
Ptp36DE-C	0.514±0.0028	0.412±0.0030	12.51±0.001	1.188±0.002	18
Ptp36DE-E	0.498±0.0019	0.412±0.0025	12.10±0.008	1.287±0.003	18
Socs36E-C	0.529±0.0022	0.411±0.0024	12.88±0.001	1.279±0.003	18
Socs36E-E	0.527±0.0019	0.411±0.0029	12.86±0.001	1.009±0.001	18

otu-Gal4/FM7, Kr>GFP; Dcr2/+ X all RNAis Summary.

Table 4: Summary of Egg length means, Tibia length means, Egg length and Tibia length correction, and Egg volume and ovariole numbers in the 16 RNAi pairs of control (C) and experimental (E). Means are reported ± standard error.

Genotype	Egg Length (mm)	Tibia length (mm)	Egg/Tibia (mm)	Egg volume (mm^3) ($x10^{-3}$)	Ovarioles
Acp36DE-E	0.503±0.0028	0.407±0.0025	12.35±0.001	0.848±0.001	17
Acp36DE-C	0.497±0.0028	0.407±0.0029	12.21±0.004	1.068±0.004	18
btv-C	0.517±0.0030	0.407±0.0019	12.73±0.001	1.460±0.001	17
btv-E	0.499±0.0019	0.407±0.0024	12.28±0.001	1.201±0.001	18
CadN-C	0.516±0.0027	0.413±0.0021	12.52±0.001	1.317±0.002	18
CadN-E	0.476±0.0014	0.413±0.0020	11.53±0.001	0.745±0.003	18
CadN2-C	0.513±0.0024	0.413±0.0026	12.44±0.001	1.242±0.002	19
CadN2-E	0.494±0.0047	0.413±0.0073	11.99±0.001	0.892±0.003	18
CG5758-C	0.511±0.0024	0.412±0.0026	12.42±0.001	2.500±0.002	17
CG5758-E	0.517±0.0027	0.412±0.0025	12.56±0.001	1.234±0.001	18
CG5783-C	0.492±0.0018	0.412±0.0027	11.97±0.001	0.875±0.003	14
CG5783-E	0.519±0.0022	0.412±0.0026	12.62±0.001	1.124±0.002	14
CG5790-C	0.507±0.0026	0.407±0.0022	12.48±0.001	1.048±0.002	15
CG5790-E	0.500±0.0027	0.408±0.0030	12.27±0.001	1.277±0.004	16
CG31741-C	0.517±0.0028	0.409±0.0027	12.68±0.001	1.137±0.003	19
CG31741-E	0.511±0.0023	0.408±0.0030	12.54±0.001	1.075±0.003	19
CG31802-C	0.535±0.0037	0.413±0.0031	13.52±0.001	1.295±0.001	17
CG31802-E	0.508±0.0093	0.412±0.0025	12.36±0.001	1.145±0.004	18
Fas3-C	0.514±0.0027	0.409±0.0024	12.58±0.001	1.202±0.003	17
Fas3-E	0.518±0.0027	0.404±0.0079	15.01±0.002	1.366±0.001	16
JMJD4E-C	0.509±0.0023	0.408±0.0030	12.51±0.001	1.336±0.001	17
JMJD4E-E	0.494±0.0018	0.408±0.0029	12.14±0.001	0.967±0.002	16
Kel31251-C	0.500±0.0021	0.410±0.0021	12.20±0.001	1.012±0.004	18
Kel31251-E	0.522±0.0035	0.410±0.0027	12.74±0.001	1.194±0.002	18
Kel55612-C	0.515±0.0024	0.409±0.0029	12.60±0.001	1.237±0.004	18
Kel55612-E	0.519±0.0024	0.409±0.0029	12.71±0.001	1.353±0.004	19
ninaD-C	0.510±0.0025	0.417±0.0027	12.27±0.001	1.429±0.005	17
ninaD-E	0.503±0.0023	0.417±0.0030	12.70±0.001	1.505±0.002	18
Ptp36DE-C	0.511±0.0023	0.412±0.0028	12.43±0.001	1.120±0.003	18
Ptp36DE-E	0.513±0.0023	0.412±0.0025	12.48±0.001	1.147±0.003	18
Socs36E-C	0.531±0.0023	0.411±0.0028	12.93±0.001	1.287±0.003	18
Socs36E-E	0.529±0.0020	0.411±0.0024	12.90±0.001	1.026±0.002	18

ovo-Gal4/FM7, Kr>GFP; Dcr2/+ X all RNAis Summary.

Table 5: Summary of Egg length means, Tibia length means, Egg length and Tibia length correction, and Egg volume and ovariole numbers in the 16 RNAi pairs of control (C) and experimental (E). Means are reported ± standard error.

Genotype	Egg Length (mm)	Tibia length (mm)	Egg/Tibia (mm)	Egg volume (mm^3) ($x10^{-3}$)	Ovarioles
Acp36DE-E	0.514±0.0022	0.406±0.0027	12.70±0.001	1.012±0.001	17
Acp36DE-C	0.533±0.0020	0.407±0.0028	13.10±0.005	0.854±0.004	18
btv-C	0.484±0.0029	0.407±0.0032	11.90±0.001	0.922±0.001	17
btv-E	0.510±0.0017	0.407±0.0030	12.50±0.001	1.252±0.001	18
CadN-C	0.513±0.0031	0.412±0.0020	12.40±0.001	1.263±0.003	18
CadN-E	0.480±0.0013	0.412±0.0026	12.01±0.001	0.750±0.003	18
CadN2-C	0.510±0.0026	0.412±0.0026	12.40±0.001	1.305±0.003	19
CadN2-E	0.495±0.0017	0.412±0.0030	12.01±0.001	0.856±0.002	18
CG5758-C	0.509±0.0021	0.411±0.0025	12.40±0.001	1.365±0.001	17
CG5758-E	0.502±0.0027	0.412±0.0025	12.20±0.001	3.005±0.002	18
CG5783-C	0.510±0.0029	0.412±0.0027	12.40±0.001	1.252±0.001	14
CG5783-E	0.527±0.0019	0.411±0.0034	12.80±0.001	1.265±0.001	14
CG5790-C	0.505±0.0025	0.408±0.0023	12.40±0.001	1.072±0.001	15
CG5790-E	0.497±0.0025	0.408±0.0029	12.20±0.001	1.257±0.002	16
CG31741-C	0.513±0.0025	0.408±0.0026	12.60±0.001	1.009±0.001	19
CG31741-E	0.514±0.0023	0.407±0.0032	12.60±0.001	1.008±0.001	19
CG31802-C	0.535±0.0027	0.413±0.0027	12.90±0.001	0.890±0.001	17
CG31802-E	0.506±0.0019	0.413±0.0026	12.30±0.001	1.012±0.001	18
Fas3-C	0.513±0.0030	0.408±0.0026	12.60±0.001	1.198±0.001	17
Fas3-E	0.520±0.0025	0.408±0.0038	12.70±0.001	1.351±0.001	16
JMJD4E-C	0.490±0.0022	0.408±0.0031	12.00±0.001	0.996±0.002	17
JMJD4E-E	0.501±0.0018	0.408±0.0027	12.30±0.001	1.095±0.001	16
Kel31251-C	0.497±0.0029	0.410±0.0022	12.10±0.001	1.020±0.001	18
Kel31251-E	0.504±0.0023	0.410±0.0028	12.30±0.001	1.427±0.002	18
Kel55612-C	0.514±0.0019	0.409±0.0026	12.60±0.001	1.184±0.002	18
Kel55612-E	0.519±0.0023	0.409±0.0031	12.70±0.001	1.401±0.001	19
ninaD-C	0.511±0.0023	0.417±0.0026	12.30±0.001	1.307±0.001	17
ninaD-E	0.526±0.0035	0.417±0.0029	12.60±0.001	1.551±0.001	18
Ptp36DE-C	0.503±0.0025	0.411±0.0029	12.20±0.001	1.374±0.001	18
Ptp36DE-E	0.492±0.0056	0.411±0.0025	12.00±0.002	1.243±0.001	18
Socs36E-C	0.529±0.0023	0.411±0.0023	12.90±0.001	1.235±0.001	18
Socs36E-E	0.527±0.0019	0.411±0.0026	12.80±0.001	1.024±0.001	18

Drosophila genetics stocks

RNAi Lines	Source	Stock number	Insertion site	Vector	Other info:
Acp36E	Bloomington	67325	attP40	Valium20	HMC06429
btv	Bloomington	28373	attP2	Valium10	JF03010
CadN	Bloomington	27503	attP2	Valium10	JF02653
CadN2	Bloomington	40889	attP40	Valium20	HMS02137
CG31741	Bloomington	44518	attP2	Valium10	HMC02908
CG31802	Bloomington	53893	attP40	Valium20	HMJ21211
CG5758	Bloomington	57808	attP40	Valium20	HMJ21816
CG5783	Bloomington	61854	attP40	Valium27	HMJ23343
CG5790	Bloomington	62453	attP40	Valium20	HMJ23933
Fas3	Bloomington	77396	attP40	Valium20	HMC06528
JMJD4	Bloomington	32485	attP2	Valium20	HMS00488
Kel	Bloomington	31251	attP2	Valium1	JF01768
Kel	Bloomington	55612	attP40	Valium20	HMC03751
ninaD	Bloomington	44652	attP2	Valium20	HMC02392
Ptp36E	Bloomington	65919	attP40	Valium20	HMC06183
Socs36E	Bloomington	35036	attP2	Valium20	HMS01450

Gal4 Driver Lines	Source	Stock number
OVO-Gal4	Bloomington	62770
OTU-Gal4	Bloomington	58424
Nanos-Gal4	Bloomington	7303
Nanos-Gal4	Bloomington	4937

Fluorescent Line	This study
y,w,Nrdc1,FRT(19A)/FM7,Kr>GFP	
w/+;Drc2/Dcr2;+/	This study

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