Hormonal Regulation of Drosophila Accessory Gland Growth

A thesis submitted in partial fulfillment of the Degree of Bachelor of Science in Neuroscience with Honors

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Abstract

Hormonal signaling regulated by the brain is a vital process. Neurohemal axes, such as the hypothalamic-pituitary axis, are particularly important to modulate stress responses, metabolism, and fertility. Drosophila are a particularly useful model of this axis, through neurosecretory cells regulating endocrine glands that mediate hormonal release. An unknown component in the field is how the Drosophila accessory gland (AG), a model of the human prostate, is regulated by neurohemal axes. To examine this, our lab studied a hormone, Juvenile Hormone (JH), known to have effects on fertility in female Drosophila. To examine if JH is involved in AG growth and ploidy in males, we knocked down JH receptors and cell cycle regulators and observed the effects on gland growth. Since the AG is known to undergo an alternative cell cycle, an endocycle, we wanted to see if JH was involved in this process. It is also unknown which genes are activated in response to JH signaling. To find possible downstream targets, we utilized a Drosophila screening tool called Gal4 technique for real time and clonal expression (GTRACE) to observe expression of certain growth factors in the past and present. We saw that when JH signaling pathway components were knocked down, AG gland size and ploidy decreased, suggesting that JH signaling plays a role in AG growth and polyploidization. We also found that the growth factor Escargot was expressed throughout the gland in the past, when JH levels are highest, suggesting that Escargot could be activated in response to JH signaling.

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Scientific Acknowledgements

The experiments presented in this thesis resulted from a close collaboration with Allison Box, a graduate student in the Buttitta Lab, and she has granted me permission to include some of the data she collected and analyzed in this thesis. Dr. Laura Buttitta, my co-sponsor, created a genetic map and she has granted me permission to include this map in this thesis. The person who conducted each illustrated experiment is indicated in this table:

Figure #	Person Responsible for the Experiment
13	All of the data in this figure was collected and analyzed entirely by me
14	Canton-S and E2F2-i data was collected by Allison Box and analyzed by me, Allison Box dissected and imaged the glands for GCE-i and Tai-i but data was collected and analyzed by me
15	Genetic map created by Dr. Laura Buttitta
16	Gland dissection and images collected entirely by me

Personal Acknowledgements

Firstly, I would like to thank Dr. Laura Buttitta for welcoming me into her lab three years ago. As a freshman who was just beginning biology classes, I could not have had a more positive introduction to the field of biology and research. Dr. Buttitta provided me with such a welcoming community immediately, and has been a crucial part of why I love science and plan to continue research in my future endeavors. Even during the Covid-19 pandemic, Dr. Buttitta continued to keep me involved in the lab remotely, which I am incredibly grateful for. I would also like to thank Dr. Buttitta for guiding me throughout the writing process and giving me the pushes I needed to complete this thesis. I could not be where I am as a mature student and researcher without her support.

I would also like to thank Allison Box for being the best graduate student mentor I could have asked for. Allison has been a true inspiration to me, as she has been able to maintain being a fantastic mentor while also taking care of her newly born daughter, all in the midst of a global pandemic. Allison has taught me so much about *Drosophila* neuroscience and genetics, and has been exceptionally understanding and comforting whenever I was stressed about everyday college student worries. Allison exemplifies what it means to be resilient, and has passed this skill to me during my time under her guidance.

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An Introduction to the Field: *Drosophila* Hormone Signaling to the Accessory Gland

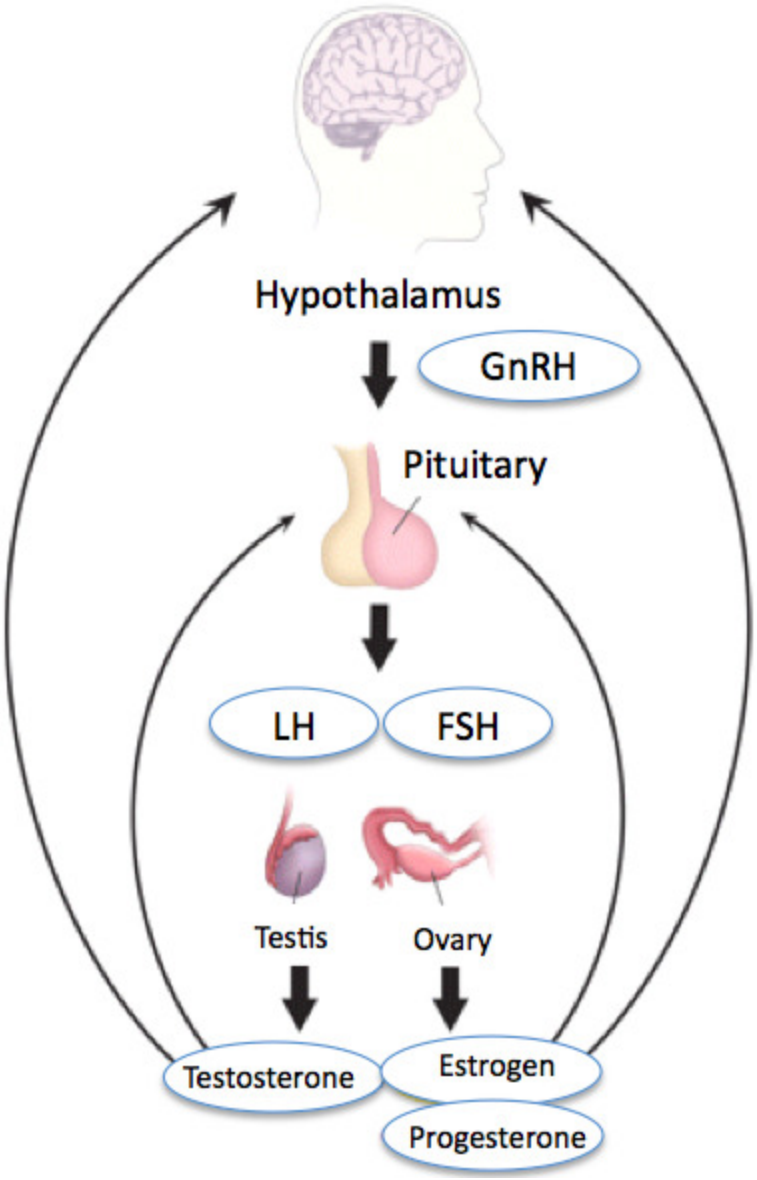
The Brain and Hormone Signaling and Regulation

Neuronal hormone signaling is a cutting edge topic in modern research due to the significant health issues that arise from these processes. The pathways for these disorders are often complex in both humans and model species, thus research to uncover the underlying mechanisms of the brain and the hormones that it regulates is crucial. Although many hormones are not conserved between *Drosophila* and humans, understanding the signaling pathways and how hormones that are released because of signals from the brain can provide valuable insight to how they act in humans. The endocrine system is implicated in many bodily processes such as growth and development, metabolism, sexual function, reproduction, and mood. Many of these processes are regulated by the brain, beginning with the hypothalamus activating the pituitary gland to release hormones targeting various tissues such as the gonads, thyroid, and kidney. Especially with hormonal diseases becoming so prominent in the 21st century, such as hyperthyroidism and hypertension, using animal models to understand neuroendocrine pathways can be extremely beneficial to society.

Remote control of tissue growth by the brain is quite relevant to humans as well, and is what makes studying the *Drosophila* accessory gland response to hormones under neuronal control so useful. When humans undergo puberty, a cascade of hormones circulate throughout the body causing various changes. Hormones are regulated not only during puberty, but throughout an individual's life, and when these processes are interrupted it can be quite debilitating. Benign prostatic hyperplasia (BPH) is a disorder that is hypothesized to be hormonally related. In 2010, around 14 million men in the United States had lower urinary tract symptoms that may have been due to BPH (Thorpe and Neal, 2003). One theory suggests that dihydrotestosterone (DHT), a hormone involved in prostate development and growth, is what causes BPH to arise. Although more research needs to be conducted, it has been shown that men who do not produce DHT do not develop benign prostatic hyperplasia. DHT is indirectly under neuronal control, as it is a metabolite of testosterone that is heavily regulated under neuronal control (Carson III and Rittmaster, 2003). This is only one example of a remotely

controlled hormonal disorder, with others including polycistronic ovary syndrome in puberty, hormonally related eating disorders, and delayed puberty (Blondell, Foster, and Dave, 1999). Thus, using *Drosophila* as a model for tissue mediated hormone growth and regulation is quite relevant in the larger scope of health and wellbeing.

In mammals, the main component of our brains that regulates hormones throughout the body is the pituitary gland. The hypothalamus' control over the pituitary gland and the pituitary's subsequent control over many remote organs can be described as neurohemal regulation. Simply put, neurohemal axes are classified as a system in which the central nervous system of an organism communicates with the rest of the body via circulating hormones. In addition to many other organs such as the thyroid gland, the adrenal glands, and more, the hypothalamus-pituitary axis has control over mammalian gonads. Under strict temporal control, the hypothalamus is able to control ovarian cycles and testicular functions which are both vital processes during a mammal's life with respect to fertility. This axis is controlled beginning with the hypothalamus releasing gonadotropin releasing hormone into the hypophyseal portal vessels, which are tightly networked with the anterior pituitary. The anterior pituitary then receives these hormonal signals from the hypothalamus, and releases luteinizing hormone and follicle stimulating hormone to the gonads (Ciechanowska, Łapot, Mateusiak, and Przekop, 2010). The gonads are then able to release hormones that have feedback mechanisms on the pituitary and the hypothalamus, as well as perform their various other physiological effects such as spermatogenesis, oogenesis, and more (Figure 1).



<u>Figure 1:</u> Hypothalamic-Pituitary-Gonadal Axis and Relevant Hormones: Overview of the human neurohemal axis beginning with the hypothalamus, then the pituitary, and lastly the gonads, including what hormones are involved.

(Adapted from Nedresky and Singh, 2019)

FSH and LH are major components in this neurohemal axis. For the menstrual cycle, FSH and LH are secreted at varying levels, and if these levels were to be abnormal or feedback mechanisms were improperly working, the organism's entire cycle could malfunction, and could negatively affect fertility (Mihm, Gangooly, and Muttukrishna, 2011). Abnormal function of this neurohemal axis could also

have negative impacts on male testicular function, also affecting fertility (Plant, 2015). These dysfunctions are important to study, especially in simpler organisms, such as insects, that also have neurohemal signaling.

Drosophila as a Model Organism

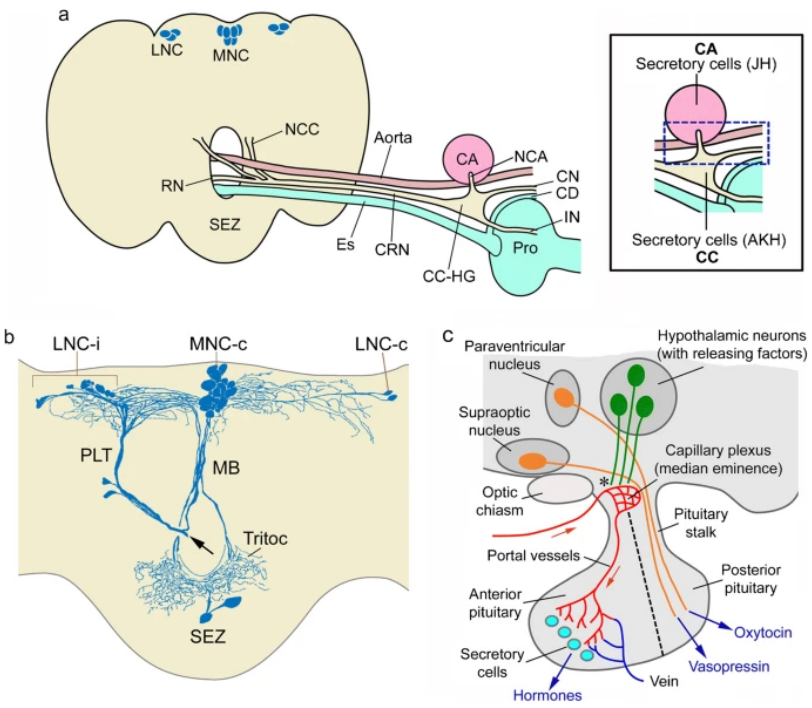
Drosophila melanogaster is one of the most common model organisms in biology. In genetics and neuroscience *Drosophila* are particularly useful due to their short life cycles, fast generation times, and relatively straightforward genetic manipulations. They are also small, easy to maintain in a laboratory, and relatively inexpensive. Studies using *Drosophila* have given rise to many genetic and developmental discoveries in scientific history. Dating back to the early twentieth century when *Drosophila* research helped advance our basic understanding of genes, chromosomes, and genetic inheritance, to 2000 where the *Drosophila* genome project was completed, so much of what is known in modern biology has arisen from studying *Drosophila*. The genome sequencing of *Drosophila* has also identified potential orthologs for nearly 75% of human-disease related genes (Yamaguchi and Yoshida, 2018).

Using *Drosophila* as a model organism also has some limitations. *Drosophila*, although with a relatively short generation time compared to monkeys or mice, have a longer generation time than smaller organisms such as yeast or *E. coli*. These organisms also produce more offspring than *Drosophila* and have smaller, less complex genomes. These organisms are much more desirable for autonomous cellular functions. *Drosophila* research is much more fitting to multicellular processes mediated by genes rather than smaller scale cellular events. *Drosophila* are also less similar to humans with respect to their organs and tissues when compared to mammals such as mice or monkeys. Since *Drosophila* are invertebrates and have a different and less complex central nervous system compared to humans, they are not as close of a match as other model species (Yamaguchi and Yoshida, 2018). With this being said, there are many homologous disease-related genes between *Drosophila* and humans, making the *Drosophila* genome still extremely useful for genetic research.

Neurohemal Signaling in Drosophila

As previously discussed, the human neurohemal signaling pathway is crucial to fertility and overall hormonal control in the body. Using *Drosophila* as a model for this axis is quite useful, as it has many analogous parts to the hypothalamic-pituitary-gonadal axis. The female *Drosophila* brain, relevant neurosecretory cells, endocrine tissues, and neurohemal release sites are depicted in Figure 2. The neurosecretory cells in *Drosophila* are primarily in three separate regions of the brain, the lateral and median neurosecretory cell groups and the subesophageal zone. The cells that receive signals from these brain regions are in the Corpora Cardiaca (CC), which produces the peptides adipokinetic hormone (AKH) and limostatin (Lst), and the Corpora Allata (CA), which produces Juvenile Hormone (JH), a sesquiterpenoid. Under conditions of low sugar, the CC releases AKH and Lst into circulation, which inhibits the release of insulin-like peptides (DILPs) (Nässel and Zandawala, 2020). DILPs act as circulating growth factors, promoting growth of various tissues (Goberdhan and Wilson, 2003). As we will discuss, Juvenile Hormone signaling affects the male *Drosophila* reproductive system, a subject on which little research has been conducted. As shown in Figure 2, the secretory cells in the pituitary correspond to the secretory cells of the *Drosophila*, as both are under neural control and ultimately produce substances that are transported throughout the organism and have profound effects on the body. Evidently, neurohemal control in the female *Drosophila* is occurring and affects its reproductive system, which is analogous to humans, making it a fantastic model species for these processes.

The research conducted in this thesis particularly focuses on how JH affects a peripheral reproductive tissue in males called the accessory gland (AG). Compared to effects of JH on reproduction in females, little is known about JH signaling and reproduction in males. Since the neurohemal axes in humans with the hypothalamus and pituitary, and in *Drosophila* with the neurosecretory cell groups and the CA, play similar roles, researching the pathways in the simpler model of *Drosophila* can be quite useful.



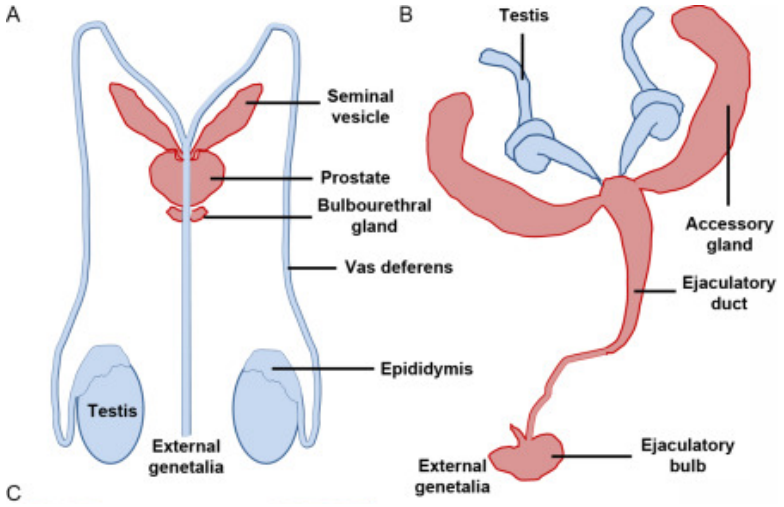
<u>Figure 2:</u> Neurohemal Axes of Humans and *Drosophila*: Parallels of the neurohemal axes in female *Drosophila* and humans.

(Adapted from Nässel and Zandawala, 2020)

The Accessory Gland as a Model of the Human Prostate

The human prostate gland located in the male reproductive system is implicated in many diseases and is a site of hormonal signaling and regulation. Using the male *Drosophila* melanogaster accessory gland (AG) as a model for the human prostate gland is an incredibly promising field (Wilson, Leiblich, Goberdhan, and Hamdy, 2017). The prostate gland is a common site for hormone related disorders in human males, so understanding the properties in analogous tissues can provide insight into the underlying processes occurring in the tissue. When human males age, their prostates enlarge, which is unlike other glands. The AG, like the human prostate gland, makes many seminal fluids components such as proteases, lectins, and cysteine-rich secretory proteins. The AG secretes the most total volume of seminal fluid, with the ejaculatory duct and ejaculatory bulb also contributing some.

Some of these proteins interact with sperm, affecting fertility (Wilson, Leiblich, Goberdhan, and Hamdy, 2017).



<u>Figure 3:</u> Human Reproductive System Compared to *Drosophila* Reproductive System:

Comparing male reproductive systems in human males and male *Drosophila*, displaying the accessory gland as an analog to the human prostate gland.

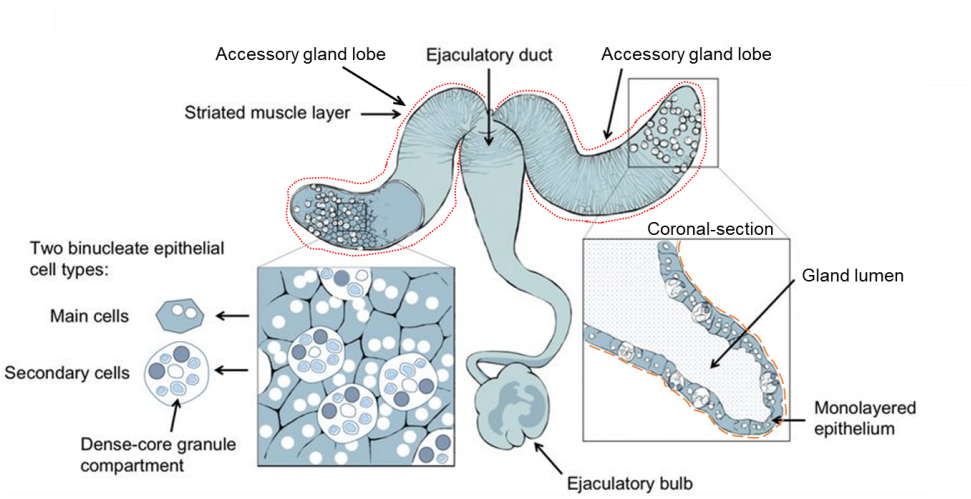
(Adapted from Wilson, Leiblich, Goberdhan, and Hamdy, 2017)

Literature Review:

The Drosophila Accessory Gland

The AG's overall functions make it particularly analogous to the male prostate. Proteases are present in both of these glands in particularly large numbers, and it has been suggested that these proteases serve to promote coagulation of the ejaculate in the female tract, which increases fertility by retaining the sperm when in the female tract. Both the AG and the prostate also secrete exosomes, which assist the sperm when fusing to the egg during fertilization. The human prostate has two major cell types: basal cells and secretory cells. Similarly, the AG is composed of two major cell types: squamous main cells and cuboidal secondary cells. Main cells are flat, hexagonal, binucleated cells that release their products into the AG lumen. Secondary cells are binucleated with large vacuoles and have a large spherical shape, primarily on the distal tips of each AG lobe. There are neurally controlled muscle cells surrounding the AG that squeeze it to release its contents into the ejaculatory duct, where they combine with the sperm made in the testis (Bertram et al, 1992). Thus, due to the similar functions and biologically useful products that the AG creates when compared to the human prostate gland, it has been used as a model for hormone regulation of the reproductive system.

The method by which the accessory gland cells become binucleate begins during metamorphosis at 50-55 hours after pupa formation. At this time, the cells arrest proliferation and begin an abbreviated variant cell cycle where nuclear division occurs, but cytokinesis does not. This results in one cell with two nuclei. About 10 hours later, the cells begin another synchronized variant cell cycle without mitoses called an endocycle that increases DNA content, resulting in about 1000 binucleate cells with two 4N nuclei (Taniguchi, Kokuryo, Imano, Minami, Nakagoshi, and Adachi-Yamada, 2014). The main cells exit the cell cycle and are thought to be largely quiescent in the adult fly (Leiblich, Marsden, Gandy, Corrigan, Jenkins, Hamdy, and Wilson, 2012), whereas the secondary cells retain the ability to cycle in certain situations such as mating signals (Leiblich et al., 2019). However, recent work from our lab has revealed another round of endocycling in the newly eclosed male and continued rare endocycles in aged males, independent of mating status, described in further detail below (Box et al. 2019).



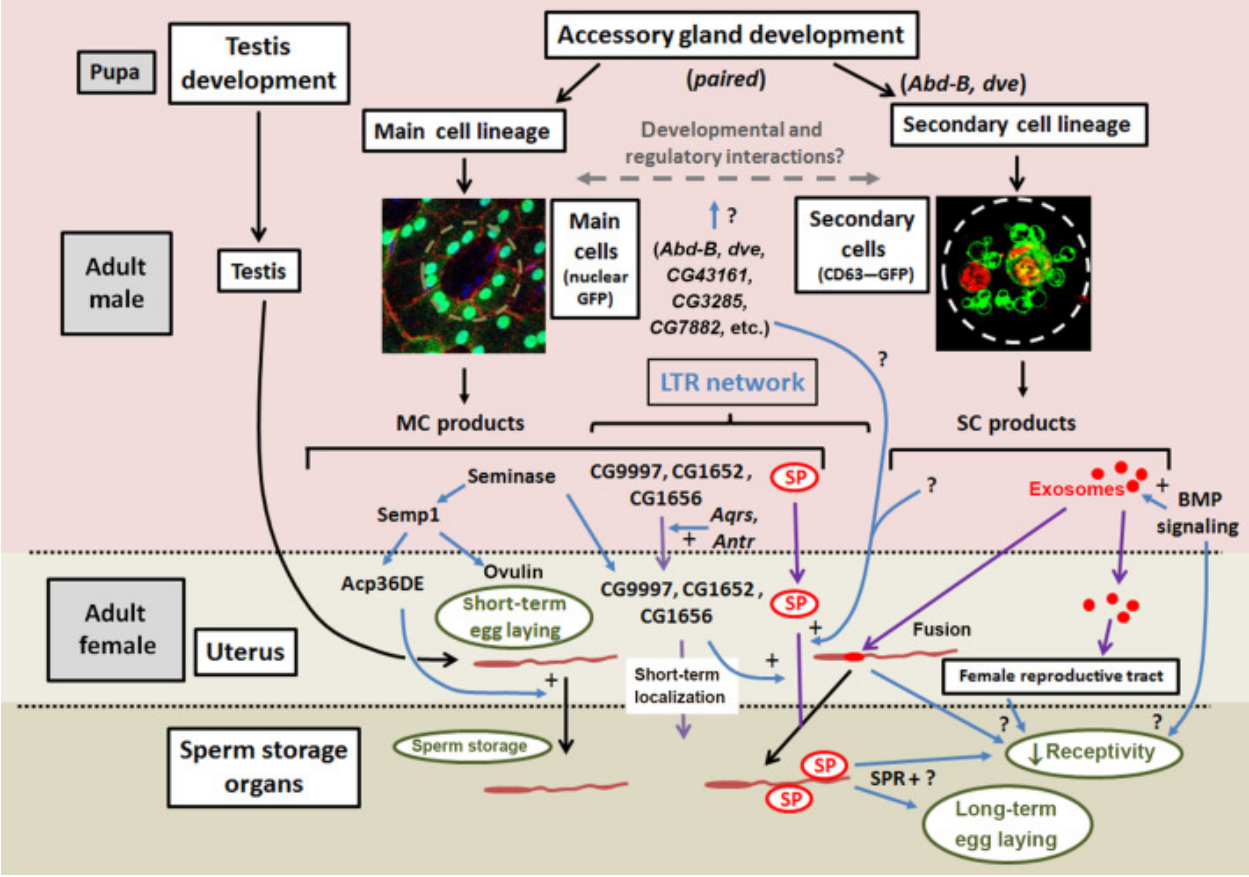
<u>Figure 4:</u> Accessory Gland Labeled Structure

(Adapted from Hopkins et al., 2019)

Main Cells and Secondary Cells

Each AG has a monolayer epithelium with about 1000 squamous main cells and about 40 cuboidal secondary cells. Main cells secrete an essential molecule to fertility called sex peptide, which is crucial to reproductive function, particularly when the ejaculate enters the female reproductive tract. When the sex peptide gene is mutated or knocked down, males are unable to elicit long term egg laying from the female and the female is less likely to remate. Many other crucial aspects are also diminished in the females after mating with these mutants/knockdowns, including the innate immune response to ejaculate, hormone and pheromone production, locomotor activity and sleep, food intake, and excretion. Also, when main cells are destroyed via diphtheria toxin, female egg laying and remating behavior are strongly suppressed. Thus, proper main cell function is crucial to male *Drosophila* fertility. Several other main cell expressed genes have been shown to induce short-term female postmating responses shown in Figure 5. Secondary cells are highly secretory, and release exosomes that fuse with the sperm after mating. Secondary cells also appear to have an effect on postmating behavior, such as

decreased female receptivity and increased long-term egg laying (Wilson, Leiblich, Goberdhan, and Hamdy, 2017).

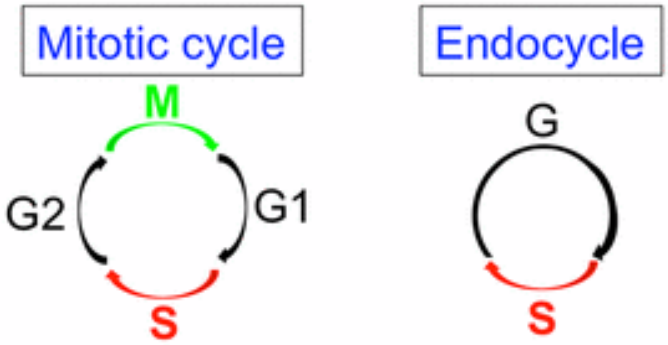


<u>Figure 5:</u> Various Effects of Main Cell and Secondary Cell Secretions on Fertility: Main cell products leading to short term egg laying in the female uterus and sperm storage in the male. Secondary cell products leading to sperm fusion in the female, decreased receptivity, and increased long-term egg laying with the assistance of sex peptide.
(Adapted from Wilson, Leiblich, Goberdhan, and Hamdy, 2017)

Accessory Gland Size Regulation with Age

The AG in *Drosophila* grows as the animal ages, just as the human prostate grows with age. Our lab has previously shown that the gland grows by virtue of cell size rather than cell number. This was shown by staining for a mitotic marker called phospho-histone H3 (PH3). When 100 AGs were stained without mating at various ages, PH3 staining was negative, indicating that the cells were

post-mitotic. This posed some uncertainty, as it is well known that the AG grows as the fly ages (Box et al. 2019), yet the cells in the AG are post-mitotic. Many cells in other tissues have shown to undergo a process called endocycling, which is a variant cell cycle that increases tissue size. Endocycling essentially is a cell cycle without any cell division via mitosis, but still with G and S phases. This process increases DNA content in the S phase and increases cell size in the G phase, leading to a larger nucleus and larger cell. Endocycling also leads to polyploidy as DNA content is increased but no cell division occurs. The increase in ploidy of AG cells can contribute to increased biosynthesis, which would be used to meet the energetic demands of the highly secretory adult AG that is responsible for making specific proteins which are transferred to the female during mating (Fox et al., 2020). To determine whether endocycling was the method that the AG was using to grow, our lab measured cell size with respect to gland size. It was confirmed that there is a correlation between AG size, main cell size, and main cell nuclear area. Our lab also performed EdU staining to see which cells in the AG were endocycling. After the EdU staining, our lab was able to see that the main cells endocycle in the fly up to 50 days of age, whereas secondary cells very rarely showed EdU staining, independent of mating status. This evidence showed that the AG grows as a result of an increase in main cell size rather than an increase in main cell number. Since the secondary cells undergo very little endocycling, this suggests that main cell endocycling is the primary driver of AG growth (Box et al. 2019).



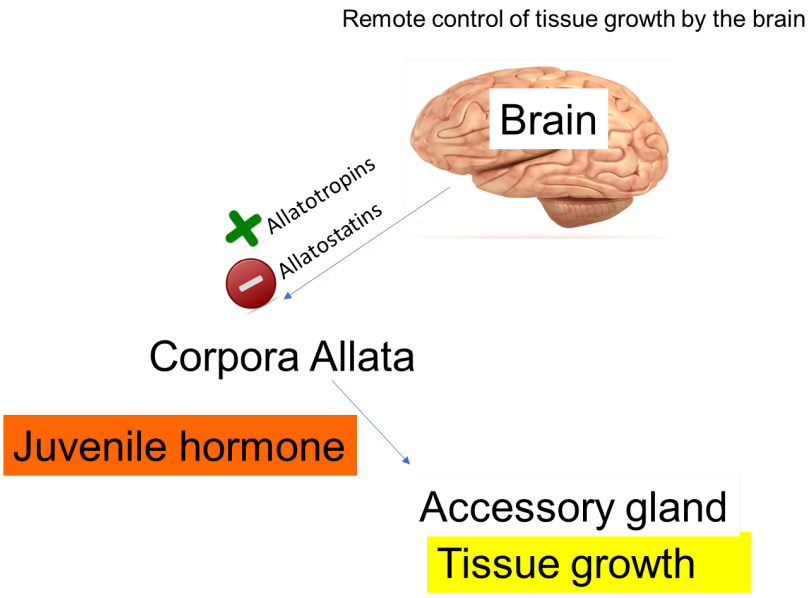
<u>Figure 6:</u> Mitotic Cycle Compared to Endocycle: Mitotic cycles have a G2 phase as well as the M phase where cell division occurs. This does not occur in endocycling; however, DNA synthesis and growth do occur, explaining why cell growth and polyploidization occur in cells that endocycle. (Adapted from Calvi, 2013)

Advantages of Accessory Gland Polyploidization

When the AG endocycles, it goes through the G and S phases, but does not divide or go through the typical M phase in the regular mitotic cycle. It is able to do this numerous times resulting in high ploidies. During the G phase, gene expression and growth take place. In the S phase, DNA synthesis occurs, increasing the cells' ploidy. Polyploidy is a normal developmental occurrence in many different organisms such as mammals, gastropods, angiosperms, and insects. When comparing polyploid cells to diploid cells with a similar mass, polyploid cells tend to have a higher metabolic rate due to the lack of mitosis and cell division occurring (Orr-Weaver, 2015). Other potential advantages of polyploid cells compared to diploid cells are that multiple genome copies in the cell provide a lower likelihood of mutations which provides the cells with damage resistance, as well as apoptosis being inactivated in certain polyploid cells which lengthen their lifespan. A unique aspect of the accessory gland is that it is able to regenerate itself. Polyploidy plays a role both in normal organogenesis, but also in tissue repair and organ regeneration (Orr-Weaver, 2015). The polyploid nature of main cells in the AG can thus be linked to its ability to regenerate when damaged.

The Corpora Allata, Juvenile Hormone, and the Accessory Gland

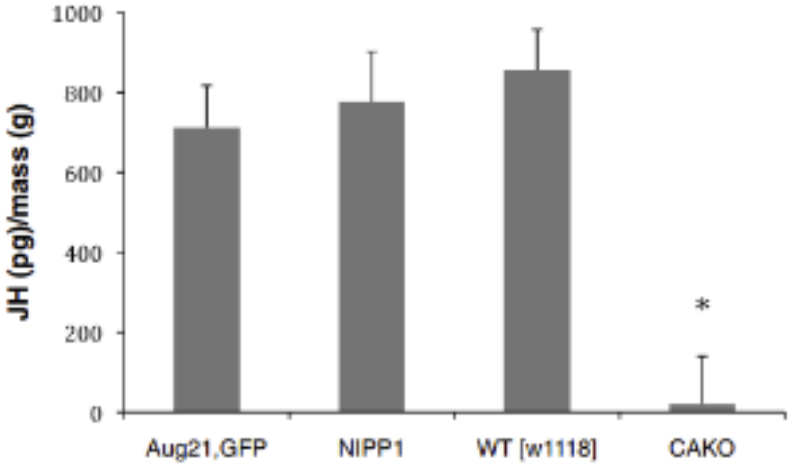
It was then unclear what was driving this variant cell cycle. Since mammalian prostates are known to be regulated by hormones, a potential explanation for the initiation of endocycling was through hormonal regulation. Juvenile hormone (JH) is a highly pleiotropic sesquiterpenoid that is produced and released by the corpora allata (CA), a group of endocrine glands located posterior to the *Drosophila* head. The CA is tightly regulated by allatotropins, which are neuropeptides that have an excitatory effect on the synthesis and release of JH, and allatostatins, which have an inhibitory effect on the synthesis and release of JH. These allatoregulatory peptides utilize rhodopsin G protein-coupled receptors to mediate their actions (Verlinden, Gijbels, Lismont, Lenaerts, Broeck, and Marchal, 2015). Unidentified brain cells send projections to the CA to trigger JH release, which is relatively analogous to the hypothalamus' control of the pituitary in humans.



<u>Figure 7:</u> Brain, Corpora Allata, Accessory Axis Control of Juvenile Hormone Release: Unidentified brain cells trigger the release of allatotropins and allatostatins, which upregulate and downregulate the CA's synthesis and release of JH respectively. The JH released by the CA then is received by the AG and, as this thesis discusses, affects the gland's growth.

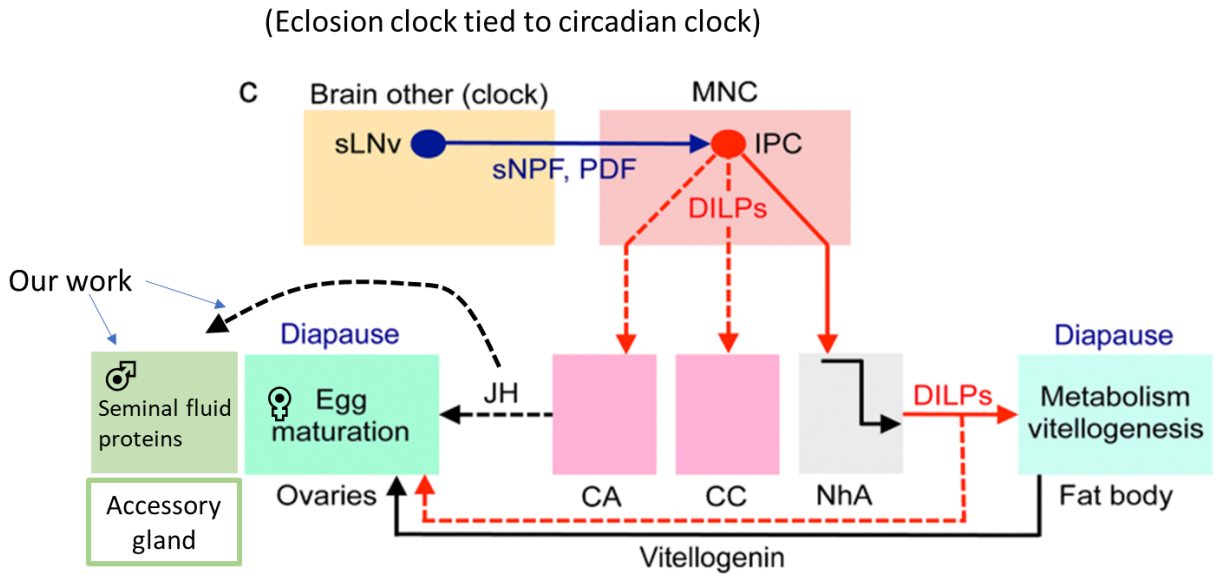
To provide more context of the CA structure and function, the CA consists of paired glands originating in the lateral ventral ectoderm in the head near the mandibles and maxillae. Eventually, this gland rests behind the brain. The CA is innervated by nerves from the brain that come from neurosecretory cells. The axons from the neurosecretory cells that project onto the CA are called corpora cardiacal nerves because they pass through and sometimes branch onto the corpora cardiaca (CC). The overall group of nerves, the CC, and CA are called the retrocerebral complex. The CA has an oval shape and is composed of a singular cell type enveloped in an extracellular sheath. As the CA cells produce hormones, their morphologies change. When hormone synthesis is upregulated, plasma membrane projections from cell surfaces increase, and mitochondrial number and size are increased (Tobe and Stay, 1985). The primary function of the CA is to synthesize and release JH, which has many targets. It has been shown that when the CA is knocked out, JH levels in *Drosophila* decrease

dramatically, implicating the CA of being crucial to JH production in this neurohemal axis (Yamamoto, Bai, Dolezal, Amdam, and Tatar, 2013).



<u>Figure 8:</u> Corpora Allata Knockout Drastically Reduces *Drosophila* Juvenile Hormone Levels: In the CAKO mutant, JH levels are reduced drastically when compared to controls, implicating the CA as a crucial component of the JH signaling pathway.
(Adapted from Yamamoto, Bai, Dolezal, Amdam, and Tatar, 2013)

JH is highly pleiotropic and has differing functions between males and females with respect to reproduction (Weaver and Audsley, 2009). In females, it has been shown that JH signaling promotes endoreplication of adult fat body cells in preparation for vitellogenesis. DILPs contribute to both the upregulation of fat body cells in preparation for vitellogenesis and egg maturation. In female *Drosophila*, JH has also been shown to be involved in the insulin signaling and neuroendocrine stress reaction (Li, Jia, and Li, 2019). Many general functions of JH have been shown in males, such as pheromone production, foraging behavior, and caste determination; however, not much is known about how JH affects fertility and the reproductive system in males. The work presented in this thesis shows an entirely new role for JH in male accessory gland growth. Figure 9 summarizes the pathways of neurohemal signaling in females that are known. The bottom left portion explains our work that uncovers unknown components of this axis in males.



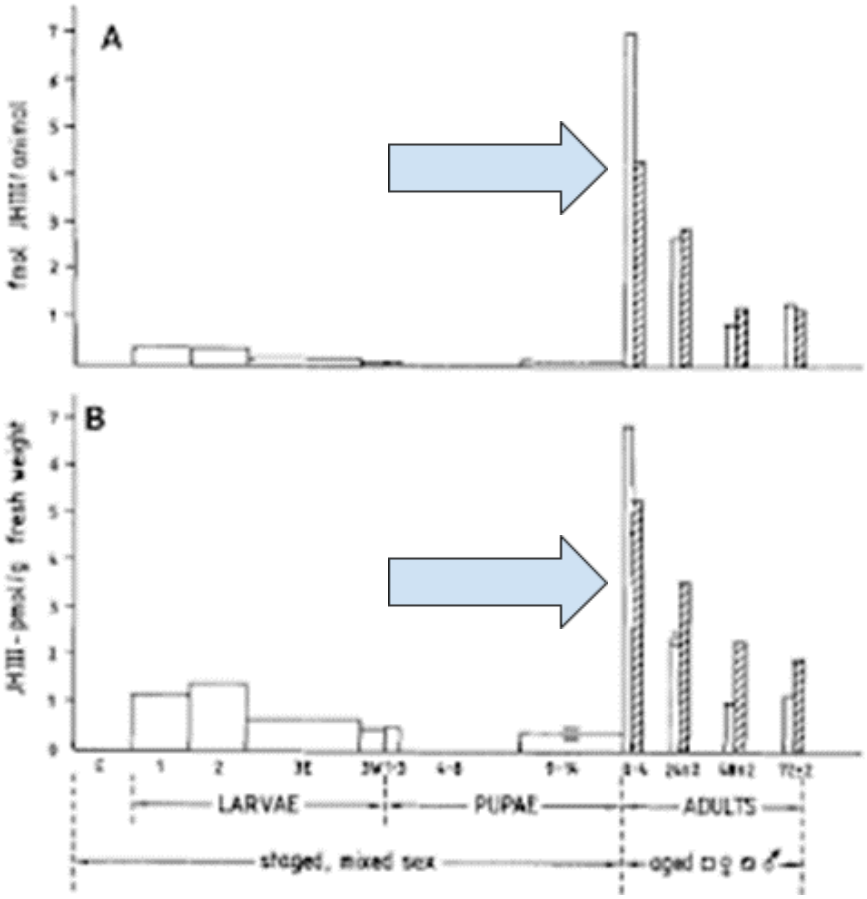
Modified From: https://link.springer.com/article/10.1007/s00441-020-03264-z

<u>Figure 9:</u> Current Knowledge of JH in Females and What This Thesis Plans to Discuss in Males: The current knowledge of JH signaling is primarily for female *Drosophila* and JH's role in fat body cell synthesis aiding in metabolism, vitellogenesis, and egg maturation. Our research is focusing on what JH does in males with respect to the AG.

(Adapted from Nässel and Zandawala, 2020)

Titer of Juvenile Hormone Throughout Fly Lifetime

If JH is implicated in the development of the accessory gland under neuronal control, it is important to examine the JH levels throughout the *Drosophila* lifespan. From the figure, we can see that JH-III has a large peak just after eclosion in both males and females, which suggests that JH plays a role in developing mature gametes. There was no significant difference between males and females in this study with respect to JH levels at different time points (Bownes and Rembold, 1987). When considering the role of JH and endocycling, the significant upregulation of JH in the fly just after eclosion further supports this notion, since tissue growth would initiate upon eclosion.

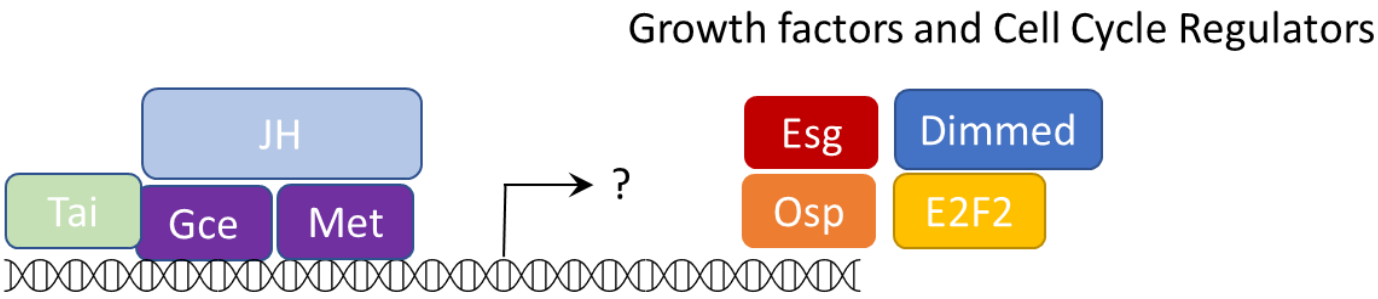


<u>Figure 10:</u> Juvenile Hormone Levels Peak Immediately After the Day of Eclosion: The arrows are signifying JH titer in *Drosophila* at the DOE, displaying its peak immediately after the DOE. (Adapted from Bownes and Rembold, 1987)

JH Signaling

When JH diffuses into the AG nuclei, there are certain proteins that play roles in its signaling. It has been shown that Methoprene (Met) mutants are resistant to JH agonists, which was the first suggestion of Met to be the JH receptor (Wilson & Fabian, 1986; Ashok et al., 1998). Met forms heterodimers with its paralog, germ-cell expressed (Gce), and JH diminishes this dimerization. Met and Gce null mutants survive, however, when there is a Met/Gce double mutant, the animal dies during the transition from larva to pupa. It was first shown in vitro that Met/Gce binds to Jh at physiological concentrations, which suggested that Met/Gce is the intracellular receptor for JH (Miura et al., 2005;

Charles et al., 2011; Jindra et al., 2015). It was then confirmed in vivo that JH binding to Met/Gce is essential for development in Drosophila (Jindra et al., 2015). Taiman (Tai) is also involved in JH, serving as a co-activator by directly interacting with Met. It is not known which specific genes are activated once JH binds to its intracellular receptors in the AG (Li, Jia, and Li, 2019).



<u>Figure 11:</u> Juvenile Hormone Intracellular Binding to Transcription Factors with Unknown Downstream Targets: Possible downstream targets when JH binds to its receptors with the assistance of its co-activators. The question mark signifies that it is unknown which genes in the AG are turned on when JH binds to its receptors.

Gce and Met have orthologs in humans called aryl hydrocarbon receptor nuclear translocator-like 1 and 2 (ARNTL and ARNTL2). These genes in humans are involved in our circadian clocks which guide the metabolic and cell-division cycles. Circadian clock genes have also been suggested to play roles in fertility and reproduction in animal models, specifically with ARNTL knockout mice. Both male and female homozygous knockout mice for ARNTL were either infertile or had significantly reduced fertility. In Finland, 511 individuals with single nucleotide polymorphisms of these genes were assessed in a health interview and an examination study, with the results indicating that women with the ARNTL mutant genotype had more miscarriages. ARNTL has also been linked to infertility in mice. In this study, ARNTL2 SNPs did not have significant impacts on fertility (Kovanen, Saarikoski, Aromaa, Lönnqvist, and Partonen, 2010). However, the Gce and Met orthologs that are involved in circadian clock rhythms certainly pose interesting implications with respect to fertility. Additionally, human circadian clocks can be analogous to the eclosion clocks that occur in

Drosophila. Since JH signaling is largest shortly after the day of eclosion, perhaps the similarity of ARNTL and ARNTL2 being involved in the circadian rhythms are how Gce and Met are related to *Drosophila* JH signaling at the day of eclosion.

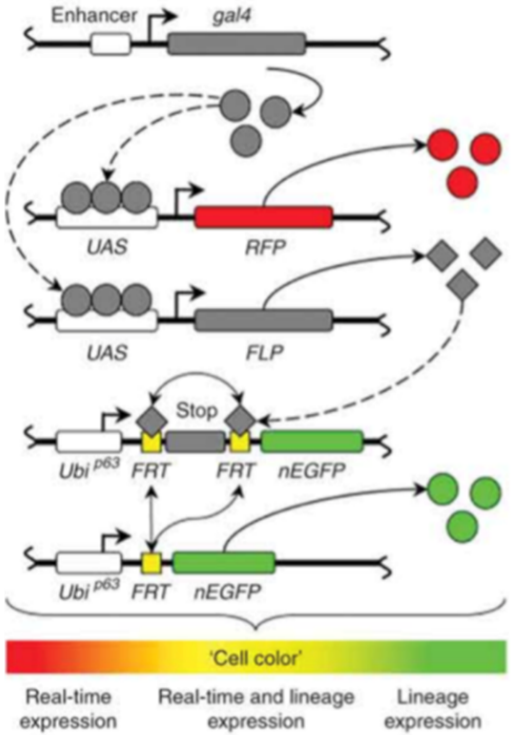
The mammalian orthologs to Taiman are nuclear receptor coactivators 1, 2 and 3 (NCOA1, 2 and 3). It has been shown in mice that NCOA1 and NCOA3 have been heavily implicated in working cooperatively to regulate placental morphogenesis and embryo survival when knockouts of each gene have been studied. When NCOA mice were heterozygous for NCOA1-3, they were either hypofertile, infertile, or lethal depending on the combination of complete knockouts and heterozygotes of the three genes (Chen, Liu, and Xu, 2010). Thus, studying Taiman in this study about JH's relation to reproductive tissue is promising, as its mammalian orthologs have shown to play roles in fertility.

Using GTRACE to Examine Gene Expression with Lineage-Tracing in the AG

Utilizing genetic screens to find drivers of tissue specific expression can be quite helpful. Since using the AG as a model for the human prostate is such a new field, it is not known what genes are activated when JH signaling is upregulated and which genes are contributing to AG growth. A *Drosophila* screening tool called Gal4 technique for real time and clonal expression (GTRACE) is especially useful with respect to identifying the genes that were expressed in the past and currently in the fly. Using the Gal4-UAS system, GTRACE flies express RFP if a gene of interest is active currently. The Gal4-UAS system works by using a tissue specific promoter activating the transcription and translation of Gal4. Gal4 then binds to the DNA sequence UAS, which is placed upstream of a gene of choice. The gene is only transcribed when Gal4 is bound, which allows genetically controlled expression of a certain gene exclusively in a certain tissue. Using the FLP recombinase-FRT construct, it is possible to conduct lineage tracing, as the cells that had past expression of a gene of interest would express GFP and fluoresce green. The FLP recombinase-FRT construct works by producing Gal4 in a tissue of choice by being placed downstream of a tissue-specific promoter. Gal4 then binds to UAS, which activates the transcription and translation of the FLP protein. FLP then binds to FRT sites and removes the stop cassette that is flanked by the FRT sites. The gene of interest is then expressed (Figure

12). This is particularly useful to determine which cells in the AG, such as main cells, secondary cells, or both, are expressing certain genes at the time of dissection, or were previously a gene of interest (Evans et al., 2009). Thus, conducting genetic screens to see which genes were active previously, such as on the day of eclosion, and currently, can provide more insight into the genes that JH signaling affects. Although it is difficult to determine causality with this approach, visualizing which growth factors and cell cycle regulators were active at the day of eclosion, which is when JH signaling is highest, would provide more insight for future research about this pathway.

GTRACE



<u>Figure 12:</u> GTRACE Genetic Technology: Display of GTRACE genetic technology that shows past expression via GFP and current expression via RFP.
(Adapted from Evans et al., 2009)

Specific Goals of this Thesis

Understanding brain-tissue axis control of hormone in *Drosophila* can have especially promising implications. With many conserved mechanisms between humans and *Drosophila*, using model organisms to understand these biological processes is quite important. Especially with the *Drosophila* accessory gland having such similar functions as the human prostate, it is particularly interesting to see how this gland grows or is prevented from growing based on remote hormonal control. Hormones regulate development and many other crucial processes in animals. This all-encompassing nature of hormones and their relation to growth is evolutionarily old and consistent across species, making for an essential component of scientific knowledge.

The relation of hormones to gene expression is another component of this study that is a very prominent topic in science and health today. Hormones that bind to intracellular receptors have the capability to regulate the expression of genes, which can have massive implications within an organism if present at abnormal levels. Knowing what genes are under this control in *Drosophila* can allow for more precise genetic manipulations and understanding the role that certain genes have in the newly studied accessory gland. Thus, broadening the knowledge of tissue growth and ploidy mediated by hormones and the genes involved in this process are major goals of this thesis.

The accessory gland is a promising research focus with respect to all of the previously mentioned scientific concerns. In this study, we first observed the effects on gland size when crucial components of the Juvenile Hormone pathway were knocked down via RNA interference. This was able to show us how growth is affected when a neuronally regulated hormone is impeding in performing its typical functions. We then examined the ploidy of the cells when the JH pathway was interrupted, as ploidy scales with cell size as well as cell biosynthetic capabilities. Finally, since the genes that are triggered by JH binding in the accessory gland are unknown, we selected three growth related proteins, Escargot, Dimmed, and Outspread, to see if there was any past or current expression of the proteins that could be linked to JH signaling during the young fly's life.

Methods

***Drosophila* Stocks:** Canton-S was used as a control. UAS-RNAis were expressed using a Paired-Gal4 driver, which expresses throughout the accessory gland. Measurements were taken from 10 day accessory glands.

Fly stocks (Stock Numbers in parentheses):

- Canton S
- E2F2-i (36674)
- E2F1-i (36126)
- GCE-i (26323)
- Taiman-i (32285)
- Escargot-Gal4 (kyoto line, NP5153)
- Escargot-Gal4 (Bloomington line, 05730B)
- Dimmed-Gal4 (25373)
- Outspread-Gal4 (63503)
- Escargot-Gal4 (BL63437)
- Escargot-Gal4 (BL65632)
- GTRACE (28280)

Fly Rearing

All flies were raised and kept at room temperature (25°C) on Bloomington Cornmeal food. Male virgins were aged for 10 days. For experiments with mated animals: males and females were kept in ratios of 1:1.5 approximately per vial.

Slide preparation:

Flies were anesthetized with CO2 and kept on ice after. Accessory glands were dissected in 1X PBS and were fixed at room temperature in 4% paraformaldehyde and 1X PBS. The samples were

replaced on a rotator while fixing for 30 minutes. After fixing, tissues were then washed in 1x PBS+ 0.1%Triton-X twice for 10 minutes. Tissues were then incubated in DAPI (1μg/ml) and 1x PBS+ 0.1%Triton-X for 10 minutes. DAPI stock kept at 4℃. Samples then washed twice for 10 minutes in 1x PBS+ 0.1%Triton-X.

Slides for imaging were made by pipetting the stained and washed tissues onto the slide with a cut off 20 microliter pipette. Ten microliters of Vectashild were pipetted onto the tissues, and a cover slip was placed on top of the tissues. The cover slip was then sealed by nail polish.

Area Measurements:

Fluorescent images were obtained using a Leica SP5 confocal, Leica SP8 confocal, or Leica DMI6000B epifluorescence system. Images were taken at 10X magnification.

To obtain overall gland size measurements, images were imported into ImageJ. The regions of interest were drawn via touch screen around each lobe of the accessory gland. Area measurements were taken in microns. Measurements were transferred to Prism for analysis.

To obtain gland depth measurements, Z stacks were taken with a step size of 1.48 microns.

DAPI ploidy intensity measurements:

Fluorescent images were obtained using a Leica SP5 confocal, Leica SP8 confocal, or Leica DMI6000B epifluorescence system. Images were taken at 10X magnification.

DAPI intensity quantifications were measured using ImageJ. Measurements were done on binucleated cells from the mid-lobe region of the accessory gland and mononucleated, basally located, diploid muscle nuclei of the ejaculatory duct. Raw Integrated Intensity measurements were used. The average background fluorescence was subtracted from each intensity measurement to get the corrected intensity. Intensity for haploid DNA content was deduced by dividing the average intensity of the ejaculatory duct nuclei in half. Ploidy of the accessory gland nuclei was established using the following binning: 2N (0-2.9), 4N (3.0-6.9), 8N (7-12.9), 16N (13.0-24.9), 32N (>24.9)

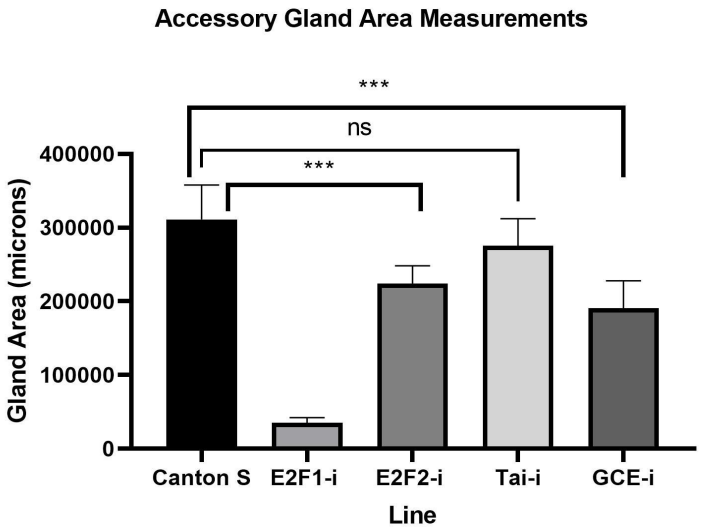
Statistics:

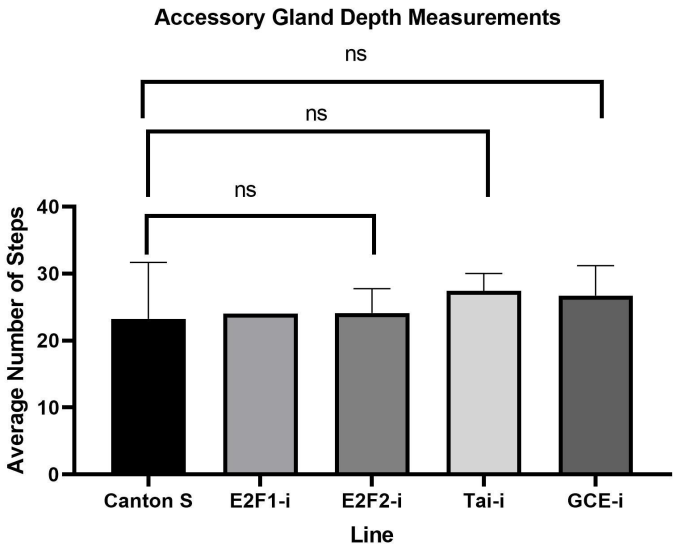
Unpaired t tests were performed since we were interested in comparing the difference between two variables of independent groups. Since the control had no knockdowns and the experimental groups had a knockdown of a gene, it is fair to assume that the groups are independent, and using an unpaired t-test would be most appropriate.

Results:

Juvenile Hormone Affecting Accessory Gland Size

In our studies, we find that knocking down the transcription factor that responds to JH, GCE, with RNA interference results in a decrease in AG size (Fig 1A). The lack of differences in gland depth suggests that the area measurements correlate with volume (Fig. 1B). We also observe that knocking down the cell cycle targets E2F2 and possibly E2F1 also reduces gland growth. This suggests roles for both JH signaling and the cell cycle in promoting adult accessory gland growth. One possibility is that JH signaling may activate cell cycle and growth transcription factors. Reducing levels of the coactivator Taiman did not significantly affect growth, which suggests Taiman may not be involved, or plays a minor role in the pathway. With this being said, a small sample size of Taiman could explain the lack of significance established, as other studies have shown Taiman to be essential in JH signaling. Some uncertainties of this experiment were that RNAi expression levels were not measured, and we do not know the extent of knockdown for each gene, so it is possible that the RNAi in some lines were more effective than others leading to stronger effects on gland size. A second caveat of these experiments is that RNAi lines were compared to Canton-S rather than the driver line alone or with a control RNAi. Future experiments should be done to match the genotype of the control as much as possible.





<u>Figure 13:</u> Area and Depth Measurements of Accessory Glands After Different RNAi: a.) Accessory gland lobes measured with n = 3,2,11, 6, and 11 for Canton S, E2F1-i, E2F2-i Tai-i, and GCE-i respectively. Statistics were only performed on samples with n=3 or more.

b.) Accessory gland depth measured by counting the number of z-sections in projections, where Z-stack size is 1.48 microns, with n = 4, 1, 10, 5, 7 for Canton S, E2F1-i, E2F2-i Tai-i, and GCE-i respectively. The similar depth of glands suggests area measurements correlate with volume.

Juvenile Hormone Affecting Main Cell Ploidy

Many studies have shown that cell and tissue size scales with ploidy. To examine whether the reduced gland size in E2F2 and Gce knockdown reflected reduced DNA content we measured the DNA content of 30 cells per gland using quantification of DAPI fluorescence intensity. We also measured Taimen knockdown flies. Control Canton S 10 day glands exhibit nuclear content of 4-8C with most nuclei containing 8C. By contrast 10 day E2F2, GCE, and Taiman knockdowns exhibited nuclear content of 2-4C with the most nuclei containing 4C (Figure 2). No cells contained a nuclear content of over 4C This suggests that the reduction in DNA content is present in the glands that showed significant reductions in overall volume as well as Taiman. The decrease in ploidy in E2F2 and GCE suggest further support for the premise that the area of the glands decreased because of disruptions in the JH pathway. This data suggests that E2F2, GCE, and Taiman respond to JH and are crucial for cell cycle initiation to increase ploidy. What remains unclear is the specific gene that is activated in this pathway, to connect JH signaling to cell cycle and growth signals, which is why future genetic screens would be useful to discover more about this pathway. Some uncertainties of this experiment were that RNAi expression levels were not measured and we do not know the extent of knockdown for each gene, so it is possible that the RNAi in some lines were more effective than others leading to stronger effects on ploidy. A second caveat of these experiments is that RNAi lines were compared to Canton-S rather than the driver line alone or with a control RNAi. Future experiments should be done to match the genotype of the control as much as possible. Also, the ploidy measurements were measured based on a range of expressions so there could be room for error when using these approximations.

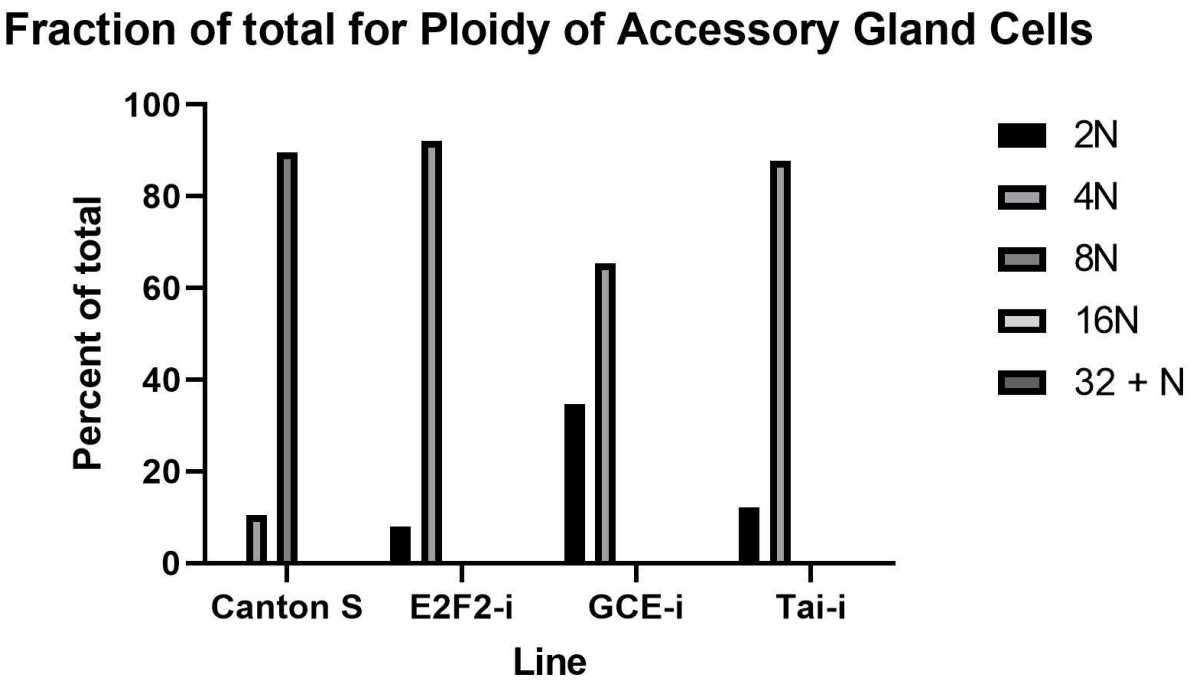


Figure 14: Ploidy of Accessory Gland Cells: DNA content measured by DAPI fluorescence intensity in lines expressing the indicated RNAi. For each genotype, 30 nuclei from 6-9 glands were measured.

Results of Size and Ploidy Measurements

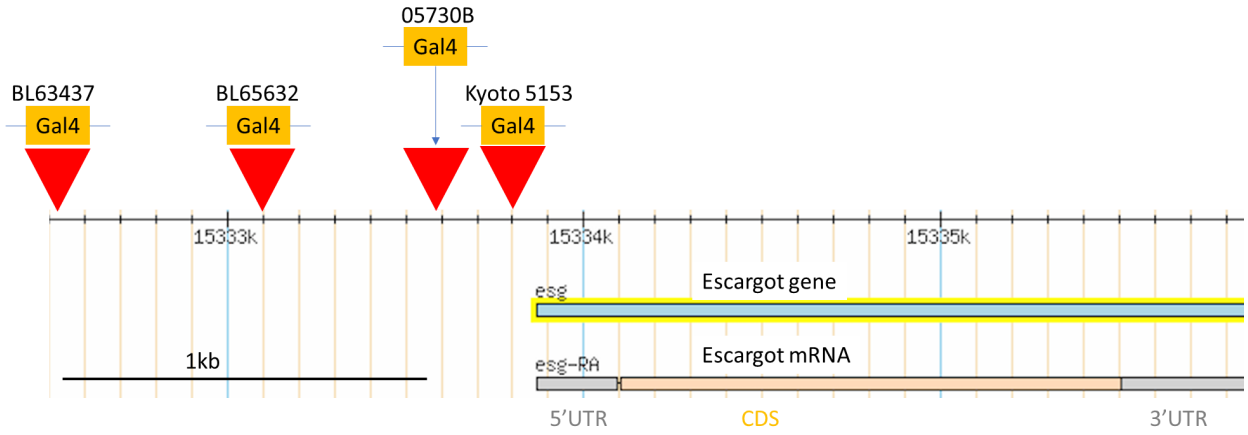
In this section, we were able to explore different knockdowns and measure whether the AG was less able to grow with these knockdowns. E2F2 and GCE showed significant decreases in gland volume when knocked down compared to the control, whereas Taiman did not show decreased gland volume, and E2F1 was unable to be statistically examined due to low sample size. We also were able to connect ploidy to gland size, as no knockdown cells expressed a nuclear content higher than 4C, yet the majority of the controls expressed mostly 8 nuclear contents. This suggests that the knockdowns in E2F2, GCE, and Taiman all affected endocycling and polyploidization, which implicates these proteins in the JH signaling pathway This provides further insight about the mechanisms that AG growth control and polyploidization are achieved and what proteins are involved under the neuronally controlled JH pathway. It is still unclear what specific gene the transcription factors that bind JH, such

as GCE, activate and how this gene then exhibits growth and cell cycle control. Our next experiment explores genetic screens with the goal of finding possible genes that are implicated in this pathway that could clarify what these crucial components to the JH signaling pathway activate to ultimately allow for more specific genetic manipulations.

GTRACE Screenings

After understanding which proteins are implicated in the JH pathway, we wanted to see which genes were active both currently and in the past using GTRACE in 10 day old flies in Escargot and Outspread, and 8 day old flies in Dimmed. Since we dissected flies at either 8 or 10 days of age, we could assume that past expression was very likely around the day of eclosion, where JH signaling is highest. The three genes that were screened were Escargot at four different Gal4 insertions, Dimmed, and Outspread. We decided to explore if Escargot was expressed in the past and currently given that Escargot is highly expressed in stem cells and progenitors, which are implicated with tissue growth and development. Using four different Gal4 insertions was able to provide us with the best insight as to which portion of the DNA was most involved in expressing Escargot, and which site would be best to explore for a potential target of the JH signaling pathway. If past expression of Escargot occurred throughout the tissue, this would suggest that Escargot was expressed around the day of eclosion, possibly in response to JH signaling (Korzelius et al., 2014). We also decided to screen for Dimmed past and current expression. It has been shown that the basic helix loop helix transcription factor Dimmed has been expressed in postmitotic neuroendocrine cells affecting neuronal growth and differentiation in multiple ways. Dimmed has been shown to inhibit apoptosis of several types of neurons that typically are programmed to eventually undergo this process. Dimmed also plays many transcriptional roles at different stages of neuronal development, affecting neuronal differentiation. Lastly, when Dimmed was expressed along with its receptor in Dimmed negative neurons it allowed for cell growth; however, when just its receptor was expressed, cell growth was not initiated. Since the AG is composed of postmitotic endocrine cells, although not neurons, it is plausible that Dimmed plays a role in AG growth and JH signaling given its growth promoting function in neuroendocrine cells (Liu, Luo, and

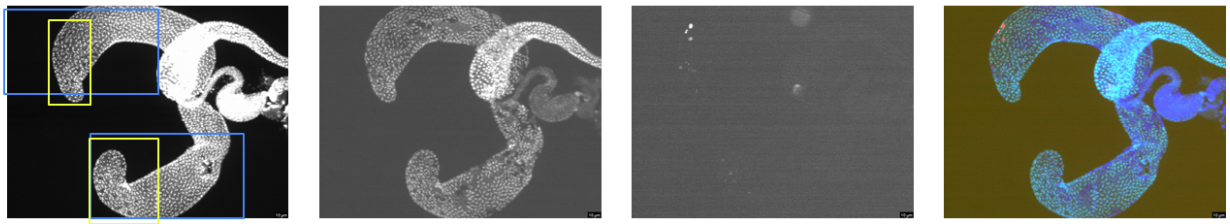
Nässel, 2016). The final gene that we tested was Outspread (Osp). *Drosophila* Osp is an ortholog for two human genes named Trio and F-actin binding protein (Tara) and myosin phosphatase Rho interacting protein (MPRIP). In humans, Tara has been shown to play a role in regulating actin cytoskeletal reorganization and cell cycle regulation. MPRIP in humans has been shown to bind two cell cycle regulators, Polo and CDK2. It is possible that in *Drosophila*, Osp promotes actin cytoskeleton changes during tissue growth which influences cell cycle regulators that alter endocycling such as Cdk2 (Yu et al., 2008).



<u>Figure 15:</u> Different Gal4 Insertions Sites of Escargot Used

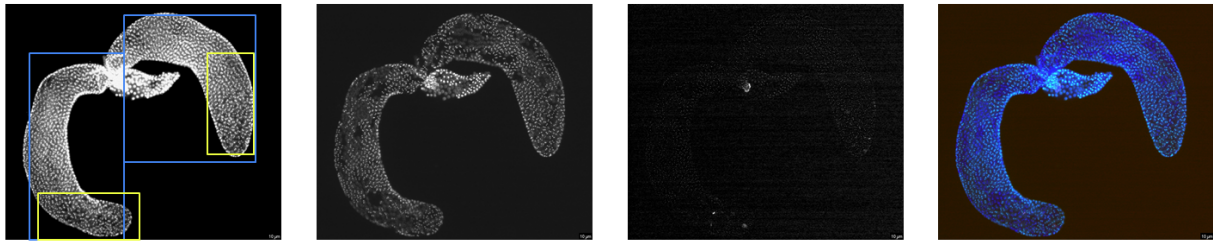
Escargot-Kyoto

DAPI GFP RFP Overlay



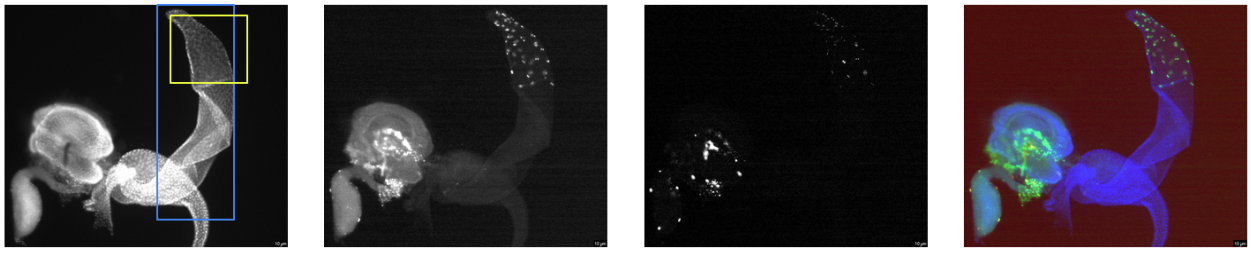
Escargot-Bloomington (05730B)

DAPI GFP RFP Overlay



Dimmed

DAPI GFP RFP Overlay



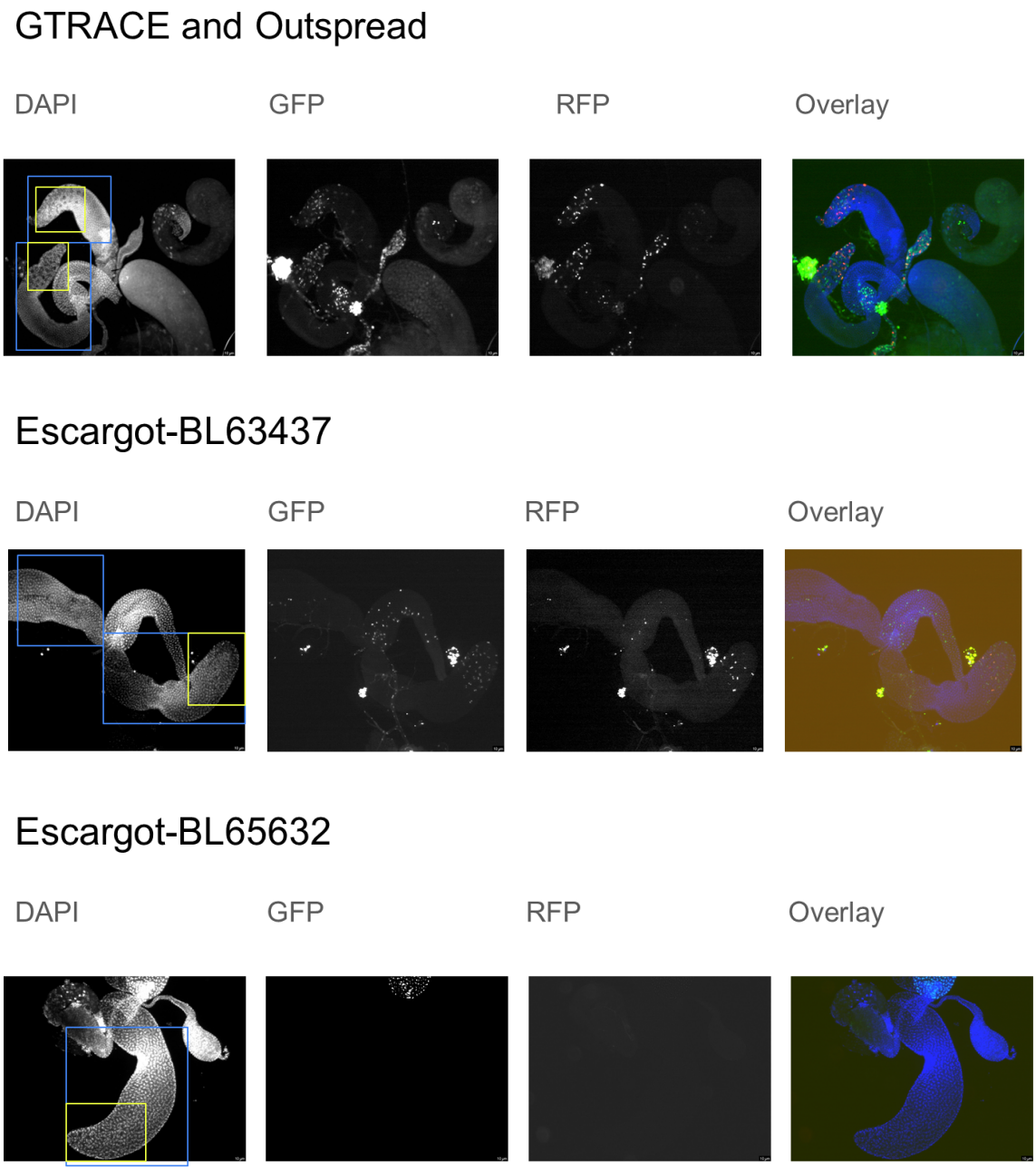


Figure 16: GTRACE Images in Different Channel: DAPI used to show nuclei of AGs. GFP used to show any past expression of the specified gene. RFP used to show any current expression of the specified genes. AG's are labeled with blue boxes, and secondary cells labeled with yellow boxes.

Genotype	GFP Whole Gland	GFP Distal Tip	GFP No Pattern	GFP No Expression	RFP Whole Gland	RFP Distal Tip	RFP No Pattern	RFP No Expression
Escargot-Kyoto	X				Anterior ejaculatory duct papillae	X (occasional secondary cells)		
Escargot-Bloomington 05730B	X				Anterior ejaculatory duct papillae	X(occasional secondary cells)		
Dimmed		X (secondary cells)				X (secondary cells)		
Outspread		X (secondary cells)				X(secondary cells)		
Escargot-BL63437		X (secondary cells)				X (secondary cells)		
Escargot-BL65632				X (in ejaculatory duct)				X

<u>Table 1:</u> Summary of GTRACE Imaging Results

GTRACE Lines Conclusion

When examining the results, the only lines that displayed GFP expression throughout the AG were Escargot-Gal4 Kyoto and Escargot-Gal4 05730B. Escargot is a gene expressed in stem cells and progenitors. Since stem cells and progenitors are highly active at the beginning of a fly's life, these results suggest that Escargot was active at the day of eclosion, which could possibly be linked to the high levels of JH in the AG at the day of eclosion. Although a causal relationship cannot be confirmed, more research should be conducted to see if Escargot is implicated in the JH signaling pathway and is the gene that is activated in response to JH binding to Gce/Met. Dimmed was not expressed in main cells in the past or currently, showing that at the day of eclosion JH signaling did not activate Dimmed expression in the main cells, suggesting that Dimmed is not a downstream target of JH signaling and endocycling. Thus, although Dimmed is expressed in neuroendocrine postmitotic cells, this data does not support that Dimmed was expressed in the postmitotic AG main cells. However, Dimmed does promote a secretory fate, which is corroborated by Dimmed past and current expression in the highly secretory secondary cells. Osp showed past and current expression only in the secondary cells of the AG, suggesting that it was not involved in the JH signaling pathway triggering endocycling in the main

cells. Outspread has other functions than cell growth, such as encoding for a protein that contributes to immune cell formation, which could explain why it was only expressed in secondary cells (Yu et al., 2008).

Discussion

In our research, we were able to implicate JH as a crucial component of AG growth. The JH signaling pathway was also shown to be important, as knockdowns of various steps of the pathway, such as Gce and E2F2, showed decreased gland size. Gce, E2F2, and Taiman knockdowns were also shown to decrease the ploidy of the gland, as Canton-S control flies exhibited mostly 8N ploidy, whereas Gce, E2F2, and Taiman knockdown flies exhibited mostly 4N ploidy. The lower ploidy suggests a role in the smaller gland sizes, as less biosynthesis is occurring with less DNA content, leading to smaller AGs. Decreased ploidy when components of the JH signaling pathway were knocked down also suggests lower levels of endocycling, which also may play a role in decreased AG size. Since JH levels peak after the day of eclosion, this signaling pattern is very likely to be affected early in the fly's life. Understanding the downstream effects of the lower ploidy and gland sizes, such as fertility and gland outputs, when the JH signaling pathway is disturbed poses targets for future research. Conducting fertility assays with Gce, E2F2, and Taiman knockdown flies would certainly be a productive next step in this research, as it would link the JH pathway that is triggered by neurohemal signaling to a decrease in fertility. Since there is very little known about JH effects on male *Drosophila* fertility, this could provide some insight into additional functions of this hormone.

Additionally, more targets of the JH signaling pathway could be examined to see if similar results are produced. E2F1, a cell cycle and growth target, did not have a high enough sample size in the experiments in this thesis to conduct statistics on, so this would certainly be a productive future project to see if it produces a similar effect as the other knockdowns that we tested. Additionally, seeing the effect of Met knockdown would also be a productive next step, as it is the other JH binding protein in addition to Gce. Other possible future targets to examine would be the genes Yorkie and Myc. Yorkie encodes a transcriptional co-activator protein and works together with DNA binding proteins to activate cell cycle and growth genes. Since the AG is known to endocycle, providing a link between JH signaling and endocycling would be an insightful piece of knowledge. Myc encodes a transcription factor that contributes to cell growth, regenerative proliferation, and cell competition. Since it is

known that the AG, and the human prostate, are able to regenerate when damaged, it would be useful to see if these processes are enabled via neurohemal axes, and if *Drosophila* JH plays a role in this unique capability. If Myc was shown to be important to this pathway, JH titer could be measured after damage to the AG, which would suggest that its signaling plays a role in the AG's regeneration.

Another question that remains is what exactly is causing the tissue to endocycle. Our research showed that when the JH pathway is disrupted, ploidy is decreased. It is known that the majority of JH signaling occurs after the day of eclosion, suggesting that endocycling contributing to polyploidization occurs at this time. Since JH receptors, Gce and Met, are basic Helix-Loop-Helix (bHLH) transcription factors, our current hypothesis is that JH signaling regulates cell cycle and growth genes which contributes to its endocycling capabilities. The bHLH transcription factor family has been shown to play vital roles in the specification of tissue type as animals develop (Moore, Barbel, Jan, and Jan, 2000). Due to these proteins' motif of being involved in development, a likely target of Met and Gce would be cell cycle and growth regulators. In female *Locusta migratoria*, it was shown that the Jh-Met/Tai receptor complex bound upstream of Mcm4, Mcm7, Cdc6, Cdk6 and E2f1. When these genes were transcribed, it led to polyploidization in the flies' fat body cells in preparation for Vitellogenin synthesis (Wu, He, Zeng, Zhou, and Zhou, 2020). Conducting a similar experiment in males to see if JH binding to these bHLH proteins would cause downstream genes to induce polyploidization would be quite useful to understand how the AG endocycles and becomes polyploid.

Since we established that E2F2 signaling is important to gland growth, understanding whether it is upstream or downstream of JH mediated transcription would be a useful next step. In order to do this, an experiment could be conducted in which Gce, Met, and Tai are all knocked down, and E2F2 is artificially added to the fly. If the reduced ploidy that would occur due to these knockdowns was rescued, this would implicate E2F2 as a downstream target of the JH pathway. This would also provide more insight into how exactly the AG achieves its endocycling capabilities, as E2F2 is a cell cycle and growth regulator. The same experiment could be conducted with other proteins as well, such as E2F1, chromosome maintenance (Mcm) genes, and cell-division-cycle 6 (Cdc6), cyclin-dependent kinase 6 (Cdk6), as these induced polyploidization in females (Wu, Guo, Yang, He, and Zhou, 2018). Since we

also found some Escargot lines to show past expression throughout the tissue this could also be a Thus, understanding how JH is able to trigger the endocycling ability of the AG remains unknown, and discovering how this is achieved in future experiments would be quite useful.

In our research, we also examined potential growth regulators using GTRACE. Only Escargot-Gal4 Kyoto and Escargot-Gal4 Bloomington (07530B) showed past expression throughout the entire gland. Since main cells are primary what endocycle in the AG, in order for a gene to implicated in contributing to the JH pathway and triggering endocycling, one would expect expression of this gene at the day of eclosion, which only Escargot-Gal4 Kyoto and Escargot-Gal4 Bloomington exhibited. This raises the question of if the two Esg loci are regulated by JH signaling at all, and if they are, then is this regulation direct or indirect. An experiment to see if Esg is implicated in the JH pathway would be to see if there is Esg expression when JH receptors such as Gce and Met are knocked down. If expression is diminished after these receptors are knocked out, this would implicate Esg as a downstream target of JH signaling. To examine whether JH regulates Esg expression directly, understanding more about the genomic region responsible for the expression of Esg would provide useful information. As we had established, the genomic region of interest is between the Kyoto and 07530B insertions. It is known that bHLH transcription factors bind to the E-box (enhancer box, CACGTG) motif of DNA (Inamoto, Sheoran, Popa, Hussain, and Shin, 2020). If an E-box were to be found in the region between Kyoto and 07530B, this would further support a direct regulation of JH signaling to transcription of Escargot, as the proteins that JH bind that regulate transcription would be in the genomic region that Escargot is expressed early in the fly's life. Thus, these experiments would be able to associate or dissociate the linkage between JH signaling and Escargot expression.

To connect the entire pathway, it would also be useful to identify the brain cells responsible for activating this signaling pathway. It is currently known that in females, neurosecretory cells in the brain trigger the CA to release JH, so starting with these cells in the males would be a good option. If these same neurosecretory cells are not what is triggering the CA to synthesize and release JH, it would be worthwhile to look at neuronal activity that corresponds to JH peak levels. To do this, observing what neurons spike in activity around the day of eclosion, are inactive before this day, and become

decreasingly active after the day of eclosion would likely correspond to a select group of neurons with this distinct firing pattern. Then, each of these neurons could be stimulated, and the ones that result in the most JH titer increase would suggest that they are what branch to the CA for JH upregulation. Thus, finding the part of the brain that triggers JH signaling would be useful to understand the entire neurohemal axis.

Infertility is an issue that affects about 15% of couples worldwide, which is about 48.5 million couples. Males have been found to be solely responsible in 20-30% of infertility cases, and overall are partially or fully responsible in 50% of cases (Agarwal, Mulgund, Hamada, and Chyatte, 2015). In a nationwide study done in Finland, infertile men and women had more psychiatric morbidity than those who did not experience infertility. Women with infertility were significantly more likely to experience dysthymia, panic disorder, and anxiety. Men with infertility had poorer qualities of life compared to men without infertility (Klemetti, Raitanen, Sihvo, Saarni, and Koponen, 2010). The mental health aspects of hormonally related disorders, specifically within the reproductive system, can be devastating, with fertility just being one example. Further research on these conditions can be quite helpful to minimize the harmful effects that reproductive disorders have on millions of people.

In summary, this thesis has provided new evidence for how hormone signaling regulates AG growth in *Drosophila*, and these findings may have large implications when studying the human prostate. As discussed, the human orthologs to various components of the JH signaling pathway, such as ARNTLs and NCOAs, have been shown to affect fertility. ARNTLs and other circadian genes such as NPAS2 and RORA have also been implicated in increased risks of aggressive prostate cancer, the most common male cancer in western countries (Wendeu-Foyet et al., 2020). Abnormal circadian rhythms of blood pressure have also been shown to correlate with patients who have benign prostatic hyperplasia, implicating another possible link to circadian genes and prostate abnormalities (Turgut et al., 2008). These are only some examples, as neurohemal signaling can be the source of many human diseases. Continued studying of these pathways in *Drosophila*, and ultimately finding the parallels to human diseases is the ultimate long-term goal of this thesis' findings.

References

Agarwal, A., Mulgund, A., Hamada, A., & Chyatte, M. R. (2015). A unique view on male infertility around the globe. Reproductive biology and endocrinology, 13(1), 1-9.

Ashok, M., Turner, C., & Wilson, T. G. (1998). Insect juvenile hormone resistance gene homology with the bHLH-PAS family of transcriptional regulators. Proceedings of the National Academy of Sciences, 95(6), 2761-2766.

Blondell, R. D., Foster, M. B., & Dave, K. C. (1999). Disorders of puberty. American Family Physician, 60(1), 209-218.

Bownes, M., & Rembold, H. (1987). The titre of juvenile hormone during the pupal and adult stages of the life cycle of *Drosophila* melanogaster. European Journal of Biochemistry, 164(3), 709-712.

Box, A. M., Church, S. J., Hayes, D., Nandakumar, S., Taichman, R. S., & Buttitta, L. (2019). Endocycles support tissue growth and regeneration of the adult *Drosophila* accessory gland. BioRxiv, 719013. https://doi.org/10.1101/719013

Calvi, B. R. (2013). Making big cells: one size does not fit all. Proceedings of the National Academy of Sciences, 110(24), 9621-9622.

Carson III, C., & Rittmaster, R. (2003). The role of dihydrotestosterone in benign prostatic hyperplasia. Urology, 61(4), 2-7.

Charles, J. P., Iwema, T., Epa, V. C., Takaki, K., Rynes, J., & Jindra, M. (2011). Ligand-binding properties of a juvenile hormone receptor, Methoprene-tolerant. Proceedings of the National Academy of Sciences, 108(52), 21128-21133.

Chen, X., Liu, Z., & Xu, J. (2010). The cooperative function of nuclear receptor coactivator 1 (NCOA1) and NCOA3 in placental development and embryo survival. Molecular endocrinology, 24(10), 1917-1934.

Ciechanowska, M., Łapot, M., Mateusiak, K., & Przekop, F. (2010). Neuroendocrine regulation of GnRH release and expression of GnRH and GnRH receptor genes in the hypothalamus-pituitary unit in different physiological states. Reproductive biology, 10(2), 85-124.

Evans, C. J., Olson, J. M., Ngo, K. T., Kim, E., Lee, N. E., Kuoy, E., ... & Banerjee, U. (2009). G-TRACE: rapid Gal4-based cell lineage analysis in *Drosophila*. Nature methods, 6(8), 603-605.

Fox, D. T., Soltis, D. E., Soltis, P. S., Ashman, T. L., & Van de Peer, Y. (2020). Polyploidy: a biological force from cells to ecosystems. Trends in cell biology, 30(9), 688-694.

Goberdhan, D. C., & Wilson, C. (2003). The functions of insulin signaling: size isn't everything, even in *Drosophila*. Differentiation, 71(7), 375-397.

Hopkins B.R., Sepil I, Bonham S, Miller T, Charles P.D., Fischer R, Kessler B.M., Wilson C, Wigby S. BMP signaling inhibition in *Drosophila* secondary cells remodels the seminal proteome and self and rival ejaculate functions. Proc Natl Acad Sci U S A. 2019 Dec 3;116(49):24719-24728.

Inamoto, I., Sheoran, I., Popa, S. C., Hussain, M., & Shin, J. A. (2020). Combining Rational Design and Continuous Evolution on Minimalist Proteins That Target the E-box DNA Site. ACS Chemical Biology, 16(1), 35-44.

Jindra, M., Uhlirova, M., Charles, J. P., Smykal, V., & Hill, R. J. (2015). Genetic evidence for function of the bHLH-PAS protein Gce/Met as a juvenile hormone receptor. PLoS genetics, 11(7), e1005394.

Klemetti, R., Raitanen, J., Sihvo, S., Saarni, S., & Koponen, P. (2010). Infertility, mental disorders and well-being–a nationwide survey. Acta obstetricia et gynecologica Scandinavica, 89(5), 677-682.

Korzelius, J., Naumann, S. K., Loza-Coll, M. A., Chan, J. S., Dutta, D., Oberheim, J., ... & Edgar, B. A. (2014). Escargot maintains stemness and suppresses differentiation in *Drosophila* intestinal stem cells. The EMBO journal, 33(24), 2967-2982.

Kovanen, L., Saarikoski, S. T., Aromaa, A., Lönnqvist, J., & Partonen, T. (2010). ARNTL (BMAL1) and NPAS2 gene variants contribute to fertility and seasonality. PLoS One, 5(4), e10007.

Leiblich, A., Hellberg, J. E., Sekar, A., Gandy, C., Mendes, C. C., Redhai, S., ... & Wilson, C. (2019). Mating induces switch from hormone-dependent to hormone-independent steroid receptor–mediated growth in *Drosophila* secondary cells. PLoS Biology, 17(10), e3000145.

Leiblich, A., Marsden, L., Gandy, C., Corrigan, L., Jenkins, R., Hamdy, F., & Wilson, C. (2012). Bone morphogenetic protein-and mating-dependent secretory cell growth and migration in the *Drosophila* accessory gland. Proceedings of the National Academy of Sciences, 109(47), 19292-19297.

Li, K., Jia, Q. Q., & Li, S. (2019). Juvenile hormone signaling–a mini review. Insect science, 26(4), 600-606.

Liu, Y., Luo, J., & Nässel, D. R. (2016). The *Drosophila* transcription factor Dimmed affects neuronal growth and differentiation in multiple ways depending on neuron type and developmental stage. Frontiers in molecular neuroscience, 9, 97.

Mihm, M., Gangooly, S., & Muttukrishna, S. (2011). The normal menstrual cycle in women. Animal reproduction science, 124(3-4), 229-236.

Miura, K., Oda, M., Makita, S., & Chinzei, Y. (2005). Characterization of the *Drosophila* Methoprene-tolerant gene product: Juvenile hormone binding and ligand-dependent gene regulation. The FEBS journal, 272(5), 1169-1178.

Moore, A. W., Barbel, S., Jan, L. Y., & Jan, Y. N. (2000). A genomewide survey of basic helix–loop–helix factors in *Drosophila*. Proceedings of the National Academy of Sciences, 97(19), 10436-10441.

Nässel, D. R., & Zandawala, M. (2020). Hormonal axes in *Drosophila*: regulation of hormone release and multiplicity of actions. Cell and tissue research, 382(2), 233-266.

Nedresky, D., & Singh, G. (2019). Physiology, luteinizing hormone.

Orr-Weaver, T. L. (2015). When bigger is better: the role of polyploidy in organogenesis. Trends in Genetics, 31(6), 307-315.

Plant, T. M. (2015). 60 YEARS OF NEUROENDOCRINOLOGY: The hypothalamo-pituitary–gonadal axis. Journal of endocrinology, 226(2), T41-T54.

Taniguchi, K., Kokuryo, A., Imano, T., Minami, R., Nakagoshi, H., & Adachi-Yamada, T. (2014). Isoform-specific functions of Mud/NuMA mediate binucleation of *Drosophila* male accessory gland cells. BMC developmental biology, 14(1), 1-18.

Thorpe, A., & Neal, D. (2003). Benign prostatic hyperplasia. The Lancet, 361(9366), 1359-1367.

Tobe, S. S., & Stay, B. (1985). Structure and regulation of the corpus allatum. In Advances in Insect Physiology (Vol. 18, pp. 305-432). Academic Press.

Turgut, F., Bayrak, O., Kanbay, M., Ozkara, A., Uz, E., Bavbek, N., ... & Akcay, A. (2008). Circadian rhythm of blood pressure in patients with benign prostatic hyperplasia. Scandinavian journal of urology and nephrology, 42(1), 47-52.

Wendeu-Foyet, M. G., Cénée, S., Koudou, Y., Trétarre, B., Rébillard, X., Cancel-Tassin, G., ... & Menegaux, F. (2020). Circadian genes polymorphisms, night work and prostate cancer risk: Findings from the EPICAP study. International Journal of Cancer, 147(11), 3119-3129.

Wilson, C., Leiblich, A., Goberdhan, D. C. I., & Hamdy, F. (2017). The *Drosophila* accessory gland as a model for prostate cancer and other pathologies. Current topics in developmental biology, 121, 339-375.

Wu, Z., Guo, W., Yang, L., He, Q., & Zhou, S. (2018). Juvenile hormone promotes locust fat body cell polyploidization and vitellogenesis by activating the transcription of Cdk6 and E2f1. Insect biochemistry and molecular biology, 102, 1-10.

Wu, Z., He, Q., Zeng, B., Zhou, H., & Zhou, S. (2020). Juvenile hormone acts through FoxO to promote Cdc2 and Orc5 transcription for polyploidy-dependent vitellogenesis. Development, 147(18), dev188813.

Verlinden, H., Gijbels, M., Lismont, E., Lenaerts, C., Broeck, J. V., & Marchal, E. (2015). The pleiotropic allatoregulatory neuropeptides and their receptors: A mini-review. Journal of Insect Physiology, 80, 2-14.

Weaver, R. J., & Audsley, N. (2009). Neuropeptide regulators of juvenile hormone synthesis: structures, functions, distribution, and unanswered questions. Annals of the New York Academy of Sciences, 1163(1), 316-329.

Wilson, T. G., & Fabian, J. (1986). A *Drosophila* melanogaster mutant resistant to a chemical analog of juvenile hormone. Developmental biology, 118(1), 190-201.

Yamaguchi, M., & Yoshida, H. (2018). *Drosophila* as a model organism. *Drosophila* Models for Human Diseases, 1-10.

Yamamoto, R., Bai, H., Dolezal, A. G., Amdam, G., & Tatar, M. (2013). Juvenile hormone regulation of *Drosophila* aging. BMC biology, 11(1), 1-14.