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Scribe Summary

Male fruit flies alter the behavior and physiology of females post-mating through seminal fluid secretions (Wolfner 1997). Seminal fluids are shown to have a wide range of effects when interacting with female reproductive tissue. The female fly develops a decreased attraction to males, a disinterest in mating, increase in egg laying, and decrease in lifespan (Wolfner 1997). In the male *Drosophila melanogaster* reproductive system, accessory glands secrete proteins that interact with sperm and affect fertility. Sperm is combined with secretory products of the accessory glands in the ejaculatory duct, creating seminal fluid. This fluid is transferred to the female during mating.

There are several transcription factors that play a role in accessory glands and their formation, such as Paired (Prd) and the *HR39* nuclear receptor, which was described in Praggastis et al., 2021. The function of *HR39* in the female reproductive system has been well characterized, but its role in the male reproductive system was not clear. Researchers induced mutations in *HR39* using CRISPR/Cas9, but the specifics of this method were not explicitly stated. How were the mutations introduced using CRISPR and when during the fly’s life cycle was this done? Did researchers introduce the mutations during the embryonic stage or the gametes?

The mutant adults that resulted form these methods had markedly lower levels of *HR39* mRNA (Praggastis et al., 2021). Researchers state that this was likely due to nonsense-mediated decay. What is “nonsense-mediated decay?” Does it happen with premature STOP codons in the coding sequence or truncated mRNA transcripts, i.e. a splicing defect? Does nonsense-mediated decay occur in all eukaryotes?

It was discovered that *HR39* mutants with a null allele developed normally and had a regular lifespan but were functionally sterile (Praggastis et al., 2021). Infertility was concluded through fecundity assays. As described in the paper, control virgin females were crossed to control or mutant flies. Why do the researchers use virgin flies in these genetic crosses? How do they know if a fly is a virgin?

Expression profiling revealed that many proteins and noncoding RNAs found in the accessory glands of males were present in mutants at decreased amounts. These results implied that *HR39* maintains normal levels of transcription in the accessory glands (Praggastis et al., 2021). To further investigate this deduction, researchers conducted genetic rescue and RNAi studies of *HR39* in the accessory glands. Researchers used a GAL4/UAS system to activate transcription of *HR39* RNAi for these experiments. There is no clear explanation of the system used in the paper, which leads us to the next question. How does the GAL4/UAS system work? It is also notable that researchers used a fly stock with a Gal4 gene that expressed Gal4 under the control of Paired. When is Prd expressed during the stages of a fly’s life? Did researchers choose a specific developmental period for efficient activation of the GAL4 construct?

The results gathered in this experiment confirmed that *HR39* is necessary for reproductive success and gene expression in the accessory gland of male fruit flies. *HR39* is required for male fertility and is a regulator of accessory gland transcription.

1. **How did researchers introduce the mutations using CRISPR? Was it at the embryonic stage or in the gametes?**

The Praggastis paper cites Gratz et al., 2014 as the method used for CRISPR/Cas9 mediated introduction of HR39 mutations. This method injects Cas9 into preblastoderm *Drosophila* embryos (Gratz et al, 2014).

1. **What is 'nonsense-mediated decay?' Does it happen with premature STOP codons in the coding sequence or truncated mRNA transcripts, i.e. splicing defect? Does nonsense-mediated decay occur in all eukaryotes?**

A mutation in the transcript in the mutant flies causes transcription to occur that disregards STOP codons. Therefore, the resulting mRNA will have a STOP codon within it. Nonsense-mediated decay is a mechanism coupled to translation that removes mRNAs that contain premature STOP codons (Brogna & Wen, 2009). Nonsense-mediated decay is linked to pre-mRNA splicing in mammals. When a premature STOP codon is located upstream of an intron, there is a noticeable reduction of mRNA. The researchers of this paper hypothesized that the reduction of *HR39* mRNA in mutant flies was a result of nonsense-mediated decay of prematurely truncated transcripts (Praggastis et al., 2021). Nonsense-mediated decay occurs in all eukaryotes that have been investigated, including, but not limited to, flies, yeast, birds, mice, and fish (Hwang & Maquat, 2011).

1. **Why was it important to use virgins in this experiment? How do researchers know when flies are virgins?**

Researchers in this paper used virgin female flies for genetic crosses. Virgin flies are used in genetic crosses because when flies mate, the female keeps the sperm of the male in her spermatheca and will use this sperm to fertilize her eggs throughout her life (Prokupek et al., 2008). Thus, when a female mates with a male, she will use that sperm long-term, and will not use the sperm of any other male. This can lead to contaminations within genetic crosses, so one must use virgin females in crosses to avoid unwanted genotypes. It is only necessary to collect virgin females.

There are two ways of collecting virgins: timed collections and anatomical collections. When female flies eclose from their pupa case, they do not sexually mature for another eight hours. One can clear a fruit fly bottle of all present adult flies, wait ideally four to six hours, and collect every female that has eclosed in that time frame, as she will be a virgin. The next method is anatomical collection. During anatomical collection, a researcher will select for typical phenotypic markers of virginity. Virgin flies are lighter in color, almost white. They have bloated abdomens with a black spot in them (their feces), as well as curled wings that have not opened yet.

1. **How does the GAL4/UAS system work?**

The GAL4/UAS system is a widely used system to study gene expression in the fruit fly (Caygill & Brand, 2016). GAL4 is the gene encoding the yeast transcription factor Gal4. and UAS stands for Upstream Activating Sequence. A UAS is an enhancer that the Gal4 protein will bind to in order to activate gene transcription.

In *Drosophila* research, the two components of the system are carried in two different fly lines, and they will combine in the progeny of their genetic cross to activate the gene of interest. One could use the GAL4/UAS system to overexpress or knockdown genes by using a UAS for the gene of interest or a UAS for an RNAi for that gene, respectively. There are methods to temporally control the activation of the GAL4/UAS system, such as temperature sensitivity or triggering the system with drugs.

Gal80 is another yeast protein that binds to Gal4 and inhibits it at room temperature. The two constructs will unbind at temperatures at 30-31ºC, allowing the Gal4/UAS system to complete its function (Weaver et al., 2020).

The inducible GeneSwitch GAL4/UAS system uses the human progesterone blocker RU-486 (administered through food) to activate the GAL4/UAS system. This specific GeneSwitch system transcribes negligible levels of the transgene in the absence of RU-486 (Scialo et al., 2016).

The researchers did not use an inducible GAL4/UAS system. They used the fly line Bloomington 1947 for prdGal4 (Praggastis et al., 2021).

1. **When is Prd expressed in the stages of the fly’s life? Did they choose a specific developmental period for efficient activation of the Gal4 construct?**

Paired is a paired-rule gene in *Drosophila melanogaster*. A paired rule gene is a gene involved in the development of the segmentation of insect embryos. The roles of paired include embryonic segmentation, accessory gland development, and male fertility (FlyBase).

Paired is expressed in multiple embryonic stages throughout *Drosophila* development (Fisher et al., 2012). It is also expressed in adult males, but not females (Arbeitman et al., 2004). Since Prd is expressed normally in adult males, researchers did not need to choose a specific developmental stage to conduct their experiments and were able to use adult males of 7-8 days old. It is important to note that while Prd is expressed in adult males, expression levels vary depending on age.

While the characterization of *HR39* as a regulator of male fertility is an exciting advancement in understanding the *Drosophila* male reproductive system, there are still challenges facing this research. This paper focused on the accessory glands of the male reproductive system. While these glands play an important role in male fertility, there are other parts of the reproductive system that were not investigated, such as the testis, vas deferens, and ejaculatory duct and bulb. It was also mentioned in the paper that the Prd-Gal4 system might have not been entirely reliable. The highest expression of Prd occurs when male flies first eclose, and expression dwindles as the flies age. Researchers used flies that were 7-8 days old.

Future research in this field could investigate the parts of the male reproductive system that were not examined. This research could be used to investigate transmissible diseases, as well as insect population control for crop protection.

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