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Male-biased retrogenes in Drosophila melanogaster: integration of novel genes into pathways governing sperm development

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Abstract

Sexual selection and co-evolution with the female reproductive tract drives the rapid evolution of the *Drosophila* sperm proteome. It is important to reveal the genetics underlying the rapid evolution, and understand that the sperm proteome often evolves via turnover in composition, including the incorporation of novel proteins. Retrotransposition is one mechanism that gives rise to novel genes; this process involves the mRNA from an ancestral gene being converted back to DNA and incorporated back into the genome. Genes originating through the processes of retrotransposition often acquire testis-specific expression, and a novel role in spermatogenesis. Further, in *Drosophila*, retrogenes with testis-specific expression are enriched for putative roles in mitochondrial energetic pathways. The mitochondria in sperm cells fuse to form the nebenkern which associates with microtubules to provide crucial support during sperm tail elongation. Evidence suggests that tail length affects competitive ability within *Drosophila melanogaster*, and that individuals that produce longer sperm have an advantage in positioning sperm relative to the female seminal receptacle. Through the use of the UAS/GAL4 system for RNA interference we targeted twelve testis-specific, mitochondrial enriched retrogenes and assessed knockdown phenotypes via sperm tail measurements, seminal vesicle imaging, and fertility assays. Two RNAi lines demonstrated significant deviation from control sperm length measurements, indicating a role for these genes during sperm elongation. Three additional RNAi lines showed a reduced ratio of hatched to laid eggs, indicating that retrogenes participate in mitochondrial roles other than supporting tail elongation. Our work helps deepen the understanding of retrogene function, and the mechanisms that drive rapid sperm evolution.

Executive Summary

My Capstone project falls within the broad field of evolutionary genetics. This field of biology focuses on the large ideas of Darwinian evolution, which include the classical idea of survival of the fittest, under the notion that it is heritable, genetic information undergoing change to produce different outcomes. Sexual selection is another idea proposed by Darwin, and revolves around the fact that the sexes select mates based on the presence of favorable characteristics. Occasionally, possession of these traits can be detrimental to one's own survival, as is seen with the male peacock's flamboyant tail feathers: on the one hand, the large tail demonstrates good genes and increases the male's attractiveness to females, but on the other, energy is spent on its production rather than being allocated to other processes, and larger tails can also attract the attention of predators. The peacock tail represents the classical example of pre-copulatory sexual selection. There also exists post-copulatory sexual selection, which involve the mechanisms that drive selection after mating has occurred. Examples of this include cryptic female choice, seen in the convoluted nature of their reproductive tracts, and through sperm competition. Sperm competition has contributed to the rapid evolution of sperm, and the development of many extreme morphologies, including species with sperm ornamentation, multiple sperm types, and a large diversity in sperm length.

Drosophila melanogaster is more commonly known as the fruit fly, and has been used as a model organism for genetic studies for more than a century. They are also an example of a species in which sexual selection has driven rapid evolution and the existence of an extreme morphology – a single sperm cell measures approximately 1.8 mm in length, while the fly's body itself is only 3-4 mm. Sperm is derived from primary germ cells, which go through multiple rounds of division as they move through the testes. At the point where the cell polarizes to form

distinct head and tail regions, all the mitochondria aggregate in the tail and fuse to form a giant structure known as the nebenkern. Studies have shown that this structure interactions with cytoplasmic microtubules to provide crucial scaffolding in the tail, which ultimately allows for the extreme elongation. Longer sperm are better competitors and are better able to situate themselves within the female reproductive tract so they have a higher probability of ultimately fertilizing the eggs.

The introduction of novel genes is one of the molecular mechanisms driving the rapid evolution of sperm morphology. For this project, we looked specifically at genes that arose via a duplication event known as retrotransposition. Genes exist in the form of DNA, which is normally turned into messenger RNA (mRNA) that is further modified, and then translated into proteins. In the case of retrotransposition, however, after the gene is converted to mRNA, it is then converted back to DNA and reinserted into the genome. The organism now has two copies of the same gene, and it is at this point where we see specialized functionalization emerge. The duplicate gene, called a retrogene, could hypothetically take on the same role as the parent, but it has been observed that retrogenes often obtain testis-specific expression and functions. Within *Drosophila* it has been shown that retrogenes are also enriched in mitochondrial energetic pathways. In conjunction with two fellow Honors students, this project involves researching eleven testis-specific, mitochondrial-enriched retrogenes. We believe that silencing these retrogenes will result in reduced tail lengths, as nebenkern function will be disrupted, and aim to investigate the consequences on total sperm production and overall male fertility.

The effect of retrogene knockdown was measured using three techniques: sperm flagellum measurements, seminal vesicle imaging, and fertility assays. In the course of our analysis, we discovered two genes whose knockdown produced significantly different tail

lengths with one shorter and one longer. These genes have inferred functions early within the mitochondrial energetic pathways. We identified three additional genes whose knockdown resulted in significant decreases in fertility. These genes have inferred functions later within the mitochondrial energetic pathways, and related more to the electron transport chain. These results indicate that some retrogenes do indeed take on mitochondrial functions related to flagellum elongation, but that they also appear to be crucial in pathways for fertility. On a larger scale, this project deepens our understanding on how novel genes contribute to the evolution of sperm morphology, and further highlights the importance of retrogenes in mitochondrial energetic pathways.

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Acknowledgements

I first want to thank Dr. Dorus for giving me the opportunity to work in his lab, which has expanded my biological knowledge and been the best kind of challenge. I would like to thank Emma Whittington for her patience and support throughout the course of this project. Many thanks to Dr. Stevens for teaching me everything I know about lab and fly rearing. My deepest gratitude to Makayla Dearborn and Zhaowei Jiang, my fellow undergraduate researchers. And finally I thank the Syracuse Biology Department, the Renée Crown Honors Program, and the Crown/Wise Awards Committee for providing funding for this project.

Introduction

Sexual selection

Charles Darwin described sexual selection as "the advantages that certain individuals have over others of the same sex and species in exclusive relation to reproduction" (Darwin, 1871). Though Darwin did not speculate specifically about the mechanisms of sexual selection, interest in the field began as researchers focused on certain extreme phenotypes present for reproductive purposes. Precopulatory sexual selection has been studied more extensively, and is manifested most evidently in female choice of male partners and in male-male competition for mating opportunities. Female choice can involve females actively selecting for mates who possess characteristics indicative of good gene to pass to offspring, have access to sufficient resources, and so forth (Hosken & Stockley, 2004). Male-male competition can be observed directly in examples of fighting for territory or access to females (León and Tumpson, 1975), or the use of alternative mating strategies to avoid dominant males. Though precopulatory mechanisms are more recognizable to the general audience, selection associated with reproductive outcomes continues beyond the act of mating. Postcopulatory selection occurs after mating and is seen through female cryptic choice and sperm competition (Parker, 1970). Studies in *Drosophila* have revealed the existence of convoluted female reproductive tracts (Miller and Pitnick, 2002). The maze-like quality allows females to "choose" the highest quality sperm by selecting only for those that are able to survive the journey through the labyrinth-like structure. Sperm competition between males has also led to the development of extreme phenotypes that include the evolution of genital morphology to include components designed for the removal of sperm from competitiors (Waage, 1979), sperm ornamentation (Lüpold et al., 2016), sperm heteromorphism (Snook, 1998), and a great diversity in sperm length (Joly et al., 1991).

The rapid diversification of sperm morphology is believed to be due to the influence of sexual selection. Nonetheless, a paradox exists between the need for sperm to respond to strong diversifying selection while also maintaining function in fertilization and karyogamy. Further, a balance must be established in the evolutionary trade-off between producing large quantities of sperm with a higher potential for defects and investing more energy into production of fewer, higher quality cells (Pitnick et al., 2008). Within the genus of *Drosophila*, a wide variety of extreme phenotypes exist due to sexual selection and coevolution with the female reproductive tract (Miller & Pitnick, 2003).

Evidence suggests that the composition of the entire sperm proteome undergoes rapid change, with the creation and expression of new genes being of particular importance in the rapid evolution of the genome composition (Dorus et al., 2006). New genes arise through many mechanisms, including duplication of entire genomes, chromosomes, or single genes; novel splicing of preexisting genes; and retrotransposition (Brown, 2002). Duplication events result in an organism possessing multiple copies of the same genes, presenting the opportunity for novel functions to arise (Chen et al., 2013). Novel splicing of existing genes produces new combinations of exons and introns, which can produce functionally significant changes (Babushok et al., 2006). Retrotransposition is the mechanism through which retrogenes arise. In this process, a parent gene in the form of DNA is transcribed into messenger RNA (mRNA) and goes through post-transcriptional modifications including the removal of introns. Rather than being translated into protein, however, the enzyme reverse transcriptase transcribes the mRNA back into DNA. This segment can then be reinserted into the same or separate region of the genome. Prior to the 1980's it was believed that retrogenes largely served as "junk" DNA (Kaessman et al., 2009) as it was not thought they could acquire the necessary regulatory

machinery for expression. It has since been discovered that retrogenes in mammals and *Drosophila* are actually expressed (Bai, 2008), and often acquire testis-specific expression (Betrán et al. 2002). The acquisition of novel proteins is thought to contribute to the rapid evolution observed in sperm.

Spermatogenesis and the function of the nebenkern

Spermatogenesis occurs in the testis. A single germ cell migrates from the apical tip of the testis and undergoes multiple rounds of mitosis and incomplete cytokinesis to form a 16-cell interconnected system, detailed in Figure 1. The cells then simultaneously enter meiosis I and II to form 64 haploid cells (Hirst & Carmichael, 2011). In the pre-meiotic cells, the mitochondria begin to aggregate and fuse to form the nebenkern in what will differentiate into the tail region, while the nucleus migrates to the head (Fabian & Brill, 2012). Components of the sperm tail include the axoneme, the central cilia composed of microtubules arranged in the 9+2 fashion; the nebenkern; cytoplasmic microtubules; and actin arrays (Aldridge et al., 2007). Previous works have demonstrated that the axoneme is not required for sperm elongation, whereas the nebenkern and cytoplasmic microtubule arrays are. This indicates that that the mitochondria has functions beyond the traditional energy production, including the crucial role as acting as a scaffold to support elongation (Noguchi, 2012).

Drosophila melanogaster as a model organism

Drosophila melanogaster have been used in laboratory settings since the beginning of the 1900s and since the work of Thomas Hunt Morgan have become the classic organism used to study genetics (Jennings, 2011). Fruit flies are ideal model organisms due to their quick generation turnover, small size, simple nutrient needs, and well-sequenced genome. *Drosophila melanogaster* have been used in studies regarding signal transduction pathways, development,

DNA mutations, and beyond (Kohler, 1994). Multitudes of genetic tools are available in flies, including the UAS/GAL4 system for RNA induced silencing. Ribonucleic acid interference, known simply as RNAi, is a molecular technique used to knockdown gene expression. In this process, a double-stranded RNA triggers the process of RNA-induced silencing such that its transcripts are degraded and overall expression level is reduced (Chen, 2013). In *Drosophila*, use of RNAi is further dependent on the UAS-GAL4 system. This system includes four key components: a tissue-specific driver; GAL4, a yeast transcription factor; the upstream activation sequence (UAS), the enhancer to which GAL4 binds; and an RNAi hairpin (Brand and Perrimon, 1993). Figure 2 provides a visual representation of how the UAS-GAL4 induces gene silencing. We utilized the vasa driver for testis-specific expression of GAL4. The transcription factor then activates the UAS, which is tied with the transcription of an RNA hairpin designed to be complementary to the mRNA of a gene of interest (GOI). The hairpin is in the form of inverted repeats of the desired sequence and is released as a double-stranded hairpin structure. The hairpin structure is cleaved and the strand complementray to the mRNA from each GOI used to locate these transcripts. The two small sequences anneal, forming double-stranded RNA, which is ultimately targeted by the RNA-induced silencing complex (RISC) for transcript degradation. With no mRNA translated into protein, the retrogene has been silenced. This powerful molecular tool ultimately allows us to examine the consequences of gene knockdown, and investigate the link between genotype and phenotype.

Significance of retrogenes

Retrogenes have been shown to frequently gain testis-specific expression (Betrán et al. 2002) and in *Drosophila* are additionally enriched for mitochondrial energetic pathways (Rettie et al. 2015). It has further been demonstrated that the mitochondria and cytoplasmic

microtubules are essential for sperm tail elongation (Noguchi 2012). Based on this information, we have selected eleven testis-specific, mitochondrial-enriched retrogenes to determine if they function in elongation and fertility. While part of a broader analysis of testis-specific, mitochondrial-enriched retrogene study, this project focuses specifically on the retrogenes *CG4701*, *CG4706*, and *CG17856*. *CG4701* is the retrogene of *no mitochondrial derivative* (*nmd*), which has been shown to be critical for tail elongation (Noguchi, 2011). Based on sequence analysis, *CG4701* is implicated in having an ATPase-like activity and inferred to be localized to the outer mitochondrial membrane. Analyses suggest that *CG17856* contains domains similar to the cytochrome C complex, a critical electron transporter found in the electron transport chain (ETC) (Hüttermann et al., 2011*). CG4706* has domains most closely aligned with the 4-iron 4-sulfur clusters of hydrogenase enzymes. Iron-sulfur clusters are integral to the ETC and are found in both Complex I and Complex II of the chain (Fonecave, 2006). Due to their mitochondrial enrichment, we hypothesize that these retrogenes contribute to axoneme elongation and that genetic manipulations of these GOIs will lead to deficits in nebenkern function. The specific aims of this research project are to knockdown the expression of testis-specific retrogenes with mitochondrial functionality and measure the phenotypic consequences on (1) sperm flagellum length, (2) sperm production, and (3) male fertility.

Materials and Methods

Stocks

RNAi lines originated from the Vienna Drosophila Resource Center and belonged to either the KK or GD library (Table 1). Insertion of the RNA hairpin in the GD library is random and can occur at chromosomes X, 2, or 3; insertion in the KK library had been mapped to the VIE-260b landing site of the second chromosome (Gramates et al., 2017). All populations were grown on a yeast, cornmeal and molasses-based food. Stock bottles were maintained at room temperature on a shelf lined with mite paper. Stock bottles were rotated on a biweekly basis.

F1 knockdown fly crosses

Flies from each line were collected directly from stock bottles within 24 hours of eclosion. Males were kept and aged for 5 days, while females were aged for 3-4 days. Knockdown males were generated by placing 2-3 three day old virigin vasa-GAL4 females and 2-3 five day old males containing their respective RNAi hairpins into vials. Adults were removed after 2 days, and vials then placed in a Percival Scientific incubator set for 28ºC. F1 generation eclosed after approximately 10 days. F1 males were removed within 24 hours of eclosion and placed in vials with extra yeast in Shel Lab LIFLY low temperature incubator set for 24.5ºC to age for 5 days.

Sperm tail measurements

F1 males were dissected at age 5 days under a Leica M125 microscope. Seminal vesicles were separated from the remainder of the reproductive tract and transferred to one drop of 1% phosphate-buffered saline (PBS) on a microscope slide. Vesicles were punctured with the point of a forcep and sperm allowed to billow out until individual cells were separating at the perimeter. The sperm bundle was manuevered across the drop of PBS in a repeated “S” pattern

to allow individual sperm cells to separate. Slides were placed into a VWR154 heater set to 54ºC until PBS evaporated. Slides were fixed in a 3:1 acetic acid-methanol solution for 1 minute, then washed with PBS three times. One drop of a 9:1 glycerol-PBS solution was used as mounting media. Slides were covered with glass cover slip with edges sealed shut with nail varnish. Samples were visualized using dark field on an Olympus B202 microscope at 40X and images of individual sperm cells taken with an attached Hamamatsu digital camera. Sperm tails were measured from the pictures by hand tracing and use of the program ImageJ. Approximately 10 cells per individual were measured and ten individuals dissected for a goal of 100 cells per RNAi line.

Seminal vesicle imaging

F1 males were dissected at age 5 days using a Leica M125 microscope. Seminal vesicles and testes were removed from the fly and placed into one drop of 1% PBS on a separate microscope slide. Vesicles and testes from 3-4 individual males were placed into each drop. Excess PBS was removed using a pipet and then approximately one drop of ProLong Diamond Antifade with DAPI mounting solution was added to samples. Cover slide were rested on top of the slides and then placed in a covered box to cure for 24 hours. The edges of the covers were sealed with nail varnish after curing, then visualized using a Zeiss Axiovert 200/Axiovert 200M inverted microscope.

Fertility assays

F1 RNAi males and virgin LHM females were paired and placed in vials to mate. After 24 hours males were discarded and females were transferred to a new vial. Females continued to be transferred to new vials after 24 hour periods for 4 days. The total number of eggs laid each

day were counted, and the total number of unhatched eggs counted the following day using a Leica EZ4 microscope.

Statistics

The programs RStudio (RStudio Team, 2015) and R (R Core Team, 2013) were used for all statistical analyses. We first used a Wilkes-Shapiro test and determine that the data was not normally distributed. A Mann-Whitney test was used to determine whether the mean tail length differed between each experimental group and their appropriate control. We adjusted the p-value according to Bonferroni correction such that $\alpha=0.002$. For fertility assay data, we calculated the ratio of hatched to laid eggs for each RNAi. Data was analyzed using a linear model to compare hatched eggs due to the variables of line, day of measurement, and number laid [lm=hatched~line+day+laid+line*day], with a fit contrast then used for single-variable comparison between each RNAi line and its appropriate control. P-values were adjusted using Bonferroni correction such that we compare against $\alpha=0.0009$. Another Mann-Whitney test which only measures single variables was utilized to corroborate the model results.

Results

Sperm flagellum length

In order to characterize phenotypic abnormalities in sperm tail morphology, we examined all F1 male progeny at 5 days of age, the onset of sexual maturity. First, we found that sperm tail from *CG4706* males had highly irregular appearances with coiled and broken tails. Despite numerous attempts at mounting, fixing, and visualizing, we were unable to obtain samples that were adequate for additional phenotypic analysis. Nonetheless, our observations are consistent with potential flagellum or axoneme structural abnormalities. Second, reliable flagellum length estimates were obtained for six genes of interest (Figure 3). We note that length measurements were not normally distributed for all lines and therefore utilized a nonparametric test to compare against the control values. Average tail length for the GD control was 1.689mm. Knockdown of two genes demonstrates significantly different tail lengths: *CG5718* had an average of 1.625 mm ($p=0.000607$) and *CG7514* had an average of 1.708 mm ($p=0.00156$). Lastly, we were unable to obtain tail measurements from *gskt* males due to obvious deformities within the reproductive tract and deficits in sperm production.

Spermatogenesis and sperm production pathways

To determine whether knockdown of genes of interest altered the number of mature sperm cells, we imaged the seminal vesicles at age 5 to assess the density of sperm stored in the organ (Figure 4). At age 5 days, *CG17856* males appeared to have less sperm located within the seminal vesicles (Figure 4c), which explains the insufficient number of mature sperm for measurements. By day 7 the seminal vesicles appeared to have typical densities of sperm. Seminal vesicles for *gskt* males showed a dramatic lack of sperm (Figure 4e). All other RNAi lines appeared to have a normal density of sperm in the seminal vesicles (Figure 4a and 4b).

Male fertility

We used single mating assays to assess whether gene knockdown produced any deficits to overall fertility. The number of laid eggs represents the total possible number of offspring that could be produced, while the number of hatched eggs represent the proportion of laid eggs that have been fertilized. The average number of eggs laid per line were larger for the later block, which we attributed to differences in season and food availability. Ultimately, we do not believe that differences in the number of eggs laid between blocks will affect the data, as the proportion of hatched-to-laid is consistent throughout. Figure 5 shows boxplots of the proportion of hatched-to-laid eggs for each RNAi line. Based on fit contrast analyses, we discovered four genes with significantly reduced fertility: *CG4701*, *CG14508*, *CG11913*, and *gskt*. *CG4701* and *CG14505* had 3.95% (p=5.6E-19) and 79.23% (p=4.5E-4) of egg hatched, respectively, compared to the KK control of 93.64%. *CG11913* and *gskt* had 26.29% (p=3.1E-23) and 17.6% (p=1.6E-27) hatched compared to the GD control of 86.32%. We did not observe any overlap between genes significant for flagellum length differences and deficits in fertility, suggesting that different mechanisms operate between genes necessary for elongation and fertility.

Discussion

In summary, we set out to study eleven testis-specific, mitochondrial-enriched retrogenes to characterize their contributions to sperm tail elongation and overall fertility. We utilized the UAS/GAL4 system for RNA interference to knockdown expression of each gene and investigated the consequences on sperm tail elongation and fertility. We discovered that two of our selected genes contribute to mechanisms underlying tail elongation, and that an additional three appear to be involved in mitochondrial functions not related to elongation.

Knockdown of *CG5718* and *CG7514* produced significant differences in sperm tail length, while all other genes lacked significant differences. The lack of significance suggests that the selected retrogenes do not have a role in tail elongation, despite mitochondrial localization. The non-significant genes have proposed functions ranging from AAA ATPase-like activity, cytochrome C involvement to NADH or quinone binding (Gaudet et al., 2010). A visual review of electron transport chain components is presented in Figure 6.

CG5718 functions in electron carrier activity (Gaudet et al., 2010). Though its function within the electron transport chain has not been identified, the ETC is critical for energy production during oxidative phosphorylation. Its parent gene, *Scs-fd*, functions as a succinate dehydrogenase in Complex II of the electron transport chain. This protein is crucial in both the Krebs cycle, where it oxidizes succinate to fumarate, as well as the ETC where it reduces ubiquinone. Though no specific localization within the ETC is suggested for the retrogene, sequence similarity to the parent indicates that it too functions as a succinate dehydrogenase. Thus, knockdown affects tail elongation due to deficient roles during both the Krebs cycle and electron transport. *CG7514* is suggested to be involved in oxoglutarate-malate antiporter activity (Sardiello et al., 2003 and Gaudet et al., 2010), important within the malate-aspartate shuttle,

which overall functions to reduce NAD^+ within the mitochondria to NADH. We noted that the tails of *CG7514* individuals were significantly longer than control males, though at this time we do not understand why gene knockdown resulted in this phenotype.

Fertility assays were run in order to determine whether knockdown of retrogenes had an effect for overall fertility. We identified three retrogenes with significantly reduced proportions of hatched-to-laid eggs as compared to their respective controls. *CG4701* is the retrogene for *nmd* and has suggested AAA ATPase activity and localization to the outer mitochondrial membrane (Ashburner et al., 1999). Mutations in its parent gene, *nmd*, result in the inability of mitochondria to aggregate and fuse to form the nebenkern. This deficiency has been attributed to *nmd* not fulfilling its role as a microtubule-severing protein. Sequence analysis suggests that *CG4701* and *nmd* share AAA ATPase activity (Spralding et al., 1999), which indicates that *CG4701* also functions between the mitochondrial and microtubules. The percentage of unhatched eggs for this line was not as drastically reduced as some of the other lines. Since both *nmd* and *CG4701* function within the testis, it is possible that elongation occurred appropriately and some fertility maintained due to the presence of the functional parent gene.

CG11913 is suggested to have a role as NADH dehydrogenase (Sardiello et al., 2003), important to complex I of the electron transport chain. This complex facilitates the removal of hydrogen from NADH coupled with the transportation of electrons to the start of the electron transport chain. *CG14508* is inferred to be involved as an electron transporter with specific activity related to transferring electrons from CoQH2-cytochrome C reductase complex to the cytochrome C oxidase complex, the role of mitochondrial Complex III (Sardiello et al., 2003 and Gaudet et al, 2010). Both Complexes are crucial for establishing the proton gradient that ultimately leads to the synthesis of ATP for the cell.

Sperm length differences observed for *CG7514* and *CG5718* knockdowns were not reflected in fertility assays. It is possible that while knockdown of these genes does not affect fertility but could influence competitive ability. Under a competitive mating scheme, the knockdown males would be at a competitive disadvantage and receive fewer fertilizations. Despite evident fertility effects, *CG4701* and *CG11913* did not demonstrate deficient tail elongation, suggesting that retrogenes are involved in function of mitochondria not related to the elongation process. Sperm tail lengths have not yet been obtained for *CG14508*, and thus we cannot conclude whether the fertility deficit was related to tail morphology, or if the gene is important for other mitochondrial processes. We note that the two genes with significant tail difference have indicated functions prior to the electron transport chain – *CG5718* potentially being involved in the Krebs cycle, and *CG7514* potentially being involved in the aspartate-malate shuttle that brings electrons into the mitochondria. Mutations in these genes could lead to difference in tail length due larger scale energetic pathway disruption, which prevents normal elongation of the sperm tails, whereas mutations later in the electron transport chain are associated with energetic defects in mature sperm which result in reduced fertility.

We return to the fact that retrogenes are enriched in sperm, with a large number of known retrogenes having testis-specific function. Further, we recognize that in *Drosophila* these retrogenes are further enriched for mitochondrial functions related to oxidative phosphorylation. Mitochondrial fusion and the formation of the nebenkern are essential for structural support during tail elongation. Our results demonstrate that in the case of two retrogenes, elongation is effected by gene knockdown. We therefore conclude that these genes of interest are involved in mechanisms that confer stability upon the elongating tail. Infertility phenotypes suggest that retrogenes are also involved in mitochondrial functions not directly related to tail elongation.

Limitations exist within our experimental procedures. Firstly, we were unable to obtain sperm tail measurements for all genes of interest. To more conclusively state whether these genes have functions related to tail elongation, it is crucial to have measurements. Secondly, we only tested fertility using a one-on-one set up between F1 males and LHM females. The use of competitive mating assays would test to see how sperm from knockdown males fared in situations where sperm competition exists, more accurately reflecting what occurs in nature. Thirdly we used *gskt* as the negative control and proof that retrogenes were being accurately knocked down. However, it is possible that the RNA hairpins did not accumulate substantially enough at the time that each gene of interest was expressed, thus allowing some cells to develop normally. To determine whether this occurred future studies should utilize quantitative polymerase chain reaction (qPCR) to measure transcript levels and measure knockdown efficiency. Future works could also utilize Mitotracker for visualization of the mitochondria (Gilmore & Wilson, 1999) and antibodies specific for each retrogene's product in order to demonstrate that the products localize specifically to the mitochondria of sperm cells and whether the nebenkern displays any structural abnormalities.

In conclusion, we were able to identify two testes-specific, mitochondrial-enriched retrogenes that function during sperm elongation. We found three additional genes that play important roles in fertility but not through tail elongation. These results support previous works that demonstrate the importance of the mitochondrial as a support structure during elongation, but also indicate that the functions of these retrogenes are not completely understood, and further work needs to be conducted. This study provides further insight into the underlying molecular mechanisms driving the rapid evolution of sperm length morphologies, and furthers the larger goal of elucidating the consequences of sexual selection.

Figures and Tables

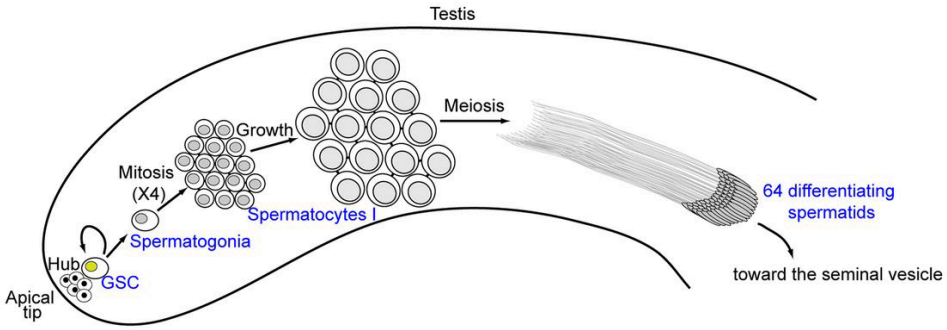

Figure 1. Spermatogenesis through the testes (Adapted from Dubruille & Loppin, 2015) Single germ cells migrate from the apical end of the testis and undergoes multiple rounds of mitosis before entering meiosis. Cells differentiate to form distinct head and elongated tail regions. Mature sperm cells are then stored in the seminal vesicle.

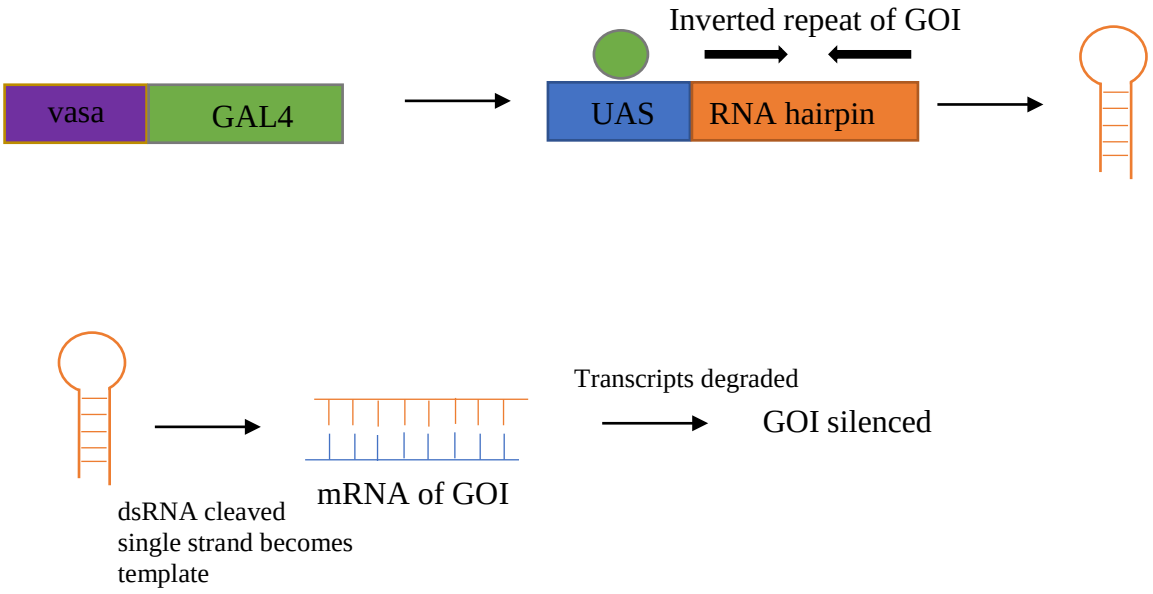

Figure 2. UAS/GAL4 system for RNA interference. Vasa drives the testis-specific expression of the GAL4 transcription factor (TF). The TF activates the UAS which then drives the transcription of the RNA hairpin. The double-stranded (ds) RNA is targeted by the RNA-induced silencing complex and cleaved to single-stranded RNA. The transcript is complementary to the mRNA produced by the GOI and the two anneal, leading to dsRNA. These transcripts are degraded and the gene silenced.

Parent gene	Retrogene name	CG number	Age	Library
Nmd	*CG4701*	CG4701	Before *Drosophila*	KK
Acon	*CG4706*	CG4706	Before *Drosophila*	GD
CG3560	*UQCR-14L*	CG17856	*D. yakuba*	GD
Sgg	*gskt*	CG31003	Before *Drosophila*	GD
CG1970	*ND-49L*	CG11913	Before *Drosophila*	GD
CG1907	*CG5714*	CG7514	Before *Drosophila*	GD
SCS-fp	*SdhAL*	CG5718	Before *Drosophila*	GD
CG7998	*CG10749*	CG10749	*D. willistoni*	GD
CG4769	*Cyt-C1L*	CG14508	-	KK
dmGlut	*CG9254*	CG9254	-	GD
CG8931	*CG5755*	CG5755	-	GD

Table 1. Retrogene information The names of the parent genes, retrogenes, and coding gene (CG) number. Age indicates at which point of the Drosophila phylogeny each retrogene emerged. Library indicates which VDRC library each RNA haipin originated from. Retrogenes, except for *gskt*, are referred to by CG number in this paper.

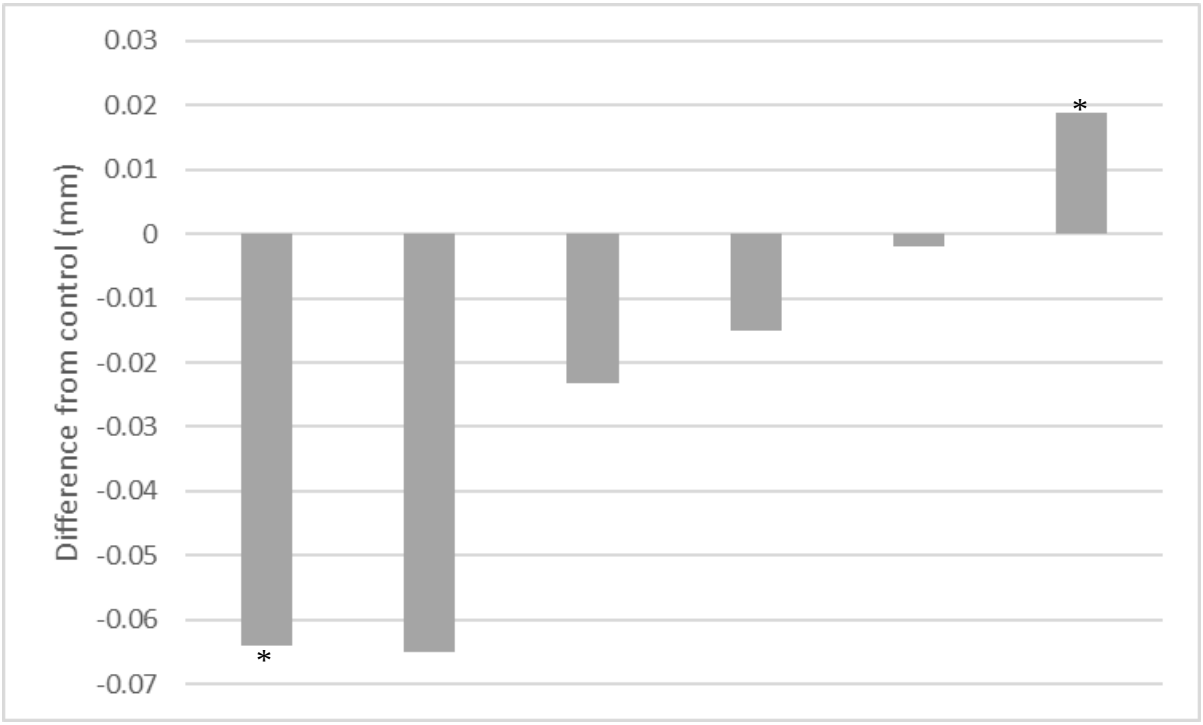

Figure 3 Effect of RNAi knockdown on sperm length. Difference in tail length determined by subtracting average tail length of control from average tail length per line. Significant values are indicated by asterisk.

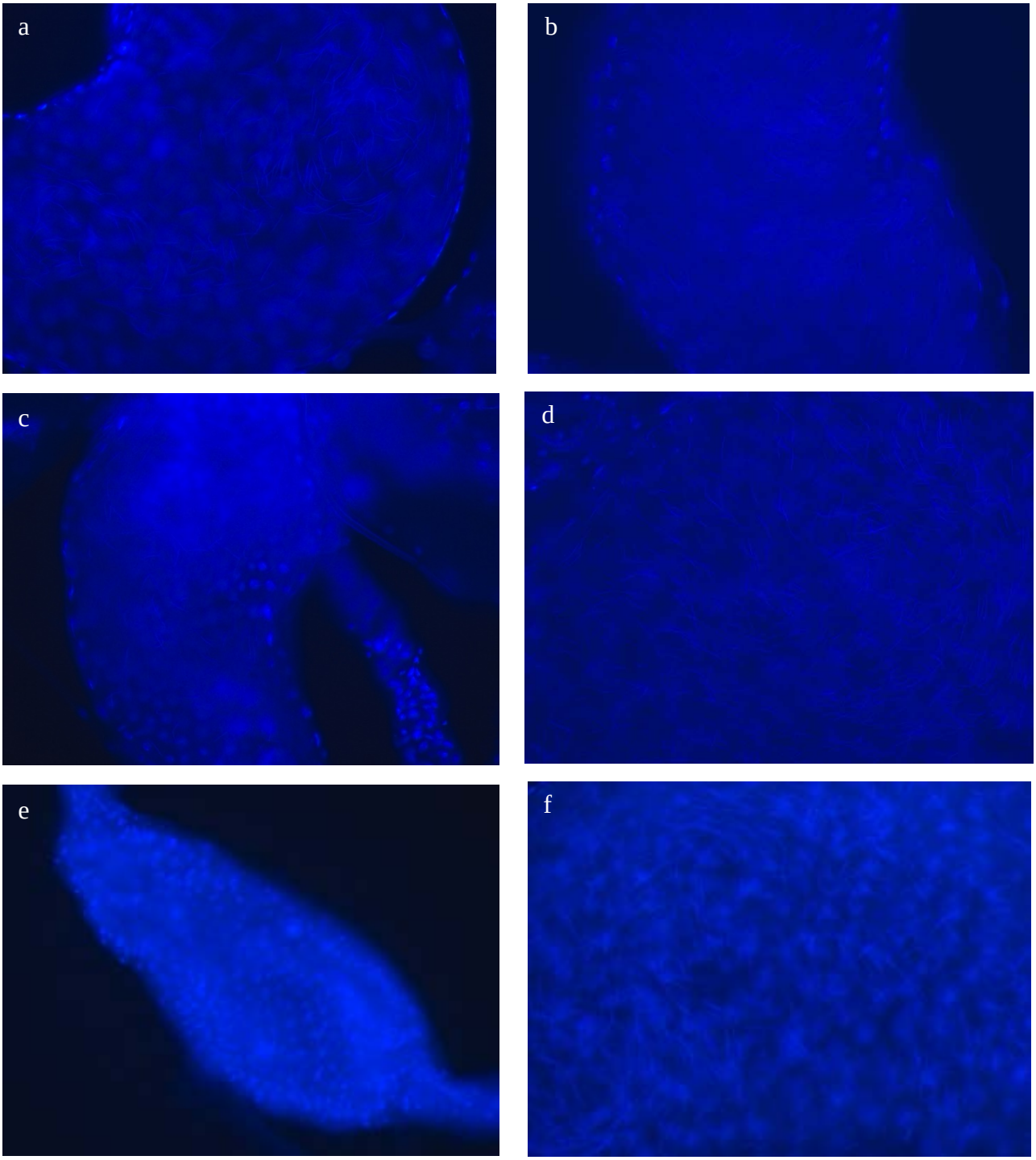

Figure 4 Miscroscopy of sperm stored in seminal vesicles. Images taken under fluorescent microscope of DAPI-stained seminal vesicles from F1 a) CG4701 b) CG4706 c) CG17856 d) KK control e) CG31003 (*gskt*) f) GD control males. Dense, rod-shaped cells are individual spermatids, while rounded cells in the fore- and background are somatic tissue.

CG number	10749	111913	14508	17856	31003	4701	5718	5755	7514	9254	4706
Estimate	-7.8	-30.9	-24.7	-6.1	-34.6	-9.3	3.8	-4.1	-9.4	-5.2	8.8
Std. error	3.0	3.0	2.6	3.1	3.05	2.6	3.0	3.1	3.1	3.0	3.1
t value	-2.6	-10.2	-9.2	-1.9	-11.3	-3.5	1.3	-1.3	-3.1	-1.7	2.8
p value	9.7E-03	3.1E-23	5.6E-19	4.9E-02	1.7E-27	4.6E-04	2.1E-01	1.8E-01	2.1E-03	8.7E-02	5.2E-03

Table 2 Fit contrast statistics. Results from the linear model and fit contrast comparisons used to analyze fertility assay data. Significant values are indicated in red.

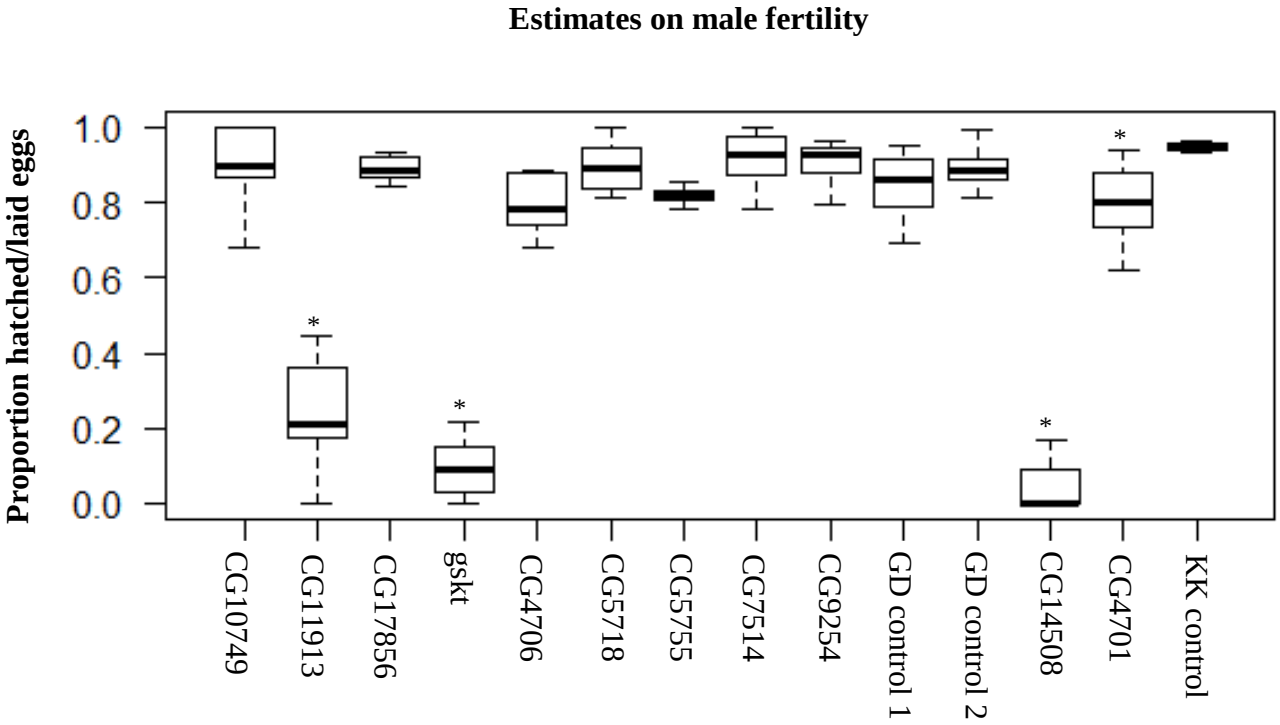

Figure 5 Estimates on male fertility. Boxplots representing the proportion of hatched/laid eggs for each RNA line. Significant values denoted by asterisks.

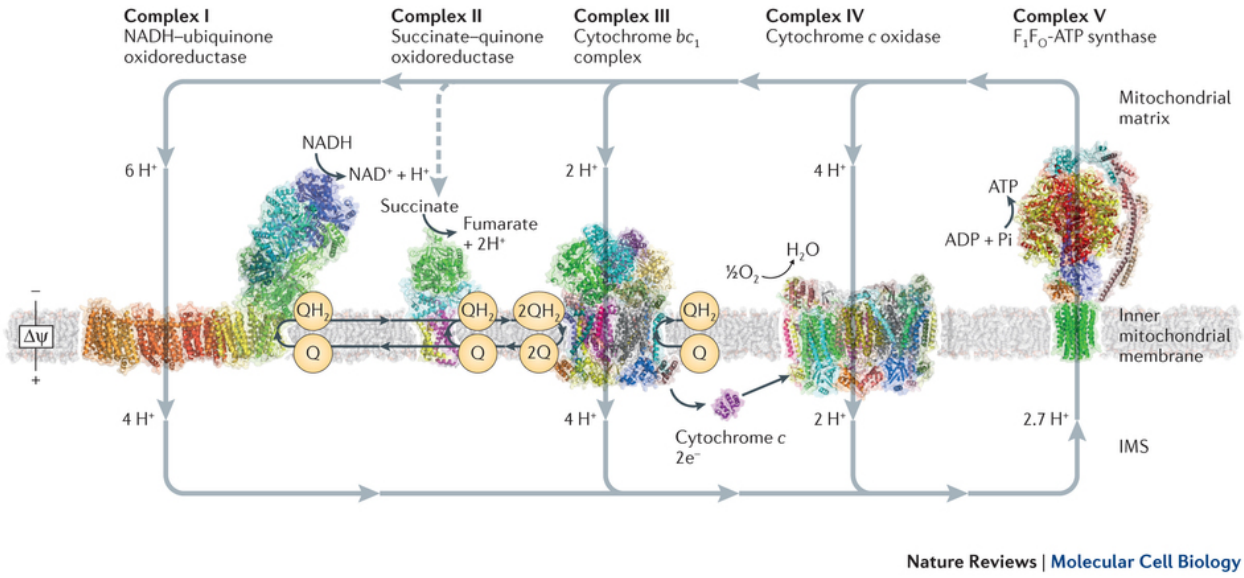

Figure 6 The electron transport chain (Adapted from Sazanov, 2015)
A review of the components of the electron transport chain. Significant genes from tail measurements and fertility assays are inferred for having functions in Complex I, Complex II, Complex III, and the electron transporter cytochrome C.

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