INVESTIGATING THE MECHANISMS BY WHICH TWO DROSOPHILA MELANOGASTER SEMINAL FLUID PROTEINS, SEX PEPTIDE AND OVULIN, ELICIT POST-MATING RESPONSES IN THE FEMALE

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INVESTIGATING THE MECHANISMS BY WHICH TWO DROSOPHILA MELANOGASTER SEMINAL FLUID PROTEINS, SEX PEPTIDE AND OVULIN, ELICIT POST-MATING RESPONSES IN THE FEMALE

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The actions of seminal fluid proteins (SFPs), which are part of the ejaculate that males transfer to females upon mating, are important for reproductive success in both sexes in a wide range of taxa. Research that has focused on identifying these proteins and studying their functions in the female has provided invaluable insight into understanding reproduction across species. For the first part of my thesis, I investigated the role of the SFP, sex peptide (SP), in the post-mating change in female nutrition and digestion. Previous work has shown that SP increases female food intake after mating and slows the rate of intestinal transit, thereby causing her to produce more concentrated excreta. SP can have both transient and long-term effects on mated females; the latter occur because of the peptide's binding to, and slow release from, sperm in the female. I used timed measures of excretion by female flies that had mated to males mutant in SP or in its regulators, to test the duration of SP's effect on excretion. I found that SP's

effect on excretion persists for at least ~1 week after mating, and that this persistence requires that SP bind to and be released from sperm.

Interactions between the sexes continue at the molecular level in the female beyond the conclusion of mating. Thus, SFPs need to interact with female proteins. Although the suite of *Drosophila* SFPs are known, a female receptor for only one *Drosophila* SFP has been identified (the sex peptide receptor). For the second part of my thesis, I focused on identifying a female receptor for the SFP, ovulin, which increases ovulation within the first 24 hours after mating by stimulating octopaminergic signaling in the female nervous system. By screening for *Drosophila* receptors that exhibit a correlated rate of evolution with ovulin, we identified 19 ovulin receptor candidates and upon further phenotypic analysis, I was able to narrow it down to one candidate, CG15744. Several assays have been initiated to confirm the ovulin-CG15744 interaction, including a cell culture-based calcium assay, split-ubiquitin yeast two hybrid, and a Tango reporter assay.

BIOGRAPHICAL SKETCH

Jennifer McGlaughon received a B.S. in Biology from Baldwin Wallace University, Berea, Ohio in 2007 and a M.S. in Molecular Biology and Biotechnology from East Carolina University, Greenville, North Carolina in 2010.

For Henry.

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CHAPTER 1

INTRODUCTION[1]

Reproduction involves not simply an interaction between individuals, but also an interaction between cells and molecules belonging to those two different organisms of the same species. Both individuals ultimately benefit from a successful reproductive interaction, because they will transmit their genes to the next generation. Although the gametes contributed by each individual will ultimately fuse to form the embryo for the next generation, production and development of this gamete is facilitated by actions and products made by tissues in the male and female reproductive tract. Much is known about those tissues, and their products, in the genetic model system, the fruit fly *Drosophila melanogaster*, which is the focus of this chapter. Male and female reproductive tracts secrete components that contribute to a complex milieu that regulates and supports essential reproductive processes such as oocyte and sperm transport, activation and maturation, fertilization, embryo development and embryo transport (Leese, 1988; Hunter, 1998; Bloch Qazi et al., 2003; Avila et al., 2010; Aviles et al., 2010; Heifetz and Rivlin, 2010; Schnakenberg et al., 2012). Male secreted components in

[1] This chapter is part of a textbook review: Avila FW, Sánchez-López JA, McGlaughon JL, Raman S, Wolfner MF, Heifetz Y (2016) Nature and functions of glands and ducts in the Drosophila reproductive tract. Extracellular Composite Matrices in Arthropods; Springer Publishing. The review was edited to include only the part to which I contributed.

animal seminal plasma include proteins and peptides, lipids, hormones, sugars, small-molecules, immune regulators, and vesicles derived from male reproductive tissues (Poiani, 2006; Avila et al., 2011; Aalberts et al., 2014; Corrigan et al., 2014; Suarez and Wolfner, 2016). Interestingly, although the specific molecules and extracellular vesicles that are present in seminal plasma differ across organisms, and even within genus *Drosophila*, there are commonalities in their types. Female animals' reproductive tract secretions include molecules in similar classes to the male ones just mentioned (i.e. proteins and peptide, lipids, hormones, sugars) and also include components of the immune response (e.g. antimicrobial peptides) that form the first line of defense against pathogenic invasion (Leese, 1988; Wira et al., 2011; Mondejar et al., 2012). Sites and/or organs that store sperm provide molecules that support sperm maintenance and may enable sperm modification (maturation and activation) to facilitate fertilization. In this chapter we review what reproductive tissues and their secretions do, and why their products are so important for reproduction. Genetic tools in *D. melanogaster* have allowed a deep understanding of these molecules' and tissues' functions.

Although both sexes benefit from successful reproduction, the strategies that promote reproductive success differ between males and females. In the simplest terms, the male, who produces large quantities of "lower cost" (small) gametes, benefits by mating with as many females as possible. Further, males will stimulate

the female's reproductive capacity as much as possible and expend energy in defending his reproductive investment from possible competition. The female, in contrast, produces a smaller number of eggs, each of which requires a significant investment. Females are thought to benefit by being "choosier" so that the best male's sperm fertilize her eggs. These differences in strategies and in reproductive physiology/needs result in both beneficial and apparent antagonistic interactions between male secretions and the female. We review these beneficial and antagonistic interactions, briefly, here.

Beneficial Interactions

The mated female insect is quite different from her unmated (virgin) self, both physiologically and behaviorally. Perhaps most dramatic, a mated female produces and lays eggs at a much higher rate than a comparable unmated female (~50 eggs/day instead of ~2/day in our lab-strain of *D. melanogaster*, respectively; in some insects unmated females lay no eggs at all (Avila et al., 2011). This increase in egg production, and provisioning all those eggs, requires resources that the female must obtain not only by eating more, but also by extracting maximal nutrition from the food she consumes (Carvalho et al., 2006; Apger-McGlaughon and Wolfner, 2013; Reiff et al., 2015). Such resource allocation for egg production potentially detracts from resources needed for the female's viability and health

(Rose and Bradley, 1998; Stearns, 2000; Schwenke et al., 2016). The mated female must also find places to oviposit her eggs, requiring movement, searching, and less sleep (Isaac et al., 2010). Oviposition also potentially makes the female more vulnerable to predators. One could imagine that it would be advantageous for a *D. melanogaster* female to use the substances males' transfer during mating as a "switch", changing her from a virgin state of metabolism to the revved up state needed for egg production. In other words, in this model, the unmated female's physiology is poised to shift to what is needed for high levels of egg production, but remains in this soma-focused physiology until a signal from the male indicates to her that she contains sperm. At this point it is advantageous for her to switch to a higher level of egg production so that her eggs and the sperm she received can rapidly produce large numbers of progeny. While in physiological terms, this seems most beneficial to the female, it is also to the male's benefit to provide a signal that turns up egg production in females. Mating-induced egg production means that the male's progeny numbers are not limited by the female's basal (virgin) level of egg production. In parallel, male-induction of egg production insures females will not have exhausted resources on egg production prior to mating and are thus able to produce large numbers of progeny after each mating.

There are several examples where it has been shown that the male acts as a switch to turn on a pre-existing pathway in the female. Initial evidence came from

transcriptomics: transcriptome changes shortly after mating were very small in magnitude, suggesting that everything needed in the first few hours after mating is already expressed in females (McGraw et al., 2004; McGraw et al., 2008). Consistent with this view of the "poised" female are data on vesicle release and reuptake; the female already contains vesicles with peptide in them, ready-for-release upon mating (Heifetz and Wolfner, 2004). Later work of Heifetz et al., (2014) showed that mating changes neuromodulator levels and release, at characteristic times in each part of the female's reproductive tract (providing a way to coordinate the post-mating physiology along the tract). Finally, recent micro-CT scanning studies have shown that mating results in movements and shape changes of reproductive organs *in situ*, that initiate the coordinated release and passage of oocytes (Mattei et al., 2015).

A major class of chemical signals that the male provides are proteins/peptides in his seminal fluid. As the action of specific seminal fluid proteins (SFPs) is becoming understood, we are beginning to see how males change the female physiological state. For example, the SFP sex peptide (SP) increases juvenile hormone (JH) production in females (Moshitzky et al., 1996; Bontonou et al., 2015), a molecule essential for the post-mating increase in oogenesis (Dubrovsky et al., 2002). Since unmated females already have begun oogenesis, JH likely facilitates further maturation of these oocytes (Soller et al.,

1999), as well initiating the production of new ones. Increased JH levels also cause a remodeling of the female's gut (Reiff et al., 2015), presumably facilitating better digestion and nutrient absorption. In another example, the SFP Acp36DE is necessary for the conformational changes in the uterus of the mated female (Adams and Wolfner, 2007, Avila and Wolfner, 2009, Mattei et al., 2015), changes necessary for sperm to move into storage and for exerting tension on the oviduct to facilitate ovulation (Mattei et al., 2015). Another SFP, ovulin, increases octopaminergic signaling by increasing the number of synapses between neurons that innervate the oviduct and the associated oviduct musculature, allowing for the relaxation of the oviduct and opening its lumen to permit ovulation (Rubinstein and Wolfner, 2013). Extending beyond octopamine, SFP receipt (and mating) alters the production and/or release of different neuromodulators at different places and times along the female reproductive tract (Heifetz et al., 2014), resulting in unique combinations of neuromodulators that may help coordinate the various reproductive tract functions. Here too, the pathways are poised in the female but are turned on by the male – thus saving the female from having to run these pathways at a high level until she has mated, benefitting both her and her mate by allowing the pathways to turn up very quickly upon mating – as though a switch has been flipped on.

Antagonistic Interactions

As previously mentioned, mating is not purely a cooperative venture between the sexes at the molecular (or other) levels. Males produce large numbers of lower-cost gametes and thus benefit most from mating as many times as possible. But within a population, multiple-mating by males introduces the likelihood of competition from sperm by other males for fertilization opportunities. For females, with fewer costlier gametes, multiple mating is less beneficial; females benefit more by devoting their resources to the production of progeny from better males (although they can sometimes receive beneficial resources during mating (Markow et al., 2001). These differences in strategies can result in situations where the interest of the sexes differ, causing antagonistic effects on females, from male molecules or organs. The simplest way to imagine this is to consider egg production. As noted above, it is advantageous for both the male and female that SFPs increase egg production in females. However one could imagine that a level of egg production ideal for the male's rapid progeny production might be disadvantageous for the female, requiring her to devote more resources to egg production than might be beneficial for her survival. Thus there is a constant tension between the needs and strategies of the two sexes. This in turn can drive an evolutionary "arms race" (e.g. see Rice, 1996). If a male produces a molecule that exerts an effect beneficial for him, it will be selected for. However, if the effects of

this molecule are not beneficial for the female, females will be selected that resist the molecule's effects. [For example, if the JH induction caused by SP is at a level that is disadvantageous for females, one could imagine that selection would then favor females whose circulatory system contained higher proteolytic activity against the SP (for example by having greater amounts of specific proteases, or proteases with higher catalytic activity or stability). Or, female receptors could evolve that bind a given SFP less strongly; more below.] This in turn will select for males with forms of this SFP (or other molecules) that can overcome the female's resistance mechanisms.

There are several examples of the likelihood of such a conflict between the interests of male and female *D. melanogaster*. One example is that some SFPs decrease female lifespan (Chapman et al., 1995, Lung et al., 2002, Wigby and Chapman, 2005). The effect has likely been tuned in ways like those suggested above, so that it takes multiple matings to see a small effect, but it is real. The SP is essential for this effect on females; perhaps its effect can be understood in terms of its altering the egg production, immunity, food ingestion, and hormone balance in the female. Like SP (Wigby and Chapman, 2005), several other SFPs have been shown to be toxic to flies when ectopic amounts are present in their hemolymph (Lung et al., 2002, Mueller et al., 2007), suggesting that there may be other contributions to the decreased longevity.

In another example, the male's aedeagus punctures the vaginal intima of the female (Kamimura, 2010, Mattei et al., 2015), potentially providing a rapid way to introduce his SFPs into her circulation (Lung and Wolfner, 1999), but also causing wounding of the female (Mattei et al., 2015). In addition, this injury has the potential to introduce microbes into the female – perhaps also explaining why female flies induce expression of antimicrobial peptide genes after mating (Peng et al., 2005a; Domanitskaya et al., 2007), although their systemic immunity is not increased after mating (Fedorka et al., 2007, Short et al., 2012), perhaps as a consequence of a resource-allocation tradeoff between immunity and reproduction (see Schwenke et al., 2016 for further discussion).

In a third example of potentially antagonistic interactions, one male effect on females that likely evolved as part of his arsenal against sperm competition is that his SP reduces female receptivity to re-mating (Chapman et al., 2003). Therefore, a mated female is less likely to mate, and the male's sperm thus less likely to encounter sperm competition. While this is advantageous for the male, it is arguably less so for the female. Although decreased re-mating could potentially protect her from longevity-decreasing effects of mating/SFPs, there are some negative consequences to the female: by reducing her re-mating-receptivity, the male is decreasing the diversity of male genetic contributions to the female's progeny. Females benefit by some ability to re-mate so that they can "choose" the

best male; by decreasing her receptivity to re-mating, a male's SFPs decrease this potentially beneficial behavior by females. This conflict between interests of the male and female may also contribute to the arms race described above.

Finally, sequence examination of SFPs has shown that a remarkably large percent of them (~20%) show features of rapid evolution, consistent with the arms race described above (Swanson et al., 2001, Wagstaff and Begun, 2007, Wong et al., 2008a). Interestingly, although the primary amino acid sequences appear to be evolving rapidly, the classes of SFP that are present are highly conserved – suggesting that the same functions are needed, but that different molecules with those activities are co-opted as females become resistant or other needs drive it. It would be useful to see if the female receptors/interactors of SFPs also evolve rapidly – in step with their ligands. Unfortunately at present, only one SFP receptor is known (SPR, the receptor for SP) (Yapici et al., 2008) and this receptor has conserved non-reproductive ligands (MIPs) (Kim et al., 2010, Poels et al., 2010), so they may constrain its evolution. An SFP-specific receptor is needed to answer this question, assuming that SFPs don't just co-opt receptors that were already there.

The Male Accessory Gland

The accessory gland of the *Drosophila* male reproductive tract produces the

majority of studied seminal fluid proteins transferred to females during mating. These proteins elicit most of the behavioral and physical changes in mated female that have been described – increases in egg-laying, ovulation and feeding behavior, decreases in receptivity to re-mating, in siesta sleep and in the rate of intestinal transit, as well as formation of a mating plug, induction of immune responses, and regulation of sperm storage and sperm competition (Gillott, 2003; Avila et al., 2011). The accessory gland plays a crucial role in the fertility of *Drosophila* males given that ablation of the tissue results in sterility (Xue and Noll, 2000).

The accessory gland is a two-lobed structure that branches from the anterior ejaculatory duct. Late in development, mesodermal cells that express the fibroblast growth factor (FGF) receptor, Breathless (Btl), in the male genital disc will become epithelial cells that develop into the accessory gland (Ahmad and Baker, 2002). The homeodomain transcription factor, Paired (prd), is required to promote the proliferation of the cells (Xue and Noll, 2002). Each lobe of the accessory gland is made up of ~1000 secretory cells arranged in a monolayer epithelium. There are two types of secretory cells: “main cells” and “secondary cells” (Bairati, 1968, Bertram et al., 1992, Gligorov et al., 2013). Flat, hexagonally-shaped main cells make up approximately 96% of the accessory gland. Secondary cells, which are large, spherical and filled with vacuoles, make up the remaining 4%; they are found at the distal tip of the accessory gland lobes, distributed among the main

cells. Both main cells and secondary cells of the accessory gland are binucleate, a result of progressing through mitosis without cytokinesis. This is regulated by the *Drosophila* microtubule binding protein, Mud (Taniguchi et al., 2014).

Proteins are secreted from the monolayer of secretory cells into the lumen of the accessory gland. The monolayer of secretory cells is further encased in a muscle sheath that likely constricts, facilitating the movement of the secreted proteins into the ejaculatory duct where they mix with secreted proteins from the ejaculatory duct and sperm, and are subsequently transferred to the female during copulation (Norville et al., 2010). In addition to its role in early cell proliferation of the accessory gland, prd is also required later in development for the differentiation and maturation of the accessory gland (Xue and Noll, 2002).

It was suggested that the main cells of the *Drosophila* accessory gland use merocrine secretion, whereas secondary cells secrete by holocrine mechanisms (Perotti, 1971, Chen, 1984). Merocrine secretion occurs when a cell uses exocytosis to release its secreted products via secretory vesicles, whereas holocrine secretion involves the rupture of the plasma membrane and the release of the secreted product, destroying the cell. It is unclear whether secondary cells destroyed in this process are replenished by the conversion of a main cell or by proliferation of other secondary cells. A study by Rylett et al., (2007) provides possible evidence for the holocrine secretion of secondary cells. The authors

demonstrated by in situ hybridization that the RNA of angiotensin I-converting enzyme (ANCE), a secondary cell secreted protein, was found not only in the secondary cells but also abundant in the accessory gland lumen. Moreover, the appearance of vesicles transferred from accessory glands, and of apparent secondary cells delaminated and transferred during mating (Leiblich et al., 2012, Corrigan et al., 2014), also supports the idea of holocrine secretion by those cells.

Given that the main cells make up 96% of the accessory gland, the products of these cells have been more extensively studied. Genetic disruption of the accessory gland main cells determined that these cells are responsible for the post-mating response (Kalb et al., 1993). About ~200 proteins and peptides have been reported in *D. melanogaster* seminal fluid, most of which are derived from accessory glands (Findlay et al., 2008; Yamamoto and Takemori, 2010; Findlay et al., 2014). One class of such molecules are peptide hormones or proteins that appear to act as precursors to peptide hormones. The best known among these is the 36 amino acid "sex peptide" (SP; Aigaki et al., 1991) and the 264 amino acid ovulin (Monsma and Wolfner, 1988). Both of these are found in *D. melanogaster* and closely related *Drosophila* species, but neither is detectable outside the genus *Drosophila*. SP has been shown to mediate multiple post-mating events in females: the decreases in sexual receptivity (Chapman et al., 2003), siesta sleep (Isaac et al., 2010) and longevity (Wigby and Chapman, 2005), the increases in feeding

(Carvalho et al., 2006), egg production (Chapman et al., 2003) and juvenile hormone levels (Moshitzky et al., 1996), the observed digestive changes (Apger-McGlaughon and Wolfner, 2013), a requirement for sperm release from storage (Avila et al., 2010), and the induction of antimicrobial peptide genes (Peng et al., 2005a, Domanitskaya et al., 2007). SP's effects on behavior, egg production and sperm release are due to SP's C-terminal region acting through a G-protein coupled receptor (SPR, Yapici et al., 2008) in a subset of neurons innervating the female reproductive tract that co-express *fruitless*, *doublesex*, and *pickpocket* (Yapici et al., 2008; Hasemeyer et al., 2009; Yang et al., 2009; Avila et al., 2015). To mediate the release of sperm from storage, SP acts through SPR in *pickpocket* neurons and the spermathecal secretory cells (Avila et al., 2015). The sex peptide's effects persist long after mating, because once inside the female SP is bound to, and retained on stored sperm (Peng et al., 2005b), where a network of seminal proteins is necessary to catalyze this binding (Ravi Ram and Wolfner, 2009, LaFlamme et al., 2012). The active C-terminal region of the SP is slowly cleaved from sperm, releasing this active region with time. The effects of SP on JH levels are transient, and involve its N-terminal region (Peng et al., 2005b).

Ovulin is a prohormone that upon transfer to the female stimulates a short-term (<24 hour) increase in ovulation (Herndon and Wolfner, 1995; Heifetz et al., 2000; Heifetz et al., 2005). Ovulin acts to increase ovulation by increasing

octopaminergic signaling in the reproductive tract (Rubinstein and Wolfner, 2013). The increased octopaminergic signaling is thought to relax the muscles that surround the oviduct, unlooping this duct and opening its lumen to accept the ovulated egg. Interestingly, although ovulin itself is transient, its effects include increasing the number of synaptic boutons made by octopaminergic neurons on the muscles around the oviduct. This may allow the oviduct musculature to remain relaxed long after ovulin is gone, a feature that may contribute to the mechanical regulation of subsequent ovulation by the female (Mattei et al., 2015), as described below. Ovulin's site of action in the female is currently unknown. The majority of transferred ovulin localizes to the upper oviduct and base of the ovaries; there it undergoes proteolytic cleavage that is thought to activate it (Monsma and Wolfner, 1988; Herndon and Wolfner, 1995; Park and Wolfner, 1995; Heifetz et al., 2005). Approximately 10% of the transferred ovulin enters the female's hemolymph where it remains uncleaved; from this location it could be in position to indirectly stimulate ovulation via the neuroendocrine system (Lung and Wolfner, 1999).

Several other SFPs made in the accessory gland have been associated with functions in the mated female. Accessory gland SFPs, in aggregate, induce a series of morphological changes of the mated female uterus. In virgin females, the uterus exists in a tightly closed conformation. During mating, a series of morphological changes 'open' the uterus (Adams and Wolfner, 2007, Mattei et al., 2015). These

changes have been hypothesized to move the sperm mass towards the female sperm stage organs (Adams and Wolfner, 2007). The accessory gland SFP, Acp36DE, is required for the completion of these changes: the uteri of females that do not receive Acp36DE only partially open (Avila and Wolfner, 2009, Mattei et al., 2015) resulting in a significant reduction of sperm being stored by the female (Neubaum and Wolfner, 1999; Bloch Qazi and Wolfner, 2003; Avila and Wolfner, 2009). *D. melanogaster* females will sometimes store sperm even in the absence of accessory gland SFPs, albeit in significantly reduced numbers (Xue and Noll, 2000). However, these sperm will not be used to fertilize eggs (Xue and Noll, 2000), highlighting the importance of accessory gland proteins in female sperm management. The predicted lectin, Acp29AB, which functions in sperm competition is also required for proper sperm storage, as females that do not receive this SFP during mating cannot maintain sperm in storage, leading to a reduction in fertility (Wong et al., 2008b). Finally, the previously mentioned network of SFPs that bind SP to sperm are required for sperm storage parameters—removal of any of the proteins of this network (two proteases, a cysteine-rich secretory protein (CRISP), and two C-type lectins) results in the inability of females to release sperm from storage, a similar effect to not receiving SP itself (Ravi Ram and Wolfner, 2007, 2009; Avila et al., 2010; LaFlamme et al., 2012).

Seminal fluid is also rich in proteases and their regulators. Multiple classes of proteases are found in *D. melanogaster* seminal fluid, including serine proteases and metalloproteases (LaFlamme and Wolfner, 2013). Several of these proteases have been shown to act in proteolytic cascades to regulate the cleavage of other seminal proteins (such as ovulin and Acp36DE, LaFlamme et al., 2014). Proteolysis can liberate active regions of proteins, or can regulate the stability of a protein; to date the roles of the proteolytic events in *D. melanogaster* seminal proteins are unclear. The female reproductive tract also produces proteases and protease inhibitors (Allen and Spradling, 2008, Prokupek et al., 2009, Prokupek et al., 2010), suggesting molecules that could potentially mediate the sort of interplay between the sexes. Unfortunately, the large number of proteases and protease inhibitors found in seminal fluid has made genetic dissection of their activities difficult, due to redundancy: effects of mutation or knockdown of one can be masked by the presence of another protease with similar activity. It is intriguing to think that such redundancy (and the use of proteins with similar activity but from different ancestral genes) could be the consequence of the arms race noted earlier in this article. In mammals, proteases and their inhibitors in seminal fluid play roles in the coagulation of semen. Although *D. melanogaster* seminal fluid does contain molecules that form a mating plug within the female, to date the roles of male or female proteases or protease inhibitors in this process have not been tested.

Other classes of protein in seminal fluid include lectins, which bind to carbohydrates. Carbohydrate-binding proteins are necessary for fertility and aspects of sperm storage in flies (Ravi Ram and Wolfner, 2007; Wong et al., 2008b; Ravi Ram and Wolfner, 2009) as well as in mammals (e.g. Demott et al., 1995, Tecle and Gagneux, 2015)–as are another class of protein in seminal fluid, the CRISPs (cysteine-rich secretory proteins; DaRos et al., 2015). Another class of proteins in *Drosophila* seminal fluid is odorant binding proteins (Findlay et al., 2008). Roles in seminal fluid for such proteins have not been reported, but a possibility is that they may serve to bind small-molecules from the male and present them to targets in the female. Small-molecules in seminal fluid are also not known in *Drosophila*, but they are inferred to be present and similar in function to small molecules present in other taxa including hormones such as ecdysterone (*Anopheles*, Pondeville et al., 2008), juvenile hormone (*Aedes*, Clifton et al., 2014) and prostaglandins (mammals, Robertson, 2007) all of which affect female physiology. It will be interesting to test for such molecules in *D. melanogaster* seminal fluid. Finally, in addition to proteins and other molecules, seminal fluid in *Drosophila* includes small vesicles or exosomes that are reported to be derived from the male's accessory gland's secondary cells. These exosomes are transferred to females during mating. Although their contents are as yet unknown, the vesicles have been suggested to affect the mating behavior of female *Drosophila* (Corrigan

et al., 2014). In mammals, such vesicles have been associated with transfer of small regulatory RNAs (e.g. Valadi et al., 2007; Belleannee et al., 2013; Vojtech et al., 2014) and possibly other molecules that can then affect gene expression or other traits in the female reproductive tract.

While the main cells and their secreted proteins (such as SP, ovulin and Acp36DE) have been extensively researched, much less is known about the role of the secondary cell secretions. However, three recent studies have shown that secondary cells are critical for a normal post-mating response. Minami et al. (2012) reported that the accessory glands of males mutant for the homeodomain transcriptional repressor *Defective proventriculus* (*Dve*) were small, lacked secondary cells, and had mononucleate main cells. These mutants had low fecundity and were unable to increase post-mating egg laying and to reduce post-mating receptivity – two responses that are elicited by the main cell accessory gland protein SP (Chapman et al., 2003), yet interestingly, *dve* mutants transferred normal amounts of SP to their mates. Gligorov et al. (2013) reported that loss of the homeotic transcription factor, *Abd-B*, in secondary cells caused those cells to develop abnormally, and resulted in defects in the long-term post mating responses in the mates of mutant males: without secondary cell function, the male's SP was unable to be bound to sperm and to be retained in the female. Additionally, this study provided evidence for interactions between the products of the two cell

types: expression of *Abd-B* in the secondary cells is required for glycosylation of at least three main cell secreted proteins: ovulin (Acp26Aa), which is responsible for a short-term (<24 hour) increase in egg laying after mating (Chapman et al., 2003), and CG1652 and CG1656, a pair of lectins that are part of the network of proteins that binds SP to sperm (Ravi Ram and Wolfner, 2007, 2009). Recently, Sitnik et al., (2016) identified several secondary cell-expressed genes that are necessary for the binding of SP to sperm, and the consequent persistence of the post-mating responses elicited by SP.

Fates of Male Secretions in Mated Females

The majority of the proteins secreted from the *D. melanogaster* male reproductive tract tissues that are transferred to females at copulation are only detectable within the female for ~4-6 hours after mating ends (Ravi Ram et al., 2005). During this time, seminal proteins have been found within the mating plug (Neubaum and Wolfner, 1999, Lung and Wolfner, 2001, Ravi Ram et al., 2005), the sperm mass (Neubaum and Wolfner, 1999, Lung and Wolfner, 2001), the tissues of the female reproductive tract, including the sperm storage organs (Ravi Ram et al., 2005), the oviduct (Neubaum and Wolfner, 1999) and the base of the ovary (Ravi Ram et al., 2005). Some SFPs, including ovulin and the SP, enter the hemolymph (Monsma et al., 1990, Pilpel et al., 2008) through a permeable part of

the lower uterus (Lung and Wolfner, 1999) and/or through a puncture made by the male genitalia (Kamimura, 2007, Mattei et al., 2015). This gives these SFPs access to targets outside the female reproductive tract. The mechanism(s) that allows some SFPs to enter the female circulation but prevents others is currently unknown. The example of an SFP that persists longer than a few hours in the mated female is the SP. Its binding to sperm allows it to remain within the female for >5d post-mating (Peng et al., 2005b), where it continues to "dose" her, changing her behavior and physiology as long as she contains stored sperm.

OBJECTIVES

In Chapter 2, I describe work on a post-mating response in females that involves changes to food intake and the rate of intestinal transit. Reproduction is an energetically costly event for the female and nutrition plays a key role in the process, so, not surprisingly, a female will increase her food intake (Carvalho et al., 2006) and modify her nutrient uptake (Cognigni et al., 2011). These responses are dependent on the female's receipt of the seminal fluid protein, sex peptide (SP). My work builds upon the finding that SP causes females to produce more concentrated excreta, thereby likely maximizing her nutrient uptake (Cognigni et al., 2011). I found that the change in intestinal transit is a post-mating response that lasts at least up to one week after mating, and like SP's other post-mating changes,

it requires the binding of SP to sperm and its gradual cleavage from sperm in order to persist (Peng et al., 2005b).

In Chapter 3, I describe a number of methods that are being used to identify the female receptor for the male SFP, ovulin. The ability to identify female receptors for SFPs would increase our understanding of how males are able to elicit the array of post-mating behavioral and physiological changes in the female. To date, only one SFP receptor in the female has been found, the sex peptide receptor (SPR) (Yapici et al, 2008). I have initiated a variety of approaches to identify the ovulin receptor, including an evolutionary rate covariation screen in order to find receptors whose sequences show a correlated rate of evolution with ovulin, RNAi knockdown of candidate receptors and egg-laying/ovulation assays, a biochemical assay, and an *in vivo* reporter assay. We have identified a likely candidate, CG15744, and further confirmation of this interaction is currently underway.

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CHAPTER 2

POST-MATING CHANGE IN EXCRETION BY MATED *DROSOPHILA MELANOGASTER* FEMALES IS A LONG-TERM RESPONSE THAT DEPENDS ON SEX PEPTIDE AND SPERM[2]

In *Drosophila melanogaster*, and many other insects, seminal fluid proteins transferred during mating elicit numerous changes in female behavior and physiology (reviewed in Avila et al., 2011 and Wolfner, 2009). Since nutrition plays a key role in reproductive success (Chapman and Partridge, 1996), it is not surprising that one post-mating change in the *Drosophila* female involves an increase in food intake (Carvalho et al., 2006). A recent study found that while food intake increases after mating, intestinal transit is significantly decreased (Cognigni et al., 2011). This slower intestinal transit results in more concentrated fecal deposits, referred to as reproductive oblong deposits (RODs), and is suggested to be an advantageous mechanism to increase nutrient absorption at a energetically demanding time for the female (Cognigni et al, 2011). In addition to increased food intake and slower intestinal transit, mating elicits a change in food

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preference by female *Drosophila*: virgin females prefer carbohydrates, while mated females prefer protein-rich food (Ribeiro and Dickson, 2010; Vargas et al., 2010). These responses are biologically important to the female since mating increases oogenesis and egg laying, which are energetically costly. Previous studies show that the male accessory gland protein, sex peptide (SP), is involved in each of these responses (Barnes et al., 2008; Carvalho et al., 2006; Cognigni et al., 2011; Ribeiro and Dickson, 2010; Vargas et al., 2010).

SP has multiple roles in the post-mating response, such as increasing egg laying and reducing receptivity to re-mating, both short-term (within the first 24 hours after mating) and long-term (up to 2 weeks after mating). Within the first 24 hours after mating, SP stimulates oocyte progression and accumulation of yolk proteins in the oocyte leading to increased egg production (Moshitzky et al., 1996; Soller et al., 1997). Sex peptide also plays major roles in the long-term response (LTR). As part of the LTR, SP decreases receptivity to re-mating and increases egg laying for up to 10 days after mating (Chapman et al., 2003; Liu and Kubli, 2003). SP produces these long-term effects by binding to sperm in the female reproductive tract and then being gradually released, by tryptic cleavage, from stored sperm over several days (Peng et al., 2005). Therefore, sperm are essential for the long-term persistence of SP's effects (Manning, 1962, 1967; Peng et al., 2005). For egg production and receptivity changes, SP exerts its effects on the

female through the sex peptide receptor (SPR) found on neurons that express *fruitless (fru)* and *pickpocket (ppk)* in the female reproductive tract (Häsemeyer et al., 2009; Yang et al., 2009).

Previously, it was shown that increased feeding after mating is a long-term response that is dependent upon egg production (Barnes et al., 2008). Therefore, it is likely that SP increases feeding indirectly by increasing egg production. However, Cognigni et al. (2011) found SP was also responsible for producing more concentrated excreta, independent of egg production. These data suggest that increased food consumption and slower intestinal transit are separable processes.

Cognigni et al. (2011) measured fly excreta production over a single 72 hour window, so it was not possible to determine whether the effect of SP that they observed was short-term, long-term or both. Therefore, to address this question, we measured ROD production in females at different times post-mating. By using crosses with males that produce normal or modified SP, and with males that do not transfer sperm, we investigated whether the change in intestinal transit is a long-term response that requires the release of sperm-bound SP. We also modified Cognigni et al.'s assay to indirectly measure food intake by quantifying the mean total fecal output per fly. We found that ROD production is a long-term response that requires the release of sperm-bound SP to persist. However, unlike increased

feeding behavior, the change in intestinal transit, as reflected by ROD production, is only partially dependent upon egg production.

Materials and Methods

Flies

Sex peptide null males ($SP^0/\Delta 130$) were generated by crossing the SP null line (SP^0/TM3, *Sb ry*) and the SP deficiency line (Δ130/TM3, *Sb Ser*) (Liu and Kubli, 2003). SP cleavage mutant males (*w*/Y; SP-TG^{QQ}/+; $SP^0/\Delta 130$) were obtained by crossing the SP null line (SP^0/TM3, *Sb ry*) to the SP-TG^{QQ} line (*y w*/Y; SP-TG^{QQ}/SP-TG^{QQ}; Δ130/TM3, *Sb Ser*) (Peng et al., 2005). The SP null mutant line, the SP deficiency line and the SP-TG^{QQ} lines were all kind gifts of Eric Kubli (University of Zurich, Zurich, Switzerland). Spermless and eggless flies (tud^1 *bw sp/*+) were the progeny of a cross of tud^1 *bw sp* females to Canton S (CS) males. tud^1 is a recessive maternal effect mutation that results in offspring that are unable to form a germline (Boswell and Mahowald, 1985). Sperm- and egg-producing controls for this experiment were also tud^1 *bw sp/*+, but were generated by crossing tud^1 *bw sp/*CyO females to CS males. *CG1656* and *CG1652* knockdown males were obtained by crossing a transgenic line carrying the RNAi construct for *CG1656* or *CG1652* (Ravi Ram and Wolfner, 2007) to a ubiquitous driver line (*tubulin*-Gal4/TM3, *Sb*). *CG1656* and *CG1652* are gene duplicates and off-targets

of one-another (Ravi Ram and Wolfner, 2007). Males that were UAS-RNAi/TM3, *Sb* were used as controls. Flies were reared on yeast-glucose media at room temperature (as in Ravi Ram and Wolfner, 2009), collected as virgins after eclosing, and aged in single-sex groups for 3-5 days prior to use in experiments.

Excreta assay

Each mating was carried out by placing a single 5-day old virgin female with a single 5-day old male in a glass vial containing a square piece of moistened filter paper. Matings were observed, and pairs with unusually short matings (<15 minutes) were discarded so that we only studied pairs that had adequate time to transfer ejaculate. Upon completion of mating, four females were placed into a 35mm petri dish containing a wedge of standard yeast-glucose food supplemented with 0.5% Bromophenol blue (B5525, Sigma) (Cognigni et al., 2011). Females were left undisturbed for 24 hours, after which they were aspirated to a new 35mm petri dish containing a new wedge of food. Digital images of the petri dish were obtained using an Epson Perfection 2400 Photo scanner. This was repeated every 24 hours to obtain scans at 24, 48, and 72 hours post-mating. The assay was performed in multiple sets of 3 to 4 flies for each genotype and condition tested. For longer-term tests, flies were transferred to fresh plates on days 1, 3, 5 and 7

after mating, and the plates were scanned as above to quantify excreta from days 1, 2+3, 4+5 and 6+7, respectively.

Quantitative analysis of excreta

The percentage of reproductive oblong deposits (RODs) (Cognigni et al., 2011) was calculated by determining the number of RODs relative to the total number of fecal spots. In order to quantify the total amount of excreta on each plate, Metamorph® imaging software was used. Briefly, the pixel area, optical density, and integrated optical density (IOD; pixel area multiplied by the optical density) were obtained for each fecal spot. Parameters for shape and size were set at 0.2-1.0 shape factor and 50-700 square pixels, respectively, to eliminate background noise. In order to quantify the amount of excreta per fly, the IOD for all excreta spots on one petri dish were added and the sum was divided by the number of flies in the set. This also allowed us to adjust for flies that were lost or died throughout the 72 hour or one week experiment. For statistical analysis, pair-wise comparisons of IOD of excreta per fly for the sets were performed using ANOVA in JMP to determine the statistical differences between genotype, days post-mating and mated versus virgin flies.

Results

The post-mating change in ROD production is a long-term response that persists beyond the first day after mating.

Cognigni et al. (2011) reported that mated females produce significantly more reproductive oblong deposits (RODs) than virgin females during a 72 hour window after mating. To determine whether the response occurred within the first 24 hours (short-term) or over the entire 72 hours (short-term and long-term) we subdivided this window. We mated 4 CS females to CS males and quantified the excreta in each of the first three days following mating (see Materials and Methods). We found that CS females that had mated to CS males produced significantly more RODs compared to CS virgin females at 24 hours ($p=0.01$), 48 hours ($p<0.0001$) and 72 hours ($p<0.0001$) after the start of mating (ASM) (Figure 2.1A). In order to quantify the total amount of excreta at these time points, we calculated the integrated optical density (IOD) (see Materials and Methods). Consistent with a previous report that increased feeding is dependent on increased egg laying (Barnes et al., 2008), itself a long-term post-mating response (Chapman et al., 2003; Liu and Kubli, 2003), we found that the mean IOD of excreta per mated female was significantly higher than the mean IOD of excreta per virgin female at 24 hours ($p<0.0001$), 48 hours ($p<0.0001$) and 72 hours ($p=0.0424$) ASM (Figure 2.1B). We conclude that in addition to increased feeding, ROD

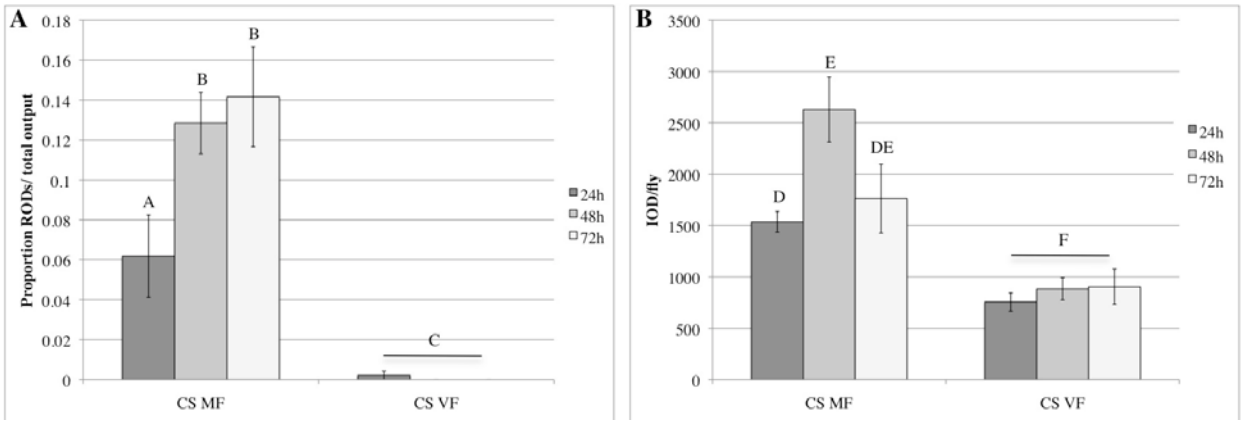

Figure 2.1. The effect of mating on excreta produced by *Drosophila* females at 24 hours, 48 hours and 72 hours post-mating. Bars with the same letter are statistically similar; MF = mated females, VF = virgin females. (A) The proportion of reproductive oblong deposits (RODs) was calculated at each time point. Females mated to CS males (CS MF) produced more RODs than CS virgin females (CS VF) at 24 hours ($p=0.01$), 48 hours ($p<0.0001$) and 72 hours ($p<0.0001$). (B) The integrated optical density (IOD) of excreta despoits per fly was calculated by totaling the IOD (pixel area x optical density) for the entire petri dish and dividing by the number of flies. The IOD of excreta per fly for CS MF was significantly higher than CS VF at 24 hours ($p<0.0001$), 48 hours ($p<0.0001$) and 72 hours ($p=0.0424$). Total numbers of flies used are as follows: for 24 hours, $n_{CSMF} = 39$ and $n_{CSVF} = 40$, for 48 hours, $n_{CSMF} = 36$ and $n_{CSVF} = 36$, for 72 hours, $n_{CSMF} = 35$ and $n_{CSVF} = 29$.

production, a measure of intestinal transit, is a long-term post-mating response that persists for at least 72 hours after mating.

The role of sex peptide in the post-mating increase in ROD production.

Sex peptide regulates ROD production for at least ~1 week post-mating.

The seminal protein, sex peptide (SP), is necessary for the post-mating change in intestinal transit (Cognigni et al., 2011). To test whether SP affects ROD production and total IOD of excreta per fly, long-term as well as short-term, we first mated females to SP-deficient (or control) males and assessed ROD production on each of the first three days post-mating (Figure 2.2A). At 24, 48 and 72 hours ASM, CS females mated to control males (that transferred SP) produced significantly more RODs than CS virgin females ($p=0.0016$, $p=0.0014$ and $p=0.0014$, respectively). In addition, at 48 hours and 72 hours ASM, control-mated females produced significantly more RODs than females mated to SP null males ($p=0.001$ and $p=0.0014$). This indicates that SP is responsible for eliciting long-term ROD production. However, in this experiment, ROD production at 24 hours was not significantly different between females mated to control males and females mated to SP null males, and in both cases was significantly greater than that of unmated females. Although this could suggest that another seminal fluid protein, besides SP, is able to elicit ROD production within the first 24 hours, we did not

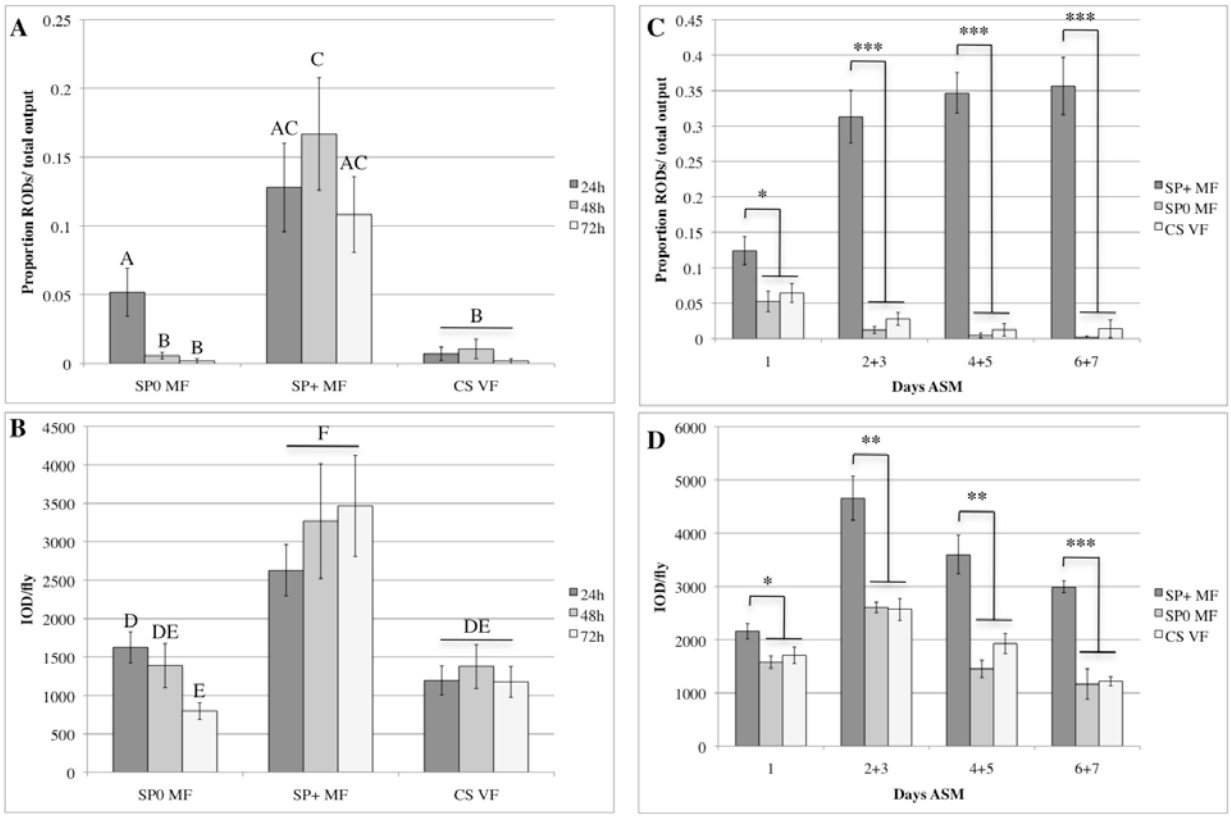
A
Proportion RODs/ total output
0.25
0.2
0.15
0.1
0.05
0
A
B
B
AC
C
AC
B
SP0 MF
SP+ MF
CS VF
24h
48h
72h
C
Proportion RODs/ total output
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
***
***
***
1
2+3
4+5
6+7
Days ASM
SP+ MF
SP0 MF
CS VF
B
IOD/fly
4500
4000
3500
3000
2500
2000
1500
1000
500
0
D
DE
E
F
DE
SP0 MF
SP+ MF
CS VF
24h
48h
72h
D
IOD/fly
6000
5000
4000
3000
2000
1000
0
*
**
**
***
1
2+3
4+5
6+7
Days ASM
SP+ MF
SP0 MF
CS VF

Figure 2.2. Sex peptide elicits long-term post-mating excretion change in females. (A) Females mated to control males (SP+ MF) produced significantly more RODs than virgin females (CS VF) at 24 hours (p=0.0016), 48 hours (p=0.0014) and 72 hours (p=0.0014) and than CS females that had mated to SP null males (SP0 MF) at 48 hours (p=0.001) and 72 hours (p=0.0014). SP null-mated females produced significantly more RODs than virgin females at 24 hours (p=0.0235). (B) The mean IOD of excreta per fly for SP+ mated females was significantly higher than SP0 mated females at 24 hours (p=0.017), 48 hours (p=0.0306) and 72 hours (p=0.001) and virgin females at each time point (p=0.0012, p=0.0299 and p=0.0042, respectively), while there was no difference between SP0 mated females and virgin females at any time point. In panels A and B, bars with the same letter are statistically similar. Total numbers of flies used are as follows: for 24 hours, $n_{SP0MF} = 40$, $n_{SP+MF} = 39$ and $n_{CSVF} = 40$, for 48 hours, $n_{SP0MF} = 38$, $n_{SP+MF} = 39$ and $n_{CSVF} = 38$, for 72 hours, $n_{SP0MF} = 38$, $n_{SP+MF} = 39$ and $n_{CSVF} = 37$. (C) Females mated to control males (SP+ MF) produced significantly more RODs than virgin females (CS VF) 1 day (p=0.0224), 2+3 days (p<0.0001), 4+5 days (p<0.0001) and 6+7 days (p<0.0001) ASM and more RODs than females mated to SP null males (SP0 MF) at each time point (p=0.0061, p<0.0001, p<0.0001, and p<0.0001, respectively). (D) The mean IOD of excreta per fly for SP+ mated females was significantly higher than SP0 mated females at 1 day (p=0.036), 2+3 days (p=0.0002), 4+5 days (p<0.0001) and 6+7 days (p<0.0001) ASM and higher than virgin females at each time point (p=0.0488, p=0.0002, p=0.0003 and p<0.0001, respectively). In panels C and D, measurements were taken at 1 day ASM, 3 days ASM, 5 days ASM and 7 days ASM. p<0.05, p**<0.01, p***<0.0001. Total numbers of flies used in C and D are as follows: for day 1, $n_{SP0MF} = 36$, $n_{SP+MF} = 39$ and $n_{CSVF} = 44$, for days 2+3, $n_{SP0MF} = 35$, $n_{SP+MF} = 39$ and $n_{CSVF} = 39$, for days 4+5, $n_{SP0MF} = 31$, $n_{SP+MF} = 38$ and $n_{CSVF} = 38$, for days 6+7, $n_{SP0MF} = 31$, $n_{SP+MF} = 38$ and $n_{CSVF} = 38$.

observe this effect in later experiments using SP null males with other wild type females (Figure 2.4C), suggesting that the 24 hour response we observed here was specific to the CS line we used.

In order to determine whether SP's effect on ROD production could persist long-term, we next mated CS females to either control males or SP null males and quantified ROD production on days 1, 2+3, 4+5 and 6+7 (Figure 2.2C). Control-mated females produced more RODs than virgins or than SP null-mated females at each time point ($p=0.0061$, $p<0.0001$, $p<0.0001$ and $p<0.0001$, respectively). Further, there were no differences between SP null-mated females and virgin females at all time points, indicating that SP is required for the increase in ROD production for at least ~1 week after mating. [Since the long-term response is already well established by 72 hours post-mating, for convenience we used a 72-hour timepoint for most of our remaining experiments.]

As expected from the known SP-dependent increase in feeding post-mating (Carvalho et al., 2006) and the coupling of increased feeding to increased egg production (Barnes et al., 2008), the IOD measurements showed that females mated to control males had a significantly higher IOD of excreta per fly than both CS virgins ($p=0.0012$, $p=0.0299$, $p=0.0042$) and females mated to SP null males ($p=0.017$, $p=0.0306$, $p=0.001$) at each time point, while there was no significant difference between CS virgins and females mated to SP null males at any time

point (Figure 2.2B). Furthermore, the SP-dependent increase in IOD persisted for at least 6-7 days after mating (Figure 2.2D).

SP binding to and release from sperm is required for the long-term increase in ROD production.

In order to determine whether the gradual cleavage of sperm-bound SP is responsible for eliciting the post-mating change in ROD production, we mated females to three different types of males: male progeny of *tudor* females (spermless males), males that produce a form of SP that cannot be cleaved from sperm (Peng et al., 2005), and males that were knocked down for seminal fluid proteins *CG1656* and *CG1652*, which are lectins that have been implicated in binding SP to sperm (Ravi Ram and Wolfner, 2009).

At all three time points that we examined (24, 48 and 72 hours ASM), females mated to spermless males produced significantly fewer RODs than females mated to control males ($p=0.0018$, $p=0.0019$ and $p<0.0001$, respectively; Figure 2.3A). At 48 hours ASM, however, females mated to spermless males produced significantly more RODs than virgin females, presumably due to unbound SP transferred in the seminal fluid. This was also true for 24 hours ASM, however it was not statistically different due to an unexpected number of RODs produced by virgin females at 24 hours ASM in this experiment. Importantly, by

72 hours ASM, females mated to spermless males did not produced more RODs than virgin females, suggesting that stored sperm is required in the female for ROD production to persist long-term. Since the transfer of sperm is necessary for the long-term increase in egg production after mating, it would be expected that this would also be true for increased feeding. As expected, at 48 and 72 hours post-mating, females mated to spermless males had a significantly lower IOD of excreta per fly than females mated to control males ($p=0.0023$ and $p<0.0001$, respectively). At 24 hours there was no significant difference in IOD between spermless- and control-mated females ($p=0.1164$), again likely due the effect of unbound SP that was transferred in the seminal fluid (Figure 2.3B). Together, these data suggest that stored sperm is required in the female for both increased feeding and ROD production to persist long-term.

In order to investigate whether the gradual release of SP from sperm is necessary for ROD production to persist, females were mated to males that produce a form of SP that cannot be released from sperm (SP-TG^{QQ} males) (Peng et al., 2005). As an independent test of whether SP binding to sperm is needed for long-term ROD production, we also examined females that had mated to males that were knocked down for *CG1656* and *CG1652*, seminal fluid proteins necessary to bind SP to sperm in the female (Ravi Ram and Wolfner, 2007, 2009). If the gradual release of sperm-bound SP is required for the post-mating increase in ROD

production to persist, females that had mated to SP-TG^{QQ} males or *CG1656/1652* knockdown males should lack this long-term response. Indeed, we found that at 48 hours and 72 hours ASM, females mated to control males produced significantly more RODs ($p=0.0179$ and $p<0.0001$) and had a higher IOD of excreta per fly ($p=0.0101$ and $p=0.0216$) than females mated to SP-TG^{QQ} males (Figure 2.3C and D). Also, females mated to *CG1656* knockdown males produced significantly fewer RODs at 48 and 72 hours post-mating than females mated to control males ($p=0.0002$ and $p=0.0003$) (Figure 2.3E). Given that *CG1656* and *CG1652* are off-targets of one another (Ravi Ram and Wolfner, 2007), similar results were obtained with *CG1652* knockdown males (data not shown). Interestingly, there was no difference in IOD of excreta between control- and knockdown-mated females at any time point ASM (Figure 2.3F). This was unexpected given the observation that the cleavage of sperm-bound SP is necessary for the increase in feeding long-term (Figure 2.3D). One possibility is that since RNAi does not completely knockdown the expression of *CG1656*, its residual expression was enough to allow sufficient SP persistence to increase the amount of excreta. However, given our results, we conclude that the binding of SP to sperm and its gradual release from sperm is essential for the long-term increase in ROD production after mating.

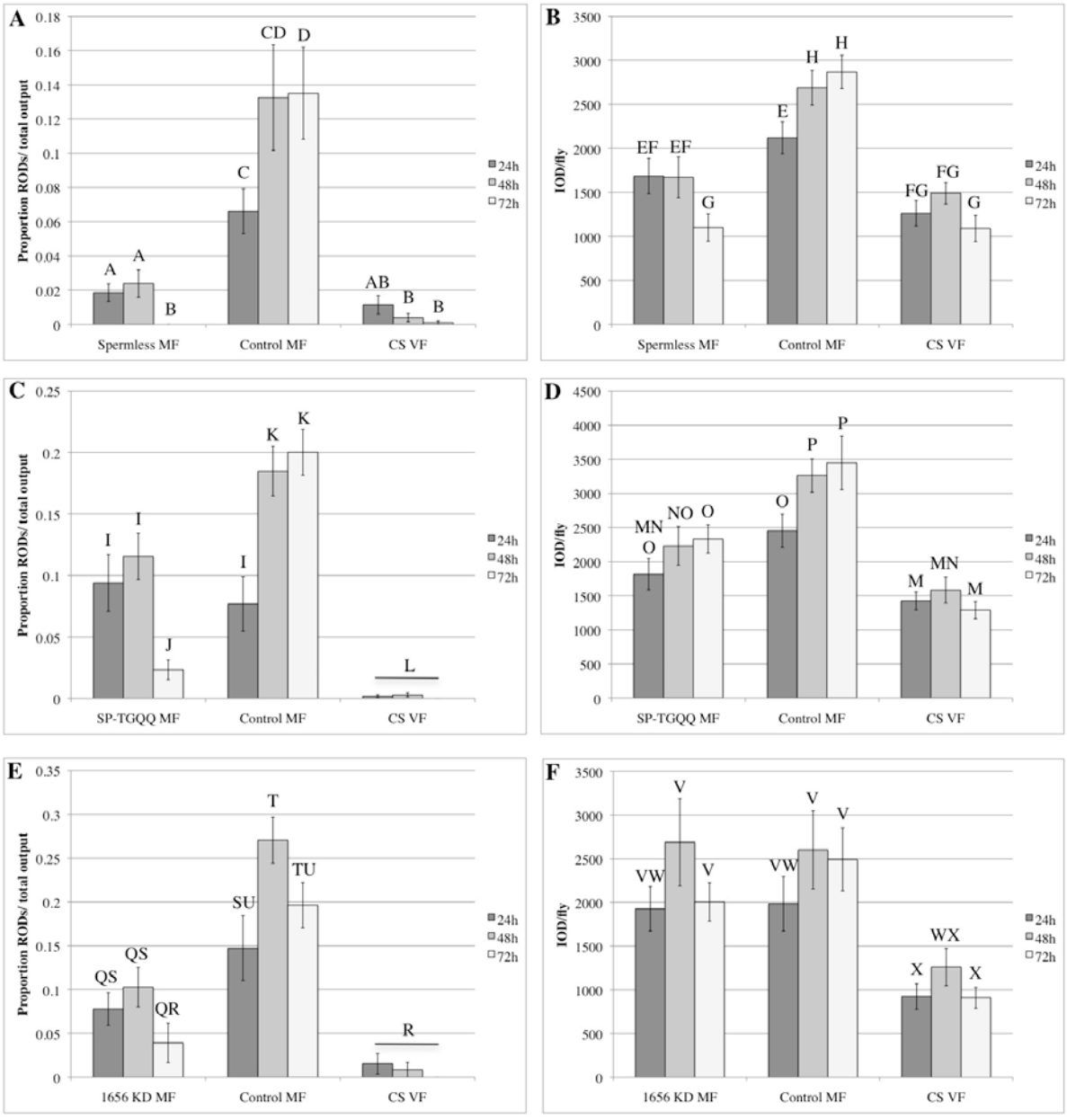
A
Proportion RODs/ total output
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
A
A
B
C
CD
D
AB
B
B
Spermless MF
Control MF
CS VF
24h
48h
72h
B
IOD/fly
3500
3000
2500
2000
1500
1000
500
0
EF
EF
G
E
H
H
FG
FG
G
Spermless MF
Control MF
CS VF
24h
48h
72h
C
Proportion RODs/ total output
0.25
0.2
0.15
0.1
0.05
0
I
I
J
I
K
K
L
SP-TGQQ MF
Control MF
CS VF
24h
48h
72h
D
IOD/fly
4500
4000
3500
3000
2500
2000
1500
1000
500
0
MN
O
NO
O
O
P
P
M
MN
M
SP-TGQQ MF
Control MF
CS VF
24h
48h
72h
E
Proportion RODs/ total output
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
QS
QS
QR
SU
T
TU
R
1656 KD MF
Control MF
CS VF
24h
48h
72h
F
IOD/fly
3500
3000
2500
2000
1500
1000
500
0
VW
V
V
VW
V
V
X
WX
X
1656 KD MF
Control MF
CS VF
24h
48h
72h

Figure 2.3. The gradual release of sperm-bound sex peptide is responsible for the long-term increase in ROD production after mating. (A) Females mated to control males (Control MF) produced significantly more RODs than females mated to spermless males (Spermless MF) and virgin females (CS VF) at 24 hours ($p=0.0018$ and $p=0.0005$), 48 hours ($p=0.0019$ and $p=0.0002$) and 72 hours after mating ($p<0.0001$ and $p<0.0001$). Spermless mated-females produced significantly more RODs than virgin females only at 48 hours ($p=0.0243$). (B) Mean IOD of excreta per fly for control-mated females was higher than virgin females at 24 hours ($p=0.0008$), 48 hours ($p<0.0001$) and 72 hours ($p<0.0001$) and higher than spermless-mated females at 48 hours ($p=0.0023$) and 72 hours ($p<0.0001$). (C) Females mated to control males produced more RODs than virgin females at all 3 time points ASM ($p=0.0026$, $p<0.0001$ and $p<0.0001$) and more RODs than females mated to males with non-cleavable SP (SP-TG^{QQ} MF) at 48 and 72 hours ($p=0.0179$ and $p<0.0001$). SP-TG^{QQ} -mated females also produced more RODs than virgin females at all 3 time points ($p=0.0004$, $p<0.0001$, $p=0.0065$). (D) Mean IOD of excreta per fly for control mated females was significantly higher than SP-TG^{QQ} -mated females at 48 and 72 hours ASM ($p=0.0101$ and $p=0.0216$). (E) Control-mated females produced more RODs than virgin females at each time point after mating ($p=0.0197$, $p<0.0001$ and $p<0.0001$) and more RODs than females mated to *CG1656* knockdown males (1656 KD MF) at 48 hours ($p=0.0002$) and 72 hours ($p=0.0003$). (F) Females mated to control males and females mated to *CG1656* knockdown males had a higher IOD of excreta per fly than virgin females at 24 hours ($p=0.007$ and $p=0.0034$), 48 hours ($p=0.0154$ and $p=0.0185$) and 72 hours ($p=0.0007$ and $p=0.0005$) while there was no significant difference between females mated to control or knockdown males. Bars with the same letter are statistically similar. Total numbers of flies used are as follows: for 24 hours, $n_{SPERMLESS} = 64$, $n_{CONTROL} = 63$, $n_{CSVF} = 64$, $n_{SP\text{-}TGQQ} = 52$, $n_{CONTROL} = 67$, $n_{CSVF} = 59$, $n_{1656KD} = 35$, $n_{CONTROL} = 36$, $n_{CSVF} = 24$, for 48 hours, $n_{SPERMLESS} = 59$, $n_{CONTROL} = 61$, $n_{CSVF} = 63$, $n_{SP\text{-}TGQQ} = 52$, $n_{CONTROL} = 67$, $n_{CSVF} = 58$, $n_{1656KD} = 31$, $n_{CONTROL} = 35$, $n_{CSVF} = 24$, for 72 hours, $n_{SPERMLESS} = 56$, $n_{CONTROL} = 53$, $n_{CSVF} = 61$, $n_{SP\text{-}TGQQ} = 51$, $n_{CONTROL} = 64$, $n_{CSVF} = 58$, $n_{1656KD} = 27$, $n_{CONTROL} = 34$, $n_{CSVF} = 24$.

The post-mating increase in ROD production is only partially dependent upon egg production.

Peng et al. (2005) found that females mated to transgenic males with SP that either could not be bound to sperm or could not be cleaved from sperm only showed only a short-term increase in post-mating egg laying; the long-term response was abolished. Additionally, previous data suggests that the post-mating increase in feeding is dependent upon egg production (Barnes et al., 2008). Therefore, it is not surprising that we observed that the increase in feeding as measured by IOD of excreta depends on SP binding to sperm. SP's effect on feeding may occur indirectly through egg production.

However, Cognigni et al. (2011) found that increased ROD production occurs after mating even in females whose oogenesis was arrested by an ovo^{D1} mutation (Oliver et al., 1987). To investigate if long-term ROD production occurs in females that cannot produce any eggs, we mated germline-less females (see Materials and Methods) to CS males and quantified their ROD production and IOD of excreta per fly at 24, 48 and 72 hours ASM. At 72 hours ASM, mated eggless females produced significantly more RODs than virgin eggless females ($p=0.0156$) (Figure 2.4A). They also produced more RODs at 48 hours, though this was not statistically significant. Therefore, these data suggest that long-term ROD production after mating can occur even without egg production. However, we

found that at each time point ASM, mated eggless females produced significantly fewer RODs than mated control females ($p=0.0054$, $p=0.0003$ and $p=0.0008$, respectively) (Figure 2.4A). This may suggest that ROD production is not completely uncoupled from egg production. Additionally, as expected, our data show that the IOD of excreta per fly is completely dependent upon egg production since mated eggless females had a significantly lower IOD of excreta per fly than mated control females at each time point ($p=0.002$, $p=0.0003$, $p<0.0001$), while mated eggless females showed no significant difference in IOD when compared to virgin eggless females ($p=0.3707$, $p=0.2186$, $p=0.0627$) (Figure 2.4B). These data suggest that total excreta upon mating (and, indirectly, food consumption) is dependent upon egg production, while long-term ROD production may be uncoupled from egg production to some extent. Our data show that ROD production is not completely independent, however, since there were still significant differences between mated control females and mated eggless females.

To further investigate the finding from Cognigni et al. (2011) that the requirement for SP in ROD production may be uncoupled from egg production, we sought to determine if SP was responsible for eliciting the long-term ROD production in eggless females. Cognigni et al. (2011) had found that sterile ovo^{D1} females produced RODs when mated to wild type males but not when mated to SP null males. Here, we show that eggless females mated to wild type males produce

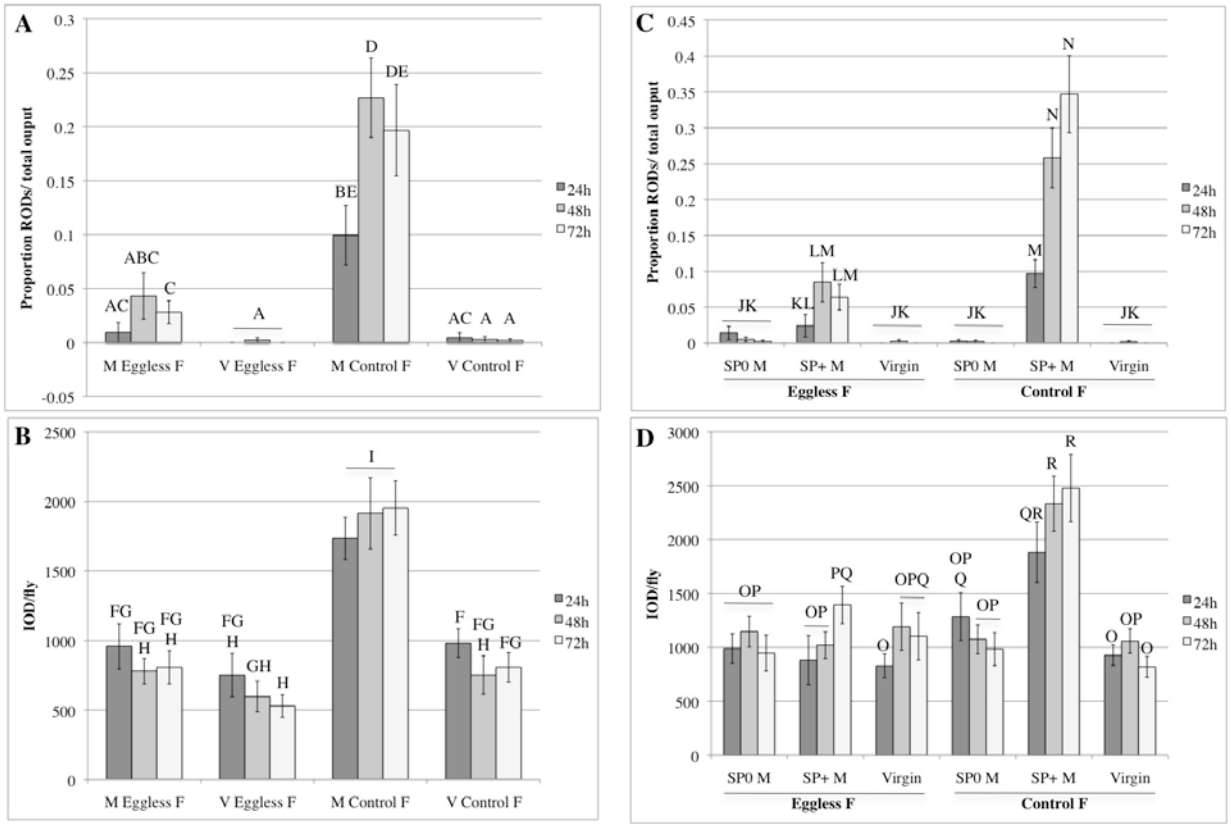
A
Proportion RODs/ total ouput
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
AC
ABC
C
A
BE
D
DE
AC A A
M Eggless F
V Eggless F
M Control F
V Control F
24h
48h
72h
B
IOD/fly
2500
2000
1500
1000
500
0
FG FG H FG H
FG H GH H
I
F FG H FG
M Eggless F
V Eggless F
M Control F
V Control F
24h
48h
72h
C
Proportion RODs/ total ouput
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
JK
KL
LM
LM
JK
JK
M
N
N
JK
SP0 M
SP+ M
Virgin
SP0 M
SP+ M
Virgin
Eggless F
Control F
24h
48h
72h
D
IOD/fly
3000
2500
2000
1500
1000
500
0
OP
OP
PQ
O
OPQ
OP
Q
OP
QR
R
R
O
OP
O
SP0 M
SP+ M
Virgin
SP0 M
SP+ M
Virgin
Eggless F
Control F
24h
48h
72h

Figure 2.4. SP elicits long-term ROD production partially independent of egg production. (A) Mated control females (M Control F) produced significantly more RODs than virgin control females (V Control F) and mated eggless females (M Eggless F) at 24 hours ($p=0.0026$ and $p=0.0054$), 48 hours ($p<0.0001$ and $p=0.0003$) and 72 hours ASM ($p=0.0001$ and $p=0.0008$), while mated eggless females produced more RODs than virgin eggless females (V Eggless F) only at 72 hours after mating ($p=0.0156$). (B) Mated control females had a significantly higher IOD of excreta per fly than virgin control females and mated eggless females at 24 hours ($p=0.0004$ and $p=0.002$), 48 hours ($p=0.0005$ and $p=0.0003$) and 72 hours ($p<0.0001$ and $p<0.0001$). There was no significant difference between eggless mated females and eggless virgin females. (C) Eggless females mated to control males (SP+ M) produced more RODs than eggless females mated to SP0 males (SP0 M) and eggless virgin females at 48 hours ($p=0.014$ and $p=0.0167$) and 72 hours ($p=0.0045$ and $p=0.0095$) ASM. Control females mated to SP+ males produced significantly more RODs than eggless females mated to SP+ males at all three time points ASM ($p=0.01$, $p=0.0034$, and $p=0.0002$, respectively). (D) There was no significant difference in IOD of excreta per fly between eggless females mated to SP0 males, eggless females mated to SP+ males and eggless virgin females at 24 hours, 48 hours and 72 hours ASM. Control females mated to SP+ males at a higher IOD of excreta per fly than control females mated to SP0 males at 48 hours ($p=0.0006$) and 72 hours ($p=0.0007$) and control virgin females at all three time points ASM ($p=0.0052$, $p=0.0003$ and $p<0.0001$, respectively). Bars with the same letter are statistically similar. Total numbers of flies used for A and B are as follows: for 24 hours, $n_{MEF} = 51$, $n_{VEF} = 52$, $n_{MCF} = 52$ and $n_{VCF} = 52$, for 48 hours, $n_{MEF} = 49$, $n_{VEF} = 50$, $n_{MCF} = 49$ and $n_{VCF} = 50$, for 72 hours, $n_{MEF} = 44$, $n_{VEF} = 48$, $n_{MCF} = 47$ and $n_{VCF} = 45$. Sample sizes for B and C are as follows: for eggless females at 24 hours, $n_{SP0M} = 30$, $n_{SP+M} = 33$, $n_V = 28$, for 48 hours, $n_{SP0M} = 29$, $n_{SP+M} = 33$, $n_V = 27$, for 72 hours, $n_{SP0M} = 28$, $n_{SP+M} = 33$, $n_V = 22$. For control females at 24 hours, $n_{SP0M} = 27$, $n_{SP+M} = 41$, $n_V = 42$, for 48 hours $n_{SP0M} = 27$, $n_{SP+M} = 40$, $n_V = 41$, for 72 hours, $n_{SP0M} = 25$, $n_{SP+M} = 39$, $n_V = 38$.

significantly more RODs than eggless females mated to SP null males at 48 and 72 hours ASM ($p=0.014$ and $p=0.0045$, respectively) (Figure 2.4C), indicating that SP elicits long-term ROD production independently of egg production. We found, however, that the proportion of RODs produced by eggless females mated to wild type males was significantly lower than control females mated to wild type males at all three time points ASM ($p=0.01$, $p=0.0034$, and $p=0.0002$). This furthermore suggests that SP elicits long-term ROD production independently of egg production, but the two are not entirely separable responses.

Discussion

We used a previously described assay (Cognigni et al., 2011) to investigate whether the post-mating change in the processing of food in the *Drosophila* female intestine elicited by SP is part of the long-term post-mating response. Cognigni et al. (2011) previously reported that over a single 72-hour window, mated females produced more RODs than virgin females and this effect required SP from the males. It was unclear, however, whether the RODs they observed derived from short-term effects of SP or also reflected its long-term effects, which are sperm-dependent.

When we examined ROD production at time points within and beyond the 72 hour window, we found that ROD production was elevated not only at 24 hours,

but also at 48 and 72 hours ASM as well for at least ~1 week after mating. This indicates that SP regulates ROD production as part of both the short-term and the long-term response to mating.

While it was also reported that SP elicits ROD production independent of egg production (Cognigni et al., 2011), this was only partially the case in our experiments (Figure 2.4A). The difference may be explained by the type of eggless females used in the two studies: the *tudor* progeny used in this study completely lack a germline while Cognigni et al. (2011) used ovo^{D1} females which have a germline, but whose oocytes arrested in development (Oliver et al., 1987). We conclude that ROD production is likely dependent upon two factors: egg production and SP. The long-term increase in feeding behavior, however, is dependent on an increase in egg production that results from the gradual cleavage of sperm-bound SP in the female reproductive tract over several days (Barnes et al., 2008; this study).

Our data and those from previous studies on post-mating feeding behavior in *Drosophila* females indicate multiple, and possibly separable, roles for SP in eliciting these changes in the female that ultimately maximize the female's nutritional intake at an energetically demanding time. First, SP increases food consumption after mating (Carvalho et al., 2006) and this is dependent on SP increasing egg production (Barnes et al., 2008). The gradual cleavage of sperm-

bound SP is responsible for the long-term increase in egg production post-mating (Liu and Kubli, 2003; Peng et al., 2005), and our data show that the cleavage of SP is also necessary for increased food consumption, consistent with SP's role in food intake being an indirect result of its role in increasing egg production (Barnes et al., 2008). Second, SP causes a change in intestinal transit post-mating, partially independent of egg production (Cognigni et al., 2011; this study). Cognigni et al. (2011) showed that the intestinal contents of mated females are more concentrated than those of virgin females; mating-dependent production of RODs, as highly concentrated excreta, further supports this conclusion (Cognigni et al., 2011). We showed here that ROD production is a long-term response that requires the gradual cleavage of sperm-bound SP to persist, and thus is likely mediated by the C terminus of SP (Peng et al., 2005). Future studies, including investigation of changes in the timing of ROD production after post-mating feeding, are needed to define the precise nature of SP's role in modifying intestinal transit and excreta characteristics. Finally, previous studies have shown that SP is involved in the switch in preference from carbohydrate-rich food to protein-rich food after mating, also independent of egg production (Ribeiro and Dickson, 2010). Therefore, it appears that SP is responsible for coordinating several behaviors that lead to the optimization of nutrient intake and processing the female upon mating, providing a reproductive advantage for maximal fertility.

Conclusions

In summary, we investigated the role of the seminal protein SP in changing the rate and characteristics of excretion by Drosophila females, during the first post-mating week. We quantified both total fecal output (as a measure of food consumption) and the proportion of highly concentrated RODs (which reflect slower intestinal transit (Cognigni et al., 2013)). We found that increased food intake/excretion and slower intestinal transit are long-term post-mating responses that require the binding of SP to sperm in females, followed by SP's gradual release by cleavage from the sperm. The region (and sperm-binding) of SP required for long-term ROD production are similar to those that regulate long-term egg production, but the long-term increase in ROD production is partially independent of egg production (in contrast to the increase in total food consumption).

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CHAPTER 3

GENETIC AND BIOCHEMICAL APPROACHES FOR IDENTIFYING THE FEMALE RECEPTOR FOR THE MALE SEMINAL FLUID PROTEIN, OVULIN

Introduction

While research has been done to identify male seminal fluid proteins and determine their effects on female physiology and behavior post-mating, a more recent focus has turned to *how* seminal fluid proteins (SFPs) exert their effects in the female. What are the receptor/ligand pathways in the female that interact with SFPs and how do the receptor/ligand interactions induce post-mating physiological changes?

Currently, only one SFP receptor has been identified in females: the Sex Peptide Receptor (SPR) (Yapici et al., 2008). SPR's ligand is the Sex Peptide (SP), which has been implicated in numerous post-mating responses including increased egg production, reduced receptivity to re-mating and changes in sperm storage dynamics (Chapman et al., 2003; Liu and Kubli, 2003). SPR's functions map to the subset of neurons that express *fruitless* (*fru*) and *pickpocket* (*ppk*) (Hasemeyer et al., 2009; Yang et al., 2009; Yapici et al., 2008). The identification of SPR has been a valuable step in understanding SFP-receptor dynamics in the female,

however, using the SP-SPR interaction as a model for other SFP-receptor interactions is not ideal for several reasons: (1) SP's post-mating effects persist over many days due to its binding to and slow release from sperm (Peng et al., 2005) during which time it causes changes in the female transcriptome (Gerrard et al., 2013; Innocenti and Morrow, 2009; Kapelnikov et al., 2008; Lawniczak and Begun, 2004; Mack et al., 2006; McGraw et al., 2004; McGraw et al., 2009; Short and Lazzaro, 2013) and (2) SPR has multiple ligands, including a non-reproductive one (Hussain et al., 2016; Isaac et al., 2014; Kim et al., 2010; Oh et al., 2014). A better candidate to study SFP-receptor dynamics would involve a rapid, direct interaction. Therefore, the ability to detect and characterize this specific interaction would not be confounded by changes in gene expression or phenotypes from interactions with other ligands.

One such candidate is ovulin (Acp26Aa), which is a 264 amino acid prohormone-like protein (Monsma and Wolfner, 1988) that elicits a short-term increase in ovulation to maximal levels after mating (Herndon and Wolfner, 1995). Specifically, ovulin acts to stimulate the release of mature oocytes from the ovary (Heifetz et al., 2000). Ovulin is detected in the female reproductive tract as early as three minutes after the start of mating (ASM) (Lung and Wolfner, 1999) and is found in the hemolymph within ten minutes ASM (Monsma et al., 1990). By 1.5 hours ASM, ovulin's effect on ovulation can be detected (Heifetz et al., 2000),

indicating a rapid, direct response that is unlikely to involve transcriptional changes in the female. This makes ovulin an ideal candidate to study SFP-receptor dynamics. Given that ovulin is found in the reproductive tract and hemolymph, its receptor may be present in either the reproductive tract itself, or in tissues accessed from the hemolymph, such as the central nervous system.

Another benefit to using ovulin in this study is that recent work has been done to elucidate the molecular pathway in the female upon which ovulin acts. In *Drosophila*, ovulation requires the release of neuromodulators octopamine and tyramine from neurons in the abdominal ganglion (Monastirioti, 2003; Monastirioti et al., 1996; Szabad and Fajszi, 1982) and the octopamine receptors, OAMB and Octβ2R (Lee et al., 2003; Li et al., 2015). Recently, it was shown that ovulin increases ovulation through octopamine signaling by relaxing the muscles of the oviduct and increasing the number of synaptic sites between the oviduct muscle and octopamine (OA) neurons (Rubinstein and Wolfner, 2013). Identifying the receptor that directly interacts with ovulin and initiates the downstream octopamine signaling would provide crucial insight into understanding how SFPs may co-opt canonical signaling pathways in the female in order to change her behavior for reproductive advantage.

Assays to detect such a rapid, transient response that likely occurs between ovulin and its putative receptor (referred to as OvR from now on) require the

identification of the OvR. In order to first narrow down the possible OvR candidates, we narrowed our search to G protein-coupled receptors (GPCRs). GPCRs make up a large group of integral cell membrane receptors across taxa [reviewed in (Brody and Cravchik, 2000)] and are responsible for transducing signals from a wide range of environmental, developmental and physiological stimuli. Analysis of the *Drosophila* genome identified ~200 genes coding for GPCRs (Adams et al., 2000) and about one- fourth of those have been characterized as peptide responsive GPCRs (Hewes and Taghert, 2001). The only other SFP receptor identified to date, SPR, falls into the GPCR family of receptors, as do OAMB and Octβ2R.

I initiated multiple approaches in an attempt to identify OvR. Given that proteins that interact tend to share similar rates of evolution, Geoff Findlay in the lab first narrowed down the OvR candidates based on their correlated rate of evolution with ovulin (Clark and Aquadro, 2010; Findlay et al., 2014). From this list, one candidate, CG15744, is a particularly strong candidate for being OvR. Here I will present the evidence and assays that have been initiated in order to verify the interaction, which includes using RNAi to knock down OvR candidates and observe their egg-laying response, further analysis of CG15744 using an ovulation assay, cell culture-based calcium assay, and an *in vivo* reporter assay, in addition to the generation of a CG15744 knockout line using CRISPR/Cas9.

Materials and Methods

Evolutionary Rate Covariation (ERC) Screen

For analysis, a comprehensive list of 193 *Drosophila melanogaster* proteins annotated with "G-protein coupled receptor" was downloaded from Flybase (flybase.org). Protein orthologs from 12 *Drosophila* species (*melanogaster, sechelia, simulans, yakuba, erecta, ananassae, pseudoobscura, persimilis, willistoni, grimshawi, virilis*, and *mojavensis*) were produced by the *Drosophila* 12 Genomes Project (flybase.org) (Drosophila 12 Genomes et al., 2007). Ovulin orthologs were identified using InParanoid and aligned using MUSCLE (Edgar, 2004; Findlay et al., 2014; Ostlund et al., 2010). Amino acid divergence for each branch on the phylogenetic tree was calculated using the 'aaml' of the PAML package (Findlay et al., 2014; Yang, 2007) and this calculation was transformed into a rate of evolution based on the expected branch length (Sato et al., 2005). These values were used to calculate correlation coefficients for ovulin with each GPCR. To correct for species-specific variance, the ERC values were converted to empirical p-values (0 to 1) based on the observed distribution of ERC values for each species (Findlay et al., 2014).

RNA interference (RNAi) and 24 hour egg-laying assay of ovulin receptor candidates

To knock down ovulin receptor candidates, RNAi lines from the Vienna *Drosophila* RNAi Center (www.vdrc.at)(Dietzl et al., 2007) and the Bloomington *Drosophila* Stock Center (Indiana University)(Ni et al., 2009) were obtained. Each line was ubiquitously knocked down using the *tubulin*-GAL4 (TG4) driver and kept at 26°C. For lines that showed lethality or a severe egg production phenotype when knocked down with *tubulin*-GAL4, they were crossed to a temperature sensitive *tubulin*-GAL80; *tubulin*-GAL4 ($TG80^{ts}$; TG4) line (McGuire et al., 2003). These crosses were kept at a permissive temperature of 25°C until the eclosion of adult females, which were then shifted to 29°C until the completion of the egg-laying assay. For controls, background strains for the knockdown lines were crossed to *tubulin*-GAL4 (TG4) or temperature sensitive *tubulin*-GAL80; *tubulin*-GAL4 ($TG80^{ts}$; TG4). Background strains are as follows: Attp2 for Bloomington lines (ID #s 28516, 25857, 28789, 31108, 27509) Attp60100 for VDRC KK lines (ID #s 110537, 103342, 110525, 104734, 109329, 105360, 104524, 100217) and w1118 for VDRC GD lines (ID #s 10171, 42524, 9362, 7852, 5451, 1783). All flies were reared on standard yeast-glucose media on a 12:12 light/dark cycle.

For the 24 hour egg-laying assay, 3-5 day old knockdown females were mated to 3-5 day old wild-type (Canton S) males in single pair matings on standard yeast glucose food that was evenly distributed with minimal imperfections to minimize substrate effects on egg-laying site preference. Matings that lasted under 15 minutes were discarded. After the completion of mating, males were removed from the vials and discarded while females remained in the vials for 24 hours after the start of mating (ASM). Females that had been knocked down with *tubulin*-GAL4 were kept at 26°C while females that had been knocked down with temperature sensitive *tubulin*-GAL80; *tubulin*-GAL4 were kept at 29°C.

Twenty-four hours ASM, the females were removed from the vials and the number of eggs laid was determined using a dissecting microscope. For statistical analysis, pair-wise comparisons of experimental vs control eggs laid was performed in JMP using a students t-test. The assay was repeated at least twice for each line.

Ovulation assay of CG15744 knockdown females

Knockdown of CG15744 (Bloomington #28516) was performed as described above with *tubulin*-GAL4. As a control, the background strain Attp2 was also crossed to *tubulin*-GAL4. Matings were performed as described above with Canton S males. After mating, males were discarded and females were allowed to

remain on the food for 3 hours ASM at 26°C. Within the first 3 hours ASM, ovulin's effects on egg-laying and ovulation have been initiated (Heifetz et al., 2000). We hypothesized that the putative ovulin receptor, CG15744, would behave similarly when knocked down. After 3 hours ASM, females were removed and frozen in liquid nitrogen. They remained at -80°C until the dissections could be performed. Females were removed from the -80°C freezer and placed on ice prior to dissection. Females were then placed under cold 1X PBS and an incision was made dorsally along the midline to expose the oviducts without disturbing the reproductive tract. The number of eggs found in the lateral oviducts, common oviducts, uterus and external opening was determined, in addition to the number of eggs laid on the food substrate during this 3 hour time period (Heifetz et al., 2000; Rubinstein and Wolfner, 2013) ($n_{control}$= 21, $n_{15744KD}$= 18).

CRISPR knockout of CG15744

The flyCRISPR Optimal Target Finder tool (flycrispr.molbio.wisc.edu) was used to identify highly specific PAM- adjacent (NGG) sites in the *CG15744* gene to target for Cas9 nuclease. To target the 5' end of *CG15744*, sense and antisense oligos were designed as follows: 5'sense (CTT CGC GAC GTC GAC CGC AGC AGA) and 5'anti (AAA CTC TGC TGC GGT CGA CGT CGC). For the 3' end: 3'sense (CTT CGG CCT CGT ACA GCT CGT CAA) and 3'anti (AAA CTT GAC

GAG CTG TAC GAG GCC). Using these oligos to target Cas9 nuclease to the 5' and 3' end of *CG15744* would result in a 5.1kb deletion.

Oligos were diluted to 100μM in water and annealed in 10X T4 ligation buffer and T4 polynucleotide kinase (New England BioLabs) at 95°C for 3 minutes, then cooled to 25°C at a rate of approximately -0.1°C/second. Annealed oligos were then ligated into the pU6-BbsI-chiRNA plasmid (Addgene #45946) (Gratz et al., 2013) that had been digested with BbsI and de-phosphorylated with Calf Intestinal Alkaline Phosphatase (New England BioLabs) using T4 DNA ligase and then transformed into *E.coli*. Inserts were confirmed by sequencing. The pU6-BbsI-chiRNA plasmids containing the 5' and 3' oligos were sent to Rainbow Transgenic Flies, Inc. (Camarillo, CA) for injection into the same nos-Cas9-attP2 embryo (y, sc, v; +/+; nos-Cas9) (Kondo and Ueda, 2013). Injected females were crossed to CSX males (w*; T(2;3)ap^{xa}, ap^{xa}/CyO; TM3, Sb^{1}) (Bloomington #2475). F1 females were then crossed to a line carrying the X-chromosome FM7 balancer (y^{1} P{SUPor-P}$wisp^{KG05287}$/$FM7^{c}$) (Bloomington #16467).

Cell culture-based calcium assay

Ovulin purification

Full length ovulin was purified using the baculovirus expression system. *Ovulin* was cloned into the pFastBac-CT-TOPO vector (Invitrogen), which

includes a C-terminal 6x-His tag. The signal sequence for ovulin (amino acids 1-18) was included so that the protein would be secreted into the cell culture media and post-translational modifications could be added. Sf9 cells were transfected with the ovulin baculovirus construct using the Cellfectin II reagent (Invitrogen) and re-infected to obtain a P3 viral titer. The ovulin protein was then purified over a Ni-NTA column (Qiagen) from 30ml of culture 72 hours post-infection using the following protoctol: 800µl of Ni-NTA resin was rinsed with 1ml of water to remove EtOH. The water was removed and the resin was incubated in 1ml of Wash Buffer (50mM Tris-HCL, pH 8.0, 300mM NaCl, 10mM imidazole, 10% glycerol) for 30 minutes. The 30ml suspension culture was centrifuged for 30 minutes at 4,000 rpm and the pellet was discarded (500µl of the cleared media was saved as the input sample for silver staining and Western blot). The 30ml culture was added to the resin in Wash Buffer and incubated for 30 minutes at room temperature with gentle shaking. The resin was then centrifuged for 10 minutes at 4,000 rpm and the supernatant was discarded (500µl of the supernatant was saved to test for proper binding of the proteins to the resin). The resin was transferred to a 10ml chromatography column and the excess media was allowed to run through. The column was washed with 10ml of Wash Buffer three times and protein was eluted with 3 1ml volumes of Elution Buffer (50mM Tris-HCL, pH8.0, 300mM NaCl, 250mM imidazole, 10% glycerol). Protein expression was confirmed by silver

staining and Western blot and protein concentration was determined by Pierce BCA Protein Assay (Thermo Scientific).

DNA encoding cleaved *ovulin* was synthesized by Genewiz (www.genewiz.com). This synthesized product is composed of DNA encoding amino acids 1-18 (ovulin's signal sequence) and amino acids 68-264 (N-terminal region). Cleaved *ovulin* was cloned into the pBacPAK8 vector (Clontech) using the primers BacOv-F (TAA GGA ATT CAT GAA CCA GAT TTT ATT ATG CTC TCC AAT), which contains an EcoRI restriction site and BacOv-R (CTT AGC GGC CGC TTA AAG TGT TGG TAG ATT C), which contains a Not1 restriction site. Both the PCR product and the vector were digested with EcoRI and NotI and ligated using T4 ligase (New England Biolabs). Ligation was verified by sequencing at the Biotechnology Resource Center (Cornell University).

Plasmid construction

For mammalian cell culture expression, the pME18S vector (Sato et al., 2008) was obtained from K. Touhara (University of Tokyo). The vector was converted to a Gateway® vector (pME18S-GW) using the Gateway® Vector Conversion System kit (Invitrogen) by inserting the Gateway® cassette into the EcoRI cloning site of pME18S.

DNA encoding *CG15744* was cloned into the pME18S-GW vector using standard Gateway® cloning techniques. PCR reactions were performed using GoTaq DNA polymerase (Promega), iProof High Fidelity DNA Polymerase (Bio-Rad) or Platinum Pfx DNA Polymerase (Thermo Fisher Scientific) and all sequences were verified by DNA sequencing. *CG15744* DNA was first amplified in four overlapping fragments from genomic DNA by fusion PCR (Shevchuk et al., 2004). Given its relatively large size (over 5kb), initial attempts at traditional PCR were unsuccessful. Fusion PCR is a useful method to produce multiple, shorter fragments at a high yield using nested primers which can then be joined together by one final PCR using primers at the 5' and 3' end of the gene (Shevchuk et al., 2004). For the fusion PCR, the following primers were used: 15744-F'4 (CGA TTG CGG ATT AAT TTG GT) and 15744-R'2 (CTT AAC CTG CCC GCT CAT CT) (fragment 2), 15744-F'2 (GCT GAC TAA GGG TCA GAA TG) and 28516R (GCA ACT CCA TTG TGG CCT AT) (fragment 3), 28516F (GAG CAG TAC AGC ACG CTG AG) and 15744-R'3 (AGT TTA AGC GCT TTG TGC CT) (fragment 4), and 15744-F'3 (CAC CAC AAT GCG ATG ACA AT) and 15744out-R (CTC ACT CAG CCC AAC CCT AGT) (fragment 5) (Figure 3.1). There are two introns within the first 710 base pairs of the *CG15744* gene so this fragment could not be amplified from genomic DNA. Therefore, this fragment was synthesized as a linear DNA product from Genewiz

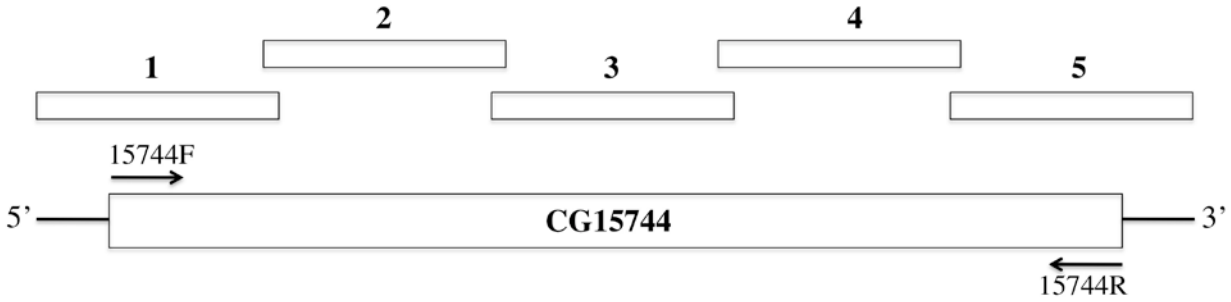

Figure 3.1. Schematic of the fusion PCR to generate full length *CG15744* cDNA. Fragments 1-5 were amplified individually and a final PCR using all 5 fragments as template with primers 15744F and 15744R was performed

(www.genewiz.com) (fragment 1) (Figure 3.1). The five fragments were fused together by PCR using primers 15744F (CAC CAT GCC GAC AGC GAC GGC GAC GT) and 15744R (CAA CGA GCC ACG CCC CTT TCC CAG CTA G) (Figure 3.1) and the fused product was cloned into the pENTR/D-TOPO entry vector (Invitrogen) followed by the pME18S-GW destination vector.

DNA for the *sex peptide receptor (SPR)* was amplified from a cDNA clone (RE15519) obtained from the *Drosophila* Genomics Resource Center (Bloomington, IN) by PCR using primers SPR-F (CAC CAT GGA CAA CTA TAC GGA CGT AC) and SPR-R (CTA GAG GAC CGT CTC GTT GGT G). The PCR product was cloned into the pENTR/D-TOPO entry vector (Invitrogen) followed by the pME18S-GW destination vector.

Cell culture assay for receptor function

RBL-2H3 cells (Eccleston et al., 1973) were plated in 2 ml suspensions at 0.5 x 10^6 cells per well in a 6 well plate and kept at 37°C overnight to allow the cells to adhere to the bottom of the well. The next day, cells were transfected with either pME18S-GW>CG15744 or pME18S-GW>SPR using Fugene HD (Promega) at a concentration of 3μg of DNA per well in Opti-MEM (Gibco). Cells were also co-transfected with 2μg per well of DNA encoding the red fluorescent, genetically encoded Ca^{2+} indicator, R-GECO (Zhao et al., 2011) and incubated at

37°C for 15 minutes at room temperature. Cells were washed with 1ml of Opti-MEM and IgE was added to a final concentration of 1μg/ml to the medium. Cells were incubated overnight at 37°C. The next day, the cells were washed with PBS with 1mM EGTA, centrifuged, and resuspended in Tyrode's buffer (137mM NaCl, 2.7mM KCl, 1mM $MgCl_2$, 1.8mM $CaCl_2$, 0.2mM Na_2HPO_4, 12mM $NaHCO_3$, 5.5mM D-glucose).

To measure changes in intracellular calcium, 300μl of the cell suspension was added to a cuvette and baseline fluorescence was measured in a fluorimeter. After the baseline fluorescence was established, 180μl of purified ovulin (10^{-7}M diluted in Tyrode's buffer) was added to the pME18S-GW>CG15744 transfected cells and the fold change in fluorescence was observed. As a positive control, 180μl of purified SP (CanPeptide) (10^{-7}M diluted in Tyrode's buffer) was added to pME18S-GW>SPR transfected cells and as a negative control, each protein was added to pME18S-GW>empty-transfected cells and the fold change in fluorescence was observed.

Split-ubiquitin yeast two-hybrid

Plasmid construction

The following split-ubiquitin yeast two-hybrid (SU-Y2H) plasmids were obtained from the *Arabidopsis* Biological Resource Center (Ohio State University):

XN21_GW (#CD3-1734), NX32_GW (#CD3-1737), NWT-X_GW (#CD3-1739), and MetYC_GW (#CD3-1740) (Lalonde et al., 2010). pENTR-CG15744 (see previous section for cloning method) and pENTR-ovulin were cloned into the split-ubiquitin plasmids using standard Gateway® cloning methods (Invitrogen). *CG15744* was cloned into the bait vector, MetYC_GW and *ovulin* was cloned into both prey vectors, XN21_GW and NX32_GW. Successful cloning was verified by sequencing at the Biotechnology Resource Center (Cornell University).

Co-transformation of yeast with bait and prey (Bashline and Gu, 2015)

The host strain for the split-ubiquitin yeast two-hybrid (SU Y2H) system, THY.AP4 (#CD3-808) {MATa *ura3*$^{-}$ *leu2*$^{-}$ *trp1*$^{-}$ *his3*$^{-}$ *ade2*$^{-}$ lexA::lacZ::trp1 lexA::HIS3 lexA::ADE2} was obtained from the *Arabidopsis* Biological Resource Center (Ohio State University) (Obrdlik et al., 2004).

50ml of YPD media (10 g/L yeast extract, 20 g/L peptone, 2% glucose) was inoculated with THY.AP4 yeast and grown at 29°C overnight. The next day, 150ml of YPD media was inoculated with 10ml of the overnight culture and grown at 29°C until the cell density (OD_{600}) reached ~0.90. The cell culture was pelleted and resuspended in a LiOAc/TE solution (1.1ml of 1M LiOAc, 1.1ml of 10x TE pH 7.5, 7.8ml water). For each transformation, the following were mixed (in order) in a 1.5ml microcentrifuge tube: 200µl LiOAc/TE/yeast, 200ng bait DNA, 200ng

prey DNA, 10µl salmon sperm DNA (ssDNA) (Sigma), and 700µl LiOAc/PEG solution (1.5ml of 1M LiOAc, 1.5ml of 10x TE pH 7.5, 12ml 50% PEG). Each transformation was incubated at 29°C for 30 minutes, heat shocked at 42°C for 15 minutes, and centrifuged. The pellets were resuspended in 500µl of a 0.9% NaCl solution, centrifuged again, and resuspended in 300µl 0.9% NaCl. 200µl of each transformation was plated on an SD/-Leu/-Trp plate (Clontech) and incubated at 29°C for several days until colonies appeared.

Detection of interaction (Bashline and Gu, 2015)

Three colonies were picked from each plate and resuspended in 50µl of water. 5µl of each was spotted onto an SD/-Leu/-Trp/-His plate and grown at 29°C for 3 days.

TANGO

Plasmid construction

The UAS-CG15744-Tango plasmid (Inagaki et al., 2012) was constructed using standard cloning and PCR methods. First, the UAS-DopR-Tango (Valium-DopR1-TEVcs-LexA-HAtag-2A-Arrestin-TEVp) plasmid was obtained from Dr. David Anderson (California Institute of Technology) (Inagaki et al. (2012). In order to replace the *DopR* coding sequence with the *CG15744* coding sequence,

the plasmid was digested with AvrII and NotI. The coding sequence for *CG15744* was amplified by fusion PCR as described in a previous section (see "Cell culture-based calcium assay" under "materials and methods"). The primers for the final fusion of the *CG15744* fragments were 15744_TANGO_F (CTA GCC TAG GAT GCC GAC AGC GAC G) and 15744-TANGO-R (TCA GGC GGC CGC AGC TGG GAA AGG GGC G). The final *CG15744* PCR product was then digested with AvrII and NotI and cloned into the UAS-Tango plasmid using T4 DNA Ligase (New England BioLabs) to create the Valium-CG15744-TEVcs-LexA-HAtag-2A-Arrestin-TEVp construct (UAS-CG15744-Tango for short). Successful cloning and the correct orientation were verified by sequencing at the Biotechnology Resource Center (Cornell University).

Fly embryo injection

UAS-CG15744-Tango DNA was sent to Rainbow Transgenic Flies, Inc. (Camarillo, CA) for phiC31-based injection into attP2 embryos (stock# R8622).

Results

Evolutionary Rate Covariation (ERC) screen reveals ovulin receptor candidates

The amino acid sequence of a protein evolves at varying rates over time and proteins that physically interact or are part of the same pathway tend to covary at a

similar rate due to evolutionary pressures acting on both (Clark et al., 2012). This has been shown in a number of organisms including yeast (Clark et al., 2012; Clark et al., 2013; Clark and Aquadro, 2010; Hakes et al., 2007), mammals (Clark et al., 2013), *Drosophila* (Findlay et al., 2014) and other invertebrates (Clark et al., 2009). ERC has recently been used in *Drosophila* to predict previously unidentified proteins in the Sex Peptide network, which plays a key role in the post-mating response in females (Findlay et al., 2014).

The rate of change between two proteins across all branches of a phylogeny is used to calculate the ERC value, which will range from -1 to +1 for a negative or positive correlation, respectively. Therefore, ERC values closer to +1 indicate an elevated rate of covariation between the pair of proteins and may indicate a possible physical interaction. Like many other reproductive proteins, ovulin is rapidly evolving (Aguade, 1998; Begun et al., 2007; Swanson and Vacquier, 2002; Tsaur et al., 1998; Tsaur and Wu, 1997) with a species amino acid divergence of ~15% between closely related *melanogaster* and *simulans* whose average amino acid divergence is only 1-2% (Begun et al., 2007). Given that evolutionary forces tend to act on both proteins in an interacting pair or pathway, it can be hypothesized that the ovulin receptor (OvR), or at least its ligand-binding region, might show similar rates of evolution.

Nineteen OvR candidates of 154 were selected from the ERC screen based on a combination of criteria (Table 3.1): a positive correlation with ovulin, orphan receptor status, and expression pattern (reproductive tract or nervous system). Additionally, receptors involved in the octopamine/tyramine signaling pathway, whether they showed a strong or weak correlation with ovulin, were included given the role of octopamine signaling in ovulin function (Rubinstein and Wolfner, 2013). It is interesting to note that out of the five octopmine/tyramine receptors, three of them showed a strong positive correlation with ovulin ($r > 0.4$), validating the screen since ovulin has already been shown to act in the octopamine pathway (Rubinstein and Wolfner, 2013).

Previous work in budding yeast has shown that the median ERC value for protein pairs without annotated functional relationships or interactions was -0.0004 while physically interacting proteins had a mean ERC value of 0.275 (Clark et al., 2012). Additionally, it was shown that between seven known proteins in the *Drosophila melanogaster* sex peptide network, there was an elevated ERC value (mean=0.3115) compared to the proteome-wide mean (0.0019) (Findlay et al., 2014). These results were taken into consideration when selecting candidates for the present screen for a positive correlation with ovulin.

The 19 OvR candidates were further tested for a 24 hour egg-laying phenotype upon knockdown in females that mimics the phenotype of females

Table 3.1. ERC values (r-value) for seven-transmembrane receptors and ovulin across 12 *Drosophila* species.

CG#	Name	r-value	p-value	Expression (FlyAtlas)
31662	Gr22a	0.8713	0.0153	minimal
4626	fz4	0.8655	0.0167	ovary
32261	Gr64a	0.8654	0.0167	minimal
42244	Octβ3R	0.9166	0.0425	brain
33083	Gr97a	0.7104	0.0695	minimal
31622	Gr39a	0.7166	0.0892	n/a
8930	Rickets	0.5607	0.1405	spermatheca
15744		0.5691	0.145	ovary
6919	OA2	0.5395	0.1514	brain
7918	mAChR-B	0.5455	0.1679	brain
31096	Lgr3	0.4812	0.1801	brain
7431	TyrR	0.4713	0.186	brain
31660	Smog	0.4249	0.2102	brain
13575		0.4088	0.2337	brain, ovary
7665	Lgr1	0.3099	0.2801	hindgut
10823	SIFaR	0.2368	0.324	brain, spermatheca
13995		0.0249	0.4672	head, brain
16766	TyrRII	-0.1745	0.6344	crop
33976	Octβ2R	-0.6786	0.944	brain, crop

mated to ovulin null males (Herndon and Wolfner, 1995). In theory, knocking down the receptor for ovulin should result in females that lay a reduced number of eggs [~10% reduction compared with controls (Heifetz et al., 2000; Herndon and Wolfner, 1995)] within the first 24 hours ASM. All genes were initially tested by ubiquitous knockdown using *tubulin*-GAL4 (TG4) (see "materials and methods"). However, five were lethal (fz4, OA2, SIFaR, TyrRII, and Gr97a) and three produced few or no eggs (Gr22a, rickets, and Octβ2R) upon ubiquitous knockdown, indicating a defect in development or egg production, respectively. Therefore, for further testing of these genes, the UAS-shRNA- carrying line was instead crossed to *tubulin*-GAL80^{ts}; *tubulin*-GAL4 (TG80^{ts}; TG4). This allowed knockdown of the receptor in adults in order to bypass any early essential developmental role of that receptor.

The 24 hour egg-laying assay revealed seven lines with an ovulin-like phenotype (Table 3.2): fz4 ($p<0.0001$), rickets ($p=0.0155$), CG15744 ($p=0.0213$), Lgr3 ($p=0.0027$), smog ($p=0.033$), SIFaR ($p<0.0001$), and Octβ2R ($p<0.0001$). One line produced significantly more eggs than the control when knocked down with *tubulin*-GAL80^{ts}; *tubulin*-GAL4: OA2 ($p=0.0002$).

As mentioned previously, three of the five octopamine/tyramine receptors showed a strong correlation with ovulin. However, the only one to show an egg-laying phenotype in this assay was Octβ2R. Although it was negatively correlated

Table 3.2. Twenty-four hour egg-laying assay with a candidate ovulin receptor knockdown mated to CS males.

CG#	Name	Driver	KD # of eggs laid (n)	Control # of eggs laid (n)	p-value
31662	Gr22a	$TG80^{ts}$; TG4	77.8 (23)	86.6 (32)	0.1668
4626	fz4	$TG80^{ts}$; TG4	70.2 (19)	95.5 (18)	<0.0001*
32261	Gr64a	TG4	68.9 (25)	73.7 (25)	0.4608
42244	Octβ3R	TG4	85.8 (24)	83.0 (32)	0.6261
33083	Gr97a	$TG80^{ts}$; TG4	88.3 (24)	86.6 (32)	0.7644
31622	Gr39a	TG4	71.7 (29)	73.7 (25)	0.6752
8930	Rickets	$TG80^{ts}$; TG4	84.6 (35)	97.7 (32)	0.0155*
15744		TG4	58.2 (14)	73.7 (25)	0.0213*
6919	OA2	$TG80^{ts}$; TG4	102 (25)	85.1 (27)	0.0002*
7918	mAChR-B	TG4	60.7 (25)	63.4 (18)	0.5503
31096	Lgr3	TG4	65.4 (25)	80.8 (23)	0.0027*
7431	TyrR	TG4	83.2 (35)	79.6 (42)	0.3755
31660	Smog	TG4	72.6 (15)	58.3 (17)	0.033*
13575		TG4	54.3 (20)	58.3 (17)	0.4622
7665	Lgr1	TG4	72.0 (29)	76.3 (26)	0.3456
10823	SIFaR	$TG80^{ts}$; TG4	59.4 (19)	95.5 (18)	<0.0001*
13995		TG4	49.5 (13)	58.3 (17)	0.2357
16766	TyrRII	$TG80^{ts}$; TG4	85.0 (26)	85.1 (27)	0.9738
33976	Octβ2R	$TG80^{ts}$; TG4	55.3 (19)	93.7 (22)	<0.0001*

with ovulin, it is still possible that Octβ2R acts downstream of ovulin and OvR in the same pathway given that it is essential for ovulation and fertilization (Li et al., 2015).

CG15744 knockdown shows a defect in ovulation

From the ERC results, one ovulin receptor candidate was especially interesting for multiple reasons. CG15744 showed a moderately strong correlation with ovulin (r=0.5691), a significant 24 hour egg-laying phenotype when knocked down (p=0.0213) (Figure 3.2A) and is moderately expressed in the adult ovary (Flyatlas.org) (Figure 3.2B). Ovulin enters the female genital tract 3 minutes ASM (Lung and Wolfner, 1999; Monsma et al., 1990) and accumulates at the base of the ovaries (Heifetz et al., 2000). It is therefore possible then that ovulin acts on a receptor directly in the female reproductive tract, like CG15744. However, ~10% of ovulin enters the hemolymph (Lung and Wolfner, 1999; Monsma et al., 1990) where it would theoretically have access to other targets. In such case, the ovulin receptor may be located elsewhere such as in the nervous system or endocrine system, rather than the female reproductive tract.

In order to further investigate the similarities between the ovulin null phenotype and the CG15744 knockdown phenotype, an ovulation assay was performed. Ovulin acts on the egg-laying process by stimulating the release of

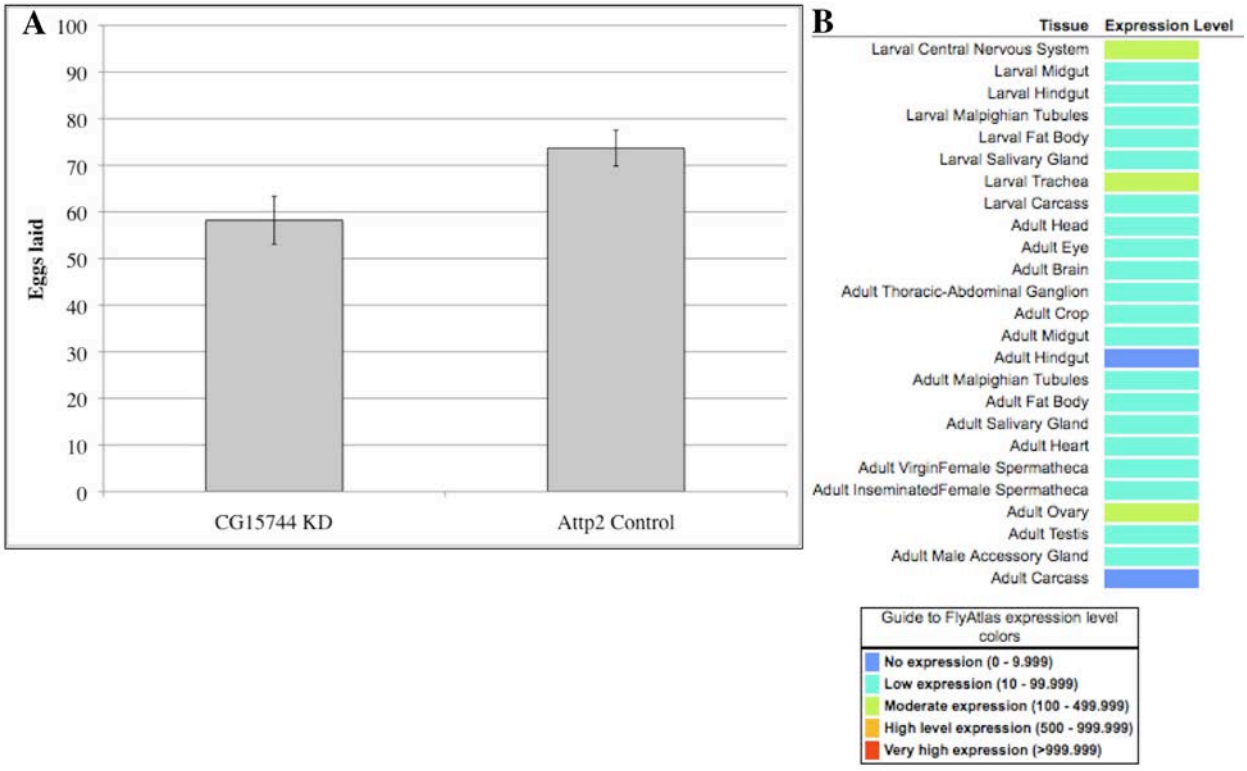

Figure 3.2. CG15744 shows a 24 hour egg-laying defect and is moderately expressed in the ovary. (A) Knockdown females (n=14) laid fewer eggs than Attp2 control females (n=24) 24 hours ASM when mated to CS males (p=0.0213). (B) Heatmap showing anatomical expression data of CG15744 from FlyAtlas (flyatlas.org).

mature oocytes by the ovary (Heifetz et al., 2000), a process that requires octopamine signaling from the central nervous system in order to increase the contractions of the ovary while simultaneously relaxing the muscles of the oviduct (Middleton et al., 2006; Rubinstein and Wolfner, 2013). This phenotype can be measured by counting the number of eggs in the lateral oviducts 3 hours ASM (Figure 3.3A), a time at which ovulin has initiated the stimulation of the release of mature oocytes from the ovary but increased post-mating oogenesis has not yet commenced (Heifetz et al., 2000). If CG15744 is OvR, it would be expected that knocking it down would cause a decrease in the rate of ovulation. When CG15744 knockdown females were mated to CS males and eggs in the lateral oviducts were counted 3 hours ASM, I found that there was a slight decrease in the number of eggs in the lateral oviducts, though not significant ($p=0.2889$) (Figure 3.3B). It is possible that the effect is small and due to incomplete knockdown, it may be difficult to detect without drastically increasing the sample size. However, this trend that was observed warrants further investigation.

Since the CG15744 knockdown was incomplete, attempts to create a CRISPR knockout of the gene have been initiated. CRISPR (clustered regularly interspaced short palindromic repeats) and the CRISPR associated Cas9 nuclease, which were identified as part of an adaptive immune response in bacteria and archaea (Barrangou et al., 2007; Ishino et al., 1987; Makarova et al., 2006), have

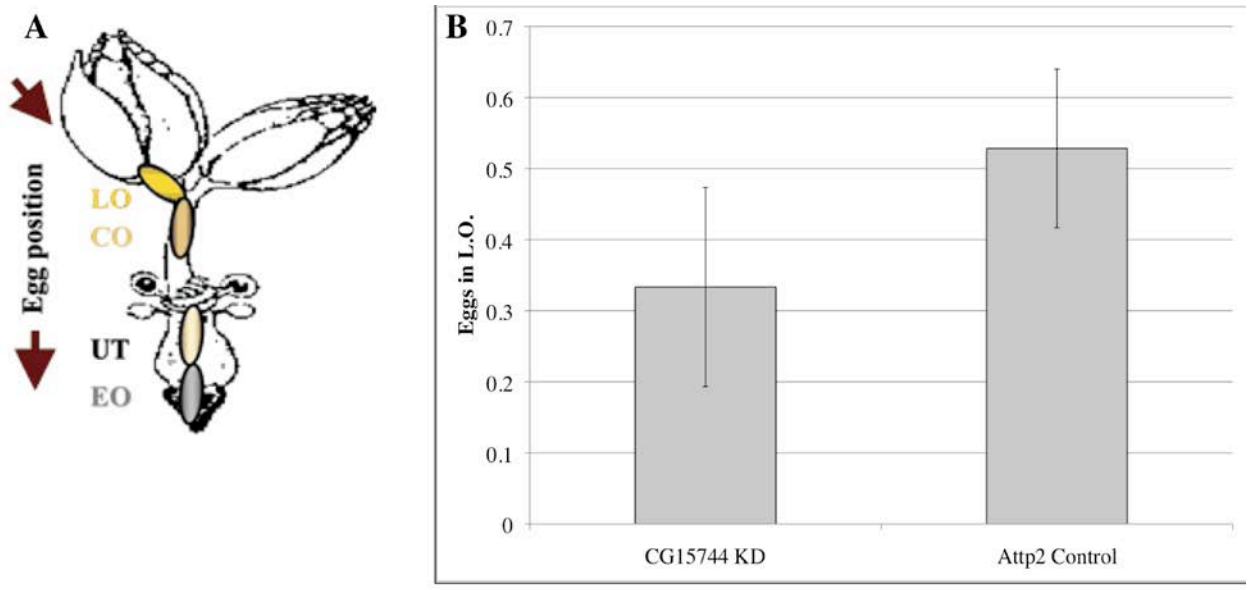

Figure 3.3. CG15744 KD females show a slight reduction in the rate of ovulation 3 hours ASM. (A) Schematic of the female reproductive tract showing the release of a mature egg from the ovary into the lateral oviduct (LO), through the common oviduct (CO) into the uterus (UT) and deposited from the external opening (EO). Figure is from Heifetz et al (2005). (B) Knockdown females (n=18) had slightly fewer eggs in the lateral oviducts (LO) than Attp2 control females (n=21) 3 hours ASM when mated to CS males, though not statistically significant (p=0.2889).

been a useful tool for precise genome editing in many organisms, including *Drosophila* where the edits can be transmitted through the germline (Gratz et al., 2013). A modified two-component CRISPR/Cas9 system was developed that is made up of Cas9 and chimeric RNA (chiRNA): CRISPR RNA (crRNA), which contains the complementary target sequence, and trans-activating CRISPR RNA (trcRNA), which interacts with Cas9 (Jinek et al., 2012) (Figure 3.4A). This system can be used to target a specific genome sequence and induce double strand breaks (DSBs). In this case, the *CG15744* gene was deleted by creating chiRNAs that target the 5' and 3' end of the gene (Figure 3.4B). The deletion of *CG15744* was confirmed by PCR using primers that annealed to either side of the gene, producing ~500bp product if the deletion was successful (Figure 3.4C). If the null is viable and fertile, further analysis of CG15744 will include testing the CRISPR-generated mutant for 24 hour egg-laying and 3 hour ovulation defects as performed with the knockdown. Similar to the knockdown, we expect to see a reduction in both egg-laying and ovulation. Therefore, we could conclude that it is likely the receptor. If we do not see a reduction in egg-laying and ovulation, it is possible that the results from the knockdown experiments were due to off-target effects of RNAi.

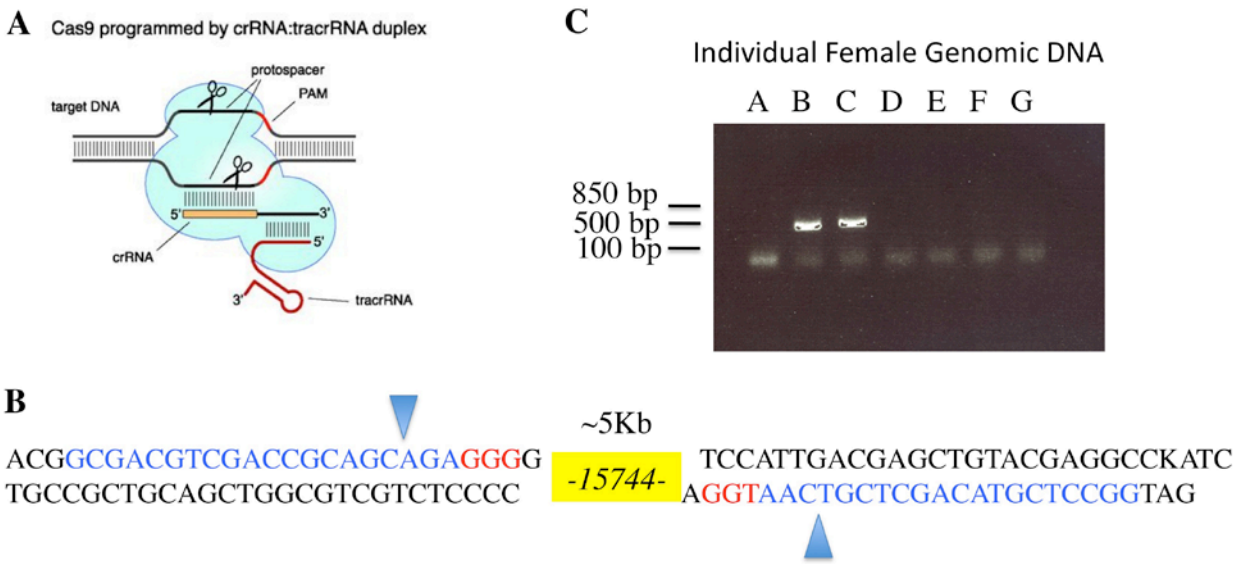

Figure 3.4. The CRISPR/Cas9 system was used to delete the *CG15744* gene. (A) Schematic of the two-component CRISPR/Cas9 system consisting of Cas9 and chimeric RNA (chiRNA). Figure is from Jinek et al. (2012). (B) Targeting of *CG15744* at the 5' and 3' ends. Blue text represents the target sequence, red text represents the PAM sequence, arrows are pointing to the Cas9 cut sites. (C) PCR on single fly genomic DNA using primers that anneal on either side of *CG15744*, producing ~500bp fragment if the deletion was successful. Lanes B and C show a successful deletion.

Attempts to verify the ovulin-CG15744 interaction using in vitro and in vivo approaches

Cell culture-based calcium assay

G protein-coupled receptors (GPCRs) are seven-transmembrane proteins (7-TM) that transduce signaling through the activation of heterotrimeric G proteins with αβγ subunits. When a ligand binds to the receptor, this causes a conformational change and interaction with the heterotrimeric G protein. This promotes the exchange of GTP for GDP on the α subunit, which causes Gα-GTP and Gβγ subunits to dissociate from the receptor and go on to further affect intracellular signaling proteins. There are two primary signaling pathways involved in GPCR function: the cyclic AMP (cAMP) and phosphatidylinositol (PIP2) pathways (Gilman, 1987). Activation of these pathways results in an increase in intracellular cAMP and calcium, respectively. Therefore, by measuring changes in intracellular calcium or cAMP using mammalian cell cultures, it is possible to determine whether a particular ligand activates a GPCR.

Using a mammalian expression vector, *CG15744* was expressed in rat basophilic leukemia (RBL) cells and after adding ovulin that was purified using the baculovirus system (Figure 3.5), the change in intracellular calcium was measured by co-transfecting the red fluorescent, genetically encoded Ca^{2+} indicator, R-GECO (Robert Campbell). As a control, this system was used to test for the

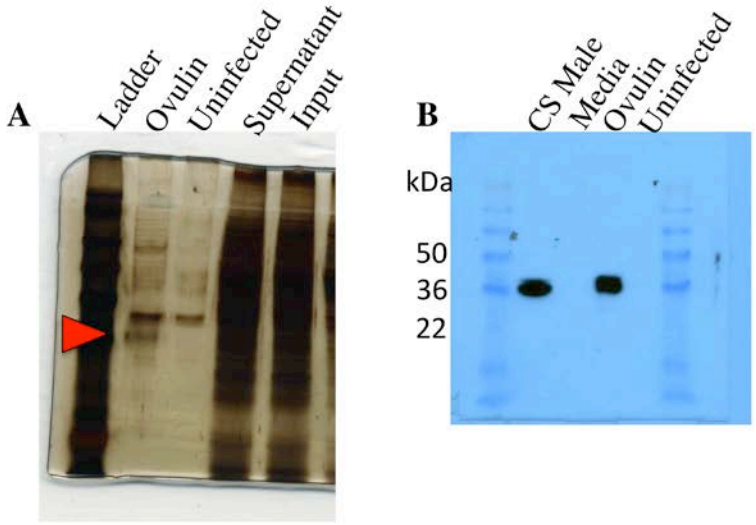

Figure 3.5. Purification of ovulin using the baculovirus system. (A) A silver stain and (B) Western blot of ovulin purified using Ni-NTA resin (Qiagen). “Ovulin” lane indicates purification using cell culture media from cells infected with the ovulin baculovirus. “Uninfected” is the negative control in which cell culture media from cells that had not been infected was used. In (A) “Input” and “Supernatant” are saved samples from the purification process. The “input” is an aliquot of the ovulin-infected cell culture media prior to Ni-NTA purification while the “supernatant” is an aliquot of media after the binding of ovulin to the resin (see “materials and methods”. The red arrow is pointing to ovulin. In (B), a primary anti-Acp26Aa antibody and secondary anti-rabbit HRP antibody was used to probe for ovulin. A Canton S (CS) male was homogenized and ran as a positive control.

positive interaction between SP and SPR (Yapici et al., 2008). If the cell culture system is able to detect the interaction, it would be expected that intracellular calcium would significantly increase upon addition of purified SP to *SPR*-expressing RBL cells compared to adding SP to cells transfected with an empty vector. Indeed, the addition of SP to *SPR*-expressing cells caused a 64.11% increase from basal fluorescence (Figure 3.6A) compared to a 1.39% increase when SP was added to cells that had been transfected with an empty vector (Figure 3.6C). However, when ovulin was added to *CG15744*-expressing cells, only a 2.93% increase occurred (Figure 3.6B) compared to a 1.83% increase with the negative control (Figure 3.6C) (results summarized in Figure 3.6D). This result could have occurred due to a number of reasons that will be discussed in the next section.

Split-ubiquitin yeast two-hybrid

The traditional yeast two-hybrid (Y2H) assay is a widely used method for detecting protein-protein interactions (Fields and Song, 1989). In the traditional Y2H assay, the protein of interest (the bait protein) is fused to the DNA-binding domain of the GAL4 transcription factor and a second protein of interest (the prey protein) is fused to the activation domain of GAL4. The two proteins are co-expressed in yeast and if they interact, GAL4 is reconstituted and elicits the

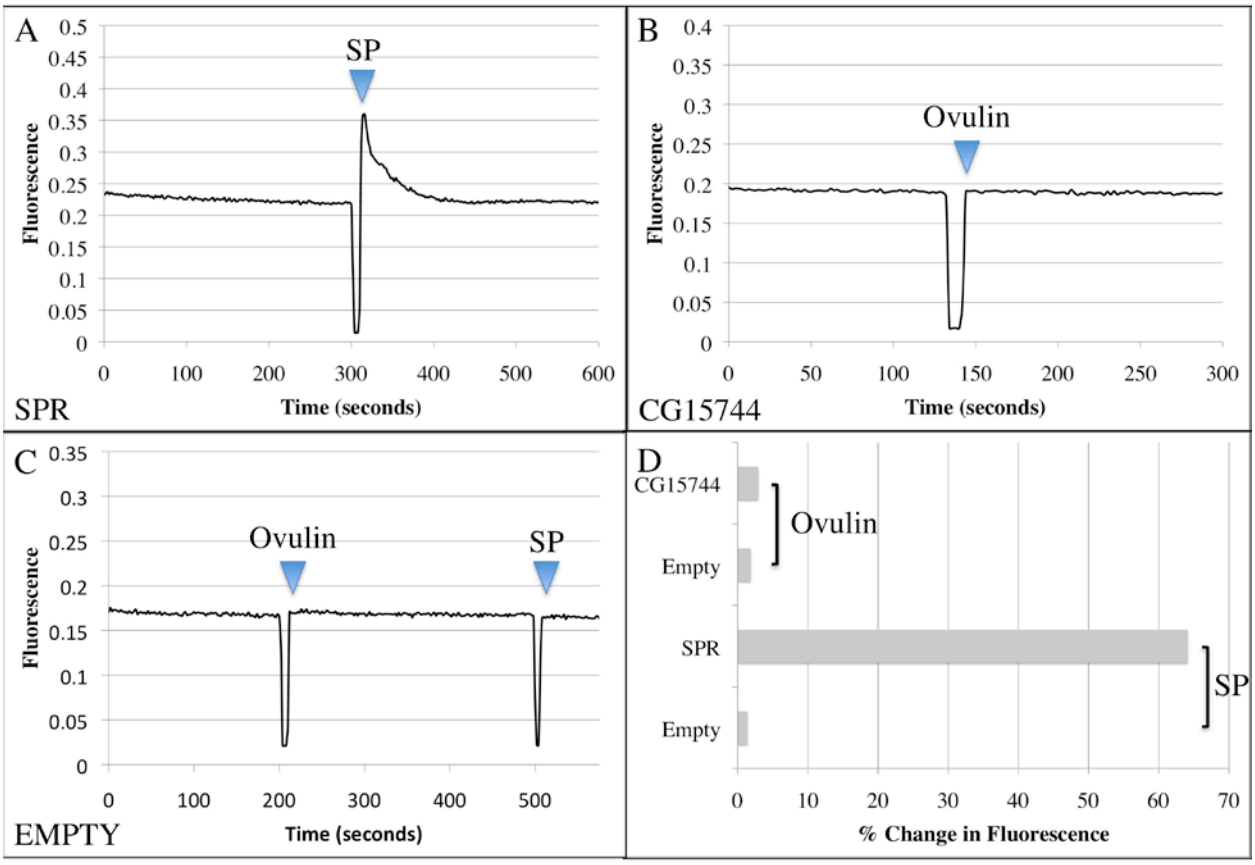

Figure 3.6. The cell culture-based calcium assay revealed activation of SPR by SP, but not the activation of CG15744 by ovulin. Plots showing the change in fluorescence when (A) SP was added to *SPR*-infected cells, (B) ovulin was added to *CG15744*-infected cells and (C) ovulin and SP were added to cells transfected with an empty vector. (D) Bar graph summarizing the % change from basal fluorescence for A-C.

expression of a reporter gene. However, one major limitation of this assay is that the interaction of the proteins must take place in the nucleus, so it would not be useful in detecting interactions between transmembrane receptors, such as CG15744, and their ligands. In order to address this issue, a modified yeast two-hybrid analysis was developed called split-ubiquitin yeast two-hybrid (SU Y2H) (Johnsson and Varshavsky, 1994; Stagljar et al., 1998). In this system, the bait protein is fused to the C-terminal half of ubiquin (Cub) with an attached ProteinA-LexA-VP16 (PLV) reporter module to create a bait-CubPLV chimeric protein. Secondly, the prey protein is fused with a mutated version of the N-terminus of ubiquitin (NubG) to create a prey-NubG (XN21_GW) or NubG-prey (NX32_GW). The mutation in the N-terminus of ubiquitin causes a reduction in the binding between Nub and Cub to prevent spontaneous reconstitution of ubiquitin. When the bait and prey fusion proteins are co-expressed in yeast, a positive interaction will reconstitute ubiquitin, which is then recognized by ubiquitin-specific proteases that will cleave the PLV module from the bait. The PLV module will then enter the nucleus and activate the transcription of reporter genes, which are LacZ, HIS3 and ADE2 (Bashline and Gu, 2015).

CG15744 was cloned into the bait vector, MetYC_GW, while *ovulin* was cloned into each prey vector, XN21_GW and NX32_GW (Lalonde et al., 2010). These were co-expressed in THY.AP4 yeast cells (Obrdlik et al., 2004), which are

his3^{-}, allowing for selection of positive interactions by plating the co-transformants on media lacking histidine. As a positive control, MetYC-CG15744 was co-transformed with a wild-type version of the N-terminus of ubiquitin, NubWT (NWTX_GW)(Lalonde et al., 2010). NubWT should interact with the Cub from the bait-CubPLV chimeric protein, reconstituting functional ubiquitin. This ensures that the CubPLV is properly translated in the yeast cells. Six negative controls were also performed: empty MetYC x empty XN21, empty MetYC x empty NX32, MetYC-CG15744 x empty XN21, MetYC-CG15744 x empty NX32, empty MetYC x XN21-ovulin, empty MetYC x NX32-ovulin. After plating co-transformants on media lacking histidine, growth was observed with MetYC-CG15744 x XN21-ovulin and MetYC-CG15744 x NX32-ovulin, in addition to the positive control. However, growth was also observed with two of the negative controls: MetYC-CG15744 x XN21 and MetYC-CG15744 x NX32 (Figure 3.7). This may indicate that nonspecific activation of the reporter occurred in the absence of a prey protein. Future experiments should include using adenine dropout media as well as histidine dropout for a more stringent assay. Also, since the Cub fusion is under the control of the methione promoter, it may be possible to increase the concentration of methione and determine whether the interactions persist.

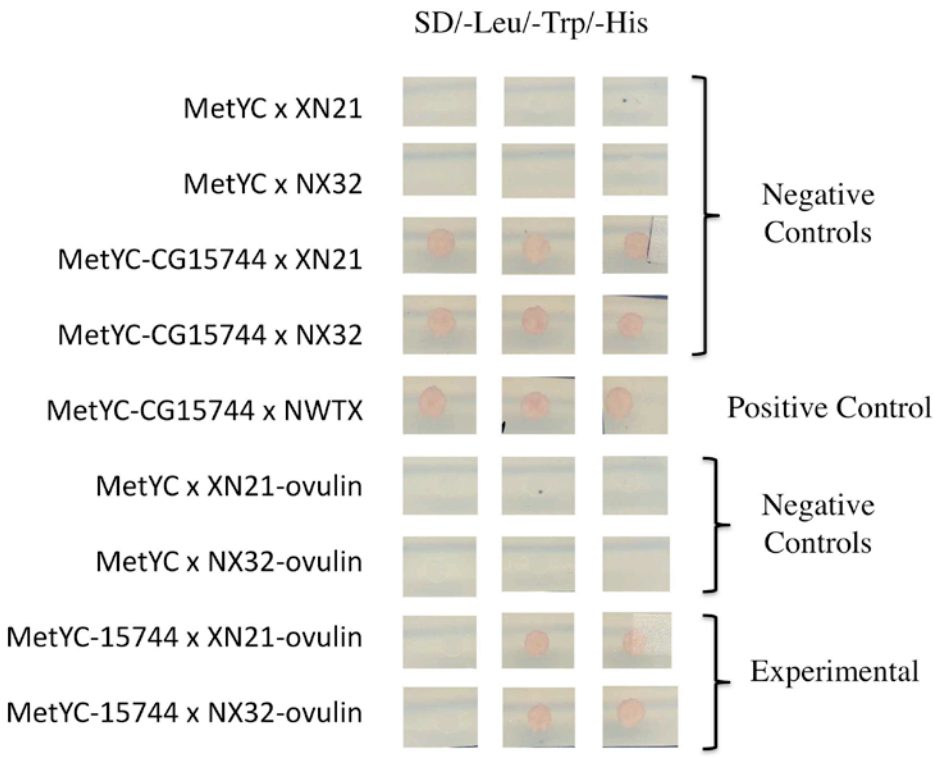

Figure 3.7. Split-ubiquitin yeast two-hybrid. *CG15744* was cloned into the SU Y2H bait vector, MetYC and *ovulin* was cloned into two different prey vectors, XN21 and NX32. Co-transformed yeast was plated on SD/-Leu/-Trp/-His plates to test for positive interactions. Combinations of bait and prey with empty vectors were also tested, in addition to a positive control, MetYC-15744 x NWTX. Growth was observed with the experimental and positive control, in addition to 2 out of the 6 negative controls.

TANGO system in flies

The Tango system was originally developed in cell culture to transform transient receptor/ligand interactions into a stable readout of reporter gene expression (Barnea et al., 2008). In the Tango system, a bacterial transcription factor is tethered to a membrane-bound receptor, such as a GPCR, by a linker containing a cleavage site for a highly specific protease. A second fusion protein consists of the protease fused to human β-arrestin2. Upon interaction of the receptor and ligand, the protease-arrestin fusion is recruited to the cleavage site and this cleavage will release the transcription factor to enter the nucleus and activate a reporter gene.

More recently, the Tango system was adapted for use in *Drosophila* (Inagaki et al., 2012). In order to test for activation of the dopamine receptor (DopR1) by dopamine (DA) *in vivo*, the authors created a construct in which DopR1 was fused to the bacterial transcription factor, LexA, by a linker that contains the cleavage site for the tobacco etch virus (TEV). The construct also encodes for a *Drosophila* arrestin1:TEV protease fusion protein. When DA binds DopR1, the arrestin-TEV protease fusion is recruited to the TEV protease cleavage site, resulting in the release of LexA and its subsequent translocation to the nucleus where it activates a lexAop-driven reporter, GFP.

Inagaki et al. established that the Tango system could be used to detect DopR1 activation by DA *in vivo* by generating flies that express the DopR1-Tango components under the control of *elav-GeneSwitch* (*elav-GS*) and also contain a *lexAop-mCD2::GFP* transgene as a reporter. *Elav-GS*, which is a pan-neuronally expressed, hormone (RU486) inducible form of GAL4 (Osterwalder et al., 2001), was used to restrict the expression of the Tango system to just 24 hours before the assay in order to reduce background signal from the accumulation of GFP. The authors were able to observe a significant increase in GFP signaling upon DA-induced activation of DopR1. We therefore hypothesized that this system could similarly be used to determine whether ovulin activates CG15744 *in vivo* (Figure 3.8A).

The DopR1-Tango transgene and the *elav-GS*; *lexAop-mCD2::GFP* flies were obtained from the lab of David Anderson. Recently, *CG15744* was successfully cloned into the vector (Figure 3.8B) by replacing DopR1 (see "materials and methods") and verified by sequencing. Once transgenic flies are obtained, they will be crossed to the *elav-GS*; *lexAop-mCD2::GFP* flies. To test for CG15744-ovulin interaction, *elav-GS*; *lexAop-mCD2::GFP/UAS-CG15744-Tango* females will be mated to either ovulin null males or wild type males and expression of GFP will be observed using confocal imaging. If CG15744 and

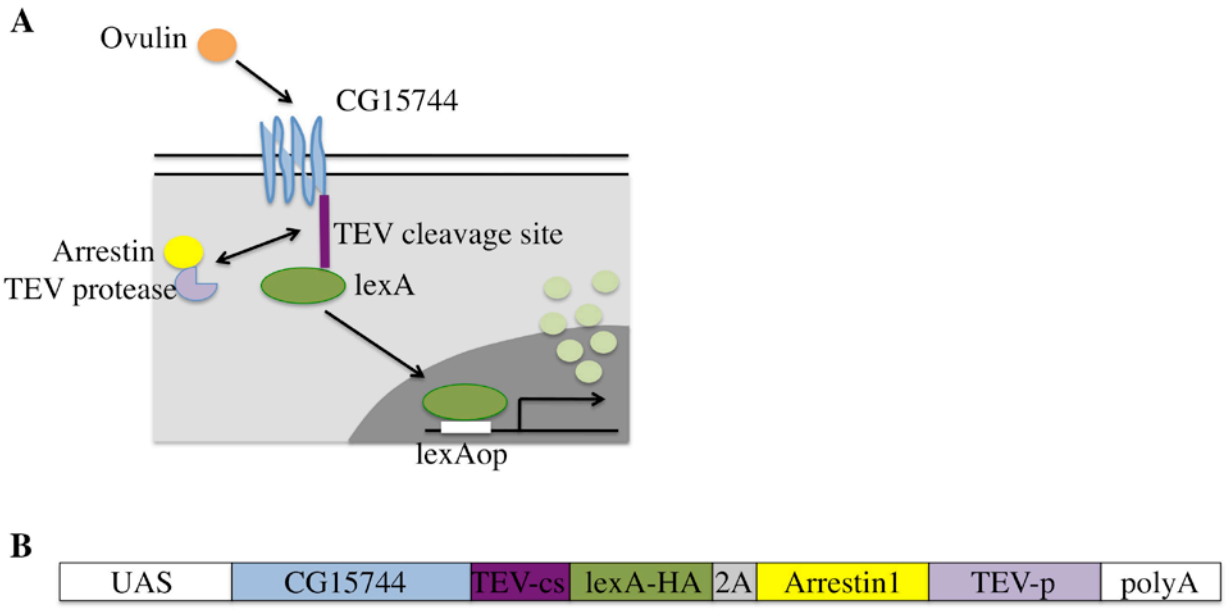

Figure 3.8. The CG15744-Tango system in flies. (A) Schematic of the Tango mechanism. (B) Design of the CG15744-Tango transgene. A and B adapted from Inagaki et al. (2012).

ovulin do interact, we would expect to see expression of GFP only when she mated with a wild type male.

Discussion

Understanding SFP-receptor interactions and the signaling pathways within the female upon which they act to induce post-mating responses is an important step in understanding reproduction in not only insects, but across species that sexually reproduce, including humans. While much work has been done to identify and characterize SFPs, we have only begun to scratch the surface of how they interact with female proteins. Here I present a range of genetic and biochemical methods that have been initiated in order to identify the female receptor for the male SFP, ovulin.

Using the evolutionary rate covariation method (ERC), we sought to identify GPCRs that had an elevated covariation rate with ovulin. We found eight candidates that showed a 24 hour egg-laying defect when knocked down in the female, of which one, CG15744, stood out as the most likely OvR candidate given its reproductive tract expression, orphan receptor status, and correlation with ovulin. Further confirmation for this interaction is underway.

First, a CRISPR-generated knockout is in the 3rd generation of crosses, so phenotypic data of the mutant, including egg-laying and ovulation data will be

available soon. Further testing will also include mating CG15744 knockout females to wild type and ovulin null males. If CG15744 is OvR, its egg-laying and ovulation phenotype should not worsen when mated to ovulin null males compared to wild type males. Additionally, ectopically expressing ovulin in CG15744 knockout females should not rescue the egg-laying and ovulation defect. In addition to generating a CRISPR knockout, another important assay is underway. The UAS-CG15744-Tango construct has been generated and flies have been injected, so data will be available soon as to whether ovulin was able activate the CG15744 receptor in flies. If CG15744 is OvR, I would expect to see expression of the GFP reporter in the female only after she received ovulin from the male.

The lack of response of CG15744 to ovulin in the *in vitro* assay was worrisome as it could indicate that CG15744 is not OvR. However, there are many technical issues that could have led to a negative response: (1) The purified *in vitro*- generated ovulin could be inactive. (2) The purified ovulin was active but not at a high enough concentration (only one concentration was tested). (3) Full-length ovulin does not bind the receptor as efficiently as its cleavage products (Heifetz et al., 2005). Soon after ovulin enters the female reproductive tract, it is proteolytically processed (Monsma et al., 1988; Monsma et al., 1990; Park and Wolfner, 1995). Three sequential cleavages occur and it has been shown that two of those cleavage products, when ectopically expressed in the female, stimulate

ovulation better than full-length ovulin (Heifetz et al., 2005). Recently, I cloned an ovulin cleavage product into a baculovirus expression plasmid in order to purify a cleaved form of ovulin. If purification is successful, the cell culture-based calcium assay will be repeated with the cleaved ovulin.

Although CG15744 shows strong genetic evidence as OvR, there is a possibility that it is not the correct receptor. First of all, an assumption was made that OvR would be a GPCR. There are three other classes of receptors in *Drosophila*: ligand-gated ion channels, single-transmembrane receptors, and nuclear receptors. It is possible that OvR falls into one of these other classes, but we believe that is very unlikely. Most receptors that respond to peptide ligands (like ovulin) are GPCRs (Hewes and Taghert, 2001) and GPCRs in *Drosophila* are very abundant with over 200 predicted (Brody and Cravchik, 2000). Secondly, the ERC method functions under the assumption that two proteins that interact will show a correlated rate of evolution. This may not be the case, however, if the receptor is under different evolutionary constraint (e.g. it has multiple ligands that are involved in conserved pathways). In fact, SP and SPR do not show a strong ERC value (Findlay et al., 2014), likely due to the fact that SPR has multiple ligands (Kim et al., 2010; Poels et al., 2010; Yapici et al., 2008) and is therefore constrained in its ability to evolve with SP (Findlay et al., 2014). Additionally, the ERC screen did not take into account whether certain domains of a protein are

more conserved than others. While the ERC screen is sometimes a helpful tool for identifying potential interactions, it is not without pitfalls. Regardless of whether the ERC was able to predict the ovulin-OvR interaction, it did reveal multiple genes that might be involved in egg-laying, which are still useful in understanding the post-mating response.

In the future, if CG15744 is not OvR, there are multiple directions that could be taken. First, there are other GPCR candidates from the ERC screen that could be tested for an ovulin interaction using the cell culture-based calcium assay or the Tango system. Additional unbiased approaches could be used to identify OvR. For instance, using CRISPR technology, male flies could be generated that have endogenous ovulin with a tandem affinity purification (TAP) tag (Puig et al., 2001). After mating, the females could be used to pull-down ovulin and any proteins that were bound to it and mass spectrometry could be used to identify OvR.

If the search for the ovulin receptor is successful, it would provide invaluable information for understanding how males and females interact on a molecular level during reproduction and how signaling pathways in the female are poised to interact with male proteins to elicit a physiological response.

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CHAPTER 4

SUMMARY AND FUTURE DIRECTIONS

In the two previous chapters, I described my work on two different seminal fluid proteins, SP and ovulin. Understanding how seminal fluid proteins (SFPs) function has broader impacts beyond *Drosophila*. For instance, SFPs may have potential as targets for controlling the reproduction of insect vectors of human diseases (e.g. the mosquitoes that transmit the viruses that cause dengue, zika, West Nile, yellow fever and chikungunya, or the parasites that cause malaria) (Colpitts et al., 2012; Morrison et al., 2008; Pimenta et al., 2015). There are currently no vaccines for these diseases, so methods for reducing their impact on human health should focus on controlling their spread. Targeting key proteins or pathways necessary for their reproduction show significant promise. Unraveling SFP functions and the signaling pathways on which they act in a model such as *Drosophila* will provide information that will help to dissect their roles in insect vectors and identify potential targets for control. Secondly, while some SFPs are rapidly evolving (Clark et al., 2006; Clark and Swanson, 2005; Haerty et al., 2007; Swanson et al., 2001; Swanson and Vacquier, 2002), the protein classes to which they belong are relatively conserved across species (Mueller et al., 2004). Elucidating the mechanisms used by SFPs in *Drosophila* can provide a more

comprehensive understanding of human reproduction and methods to improve assisted reproductive technologies. Current technologies, such as in vitro fertilization (IVF), use sperm that have been separated from the seminal plasma. Recent studies have shown the importance of seminal plasma proteins in fertility [reviewed in (McGraw et al., 2015)].

In chapter 2, I used a previously described method (Cognigni et al., 2011) of measuring nutritional output to further analyze the role of SP in the post-mating change in female intestinal transit. It had been previously established that SP causes an increase in food consumption by the female after mating as an indirect result of SP's role in stimulating egg production (Barnes et al., 2008; Carvalho et al., 2006). More recently, it had been shown that while food intake increases, intestinal transit decreases, as indicated by a production of more concentrated excreta (Cognigni et al., 2011). This response is also dependent on SP, but unlike the increase in food consumption, it is not dependent upon an increase in egg production, indicating a separate role for SP in modulating intestinal transit.

I further investigated this role of SP and determined that like many other SP-induced post-mating responses, SP's modulation of intestinal transit is part of a long-term response that requires SP to bind to sperm and subsequently be cleaved from sperm gradually over several days (Peng et al., 2005) and is partially independent of the SP-induced increase in egg production. My results indicated an

important function for SP in coordinating physiological responses that lead to optimal nutrient intake for the female post-mating.

Recently, studies have shown that after mating, the female *Drosophila* midgut dramatically increases in size presumably to enhance her ability to absorb food, helping her to produce more eggs (Reiff et al., 2015). Interestingly, the authors find that the midgut remodeling occurs prior to changes in nutrient intake and changes in energy demands, not as a result of these changes. This is consistent with my finding that SP's effect on intestinal transit is at least partially independent of egg production. The authors find that juvenile hormone (JH) is released after mating and signals the enlargement of the midgut. Given that SP has been previously linked to a post-mating increase in JH (Moshitzky et al., 1996), it is very likely that SP is involved in the post-mating remodeling of the gut. Another graduate student in the lab (M. White) is currently testing this hypothesis. The *Drosophila* midgut plays vital roles in nutrition, immunity and homeostasis and understanding how male derived proteins can stimulate its remodeling can provide insight into the intricate molecular interactions that occur between males and females after mating has ceased.

In chapter 3, I presented a number of approaches taken to identify the receptor for ovulin, an SFP that increases ovulation by the female within the first 24 hours after mating via octopamine (Heifetz et al., 2000; Rubinstein and

Wolfner, 2013). G. Findlay and I first used a method, an evolutionary rate covariation (ERC) screen, which had previously shown the ability to identify proteins that interact (Clark and Aquadro, 2010). The ERC screen provided us with a number of ovulin receptor (OvR) candidates. I subsequently knocked down each candidate by using RNAi, and measured egg production as a proxy for ovulation rate. My tests of 19 candidates revealed seven whose knockdown showed lower-than-normal egg-laying in the first 24 hours after mating; this is similar to what is seen in females after mating to males lacking ovulin (Heifetz et al., 2000; Herndon and Wolfner, 1995) Of those, one candidate, CG15744, showed expression in the female reproductive tract particularly consistent with a role as an ovulation-enhancer. Also, CG15744 has no known ligands and showed a positive ERC value with ovulin. Thus, I focused on this gene for further biochemical and *in vivo* assays to determine whether it truly is the ovulin receptor (OvR). Although I was unable to complete the assays before graduating, I have established the methods and conditions that can be used by a future graduate student to confirm the interaction.

Although our data are consistent with CG15744 being OvR, we recognize that it is possible that this gene does not encode the ovulin receptor. Our choice of CG15744 as the most likely candidate relied on several assumptions. While these were logical, they were not flawless. First, ovulin's receptor may not have a high ERC value with ovulin if it is under additional evolutionary pressures; this is the

case for the sex peptide receptor (SPR), which has a second, non-reproductive ligand (Kim et al., 2010; Poels et al., 2010; Yapici et al., 2008). Another pitfall of the ERC screen is that it only takes into account the rate of variation across the entire protein sequence. It is possible that ovulin and the OvR have different rates of variation across their entire protein sequences, while sharing correlated rates of evolution only within their interacting domains. Secondly, while our assumption that OvR will be expressed in the female's reproductive tract is very reasonable given that ovulation is mediated by neuromuscular interactions in the reproductive tract, we recognize that it is possible that OvR could act from elsewhere. Although most ovulin remains in the reproductive tract after mating, ~10% of it enters the hemolymph (Lung and Wolfner, 1999; Monsma et al., 1990), which gives it access to other targets, including the neuroendocrine system. An unbiased approach, such as TAP-tagging (Puig et al., 2001) (discussed in chapter 3), may be useful in identifying OvR if CG15744 is not it.

Additional approaches could also be taken to test other candidates from the ERC screen. The fly Tango system (Inagaki et al., 2012) shows a great deal of promise, as it is a highly specific *in vivo* system that does not rely on the ability to make functional ovulin *in vitro* and retain the ovulin-OvR interaction in tissue culture cells. New candidate receptors could be cloned into the Tango vector (described in "materials and methods", chapter 3) in order to be tested for response

to ovulin. Additionally, knockdown candidates from the ERC screen that showed an egg-laying phenotype when mated to wild type males could be mated to ovulin null males and assayed for their egg-laying response. If the egg-laying defect does not worsen upon mating the knockdown female to an ovulin null male, this may indicate that the candidate GPCR and ovulin are involved in the same pathway.

If OvR is not revealed by the ERC screen, there are still a number of receptors that showed an egg-laying phenotype in my screen (chapter 3) and may in fact have other SFP ligands. An experiment is being performed by a graduate student in the lab (S. Delbare) to investigate this possibility by using seminal fluid collected from males as the "ligand" in the cell culture-based calcium assay (chapter 3). Receptors of interest would be cloned into the pME18S-GW vector and transfected into mammalian tissues culture cells as outlined in the "materials and methods". Seminal fluid collected from males would be added to the cells and observed for changes in intracellular calcium. If the receptor has a ligand in the seminal fluid, an increase in intracellular calcium would be expected. Ovulin's effect on egg-laying is small, and there are likely other SFPs involved in the post-mating increase in egg-laying. Another interesting finding from the ERC screen is that an octopamine receptor, OA2, actually caused an increase in egg-laying when knocked down in the female. It would be intriguing to follow-up on this since ovulin (and OvR) act via the octopamine signaling pathway. Due to sexual conflict

(Sirot et al., 2015), it would be a beneficial response for females to evolve a mechanism to keep the effects of male SFPs from increasing her egg-laying to the extent that it is too energetically costly and therefore harmful to her.

Once the ovulin receptor is identified, it should be determined where it is expressed and where it is required for its activity. Using GAL4 drivers in the reproductive tract and nervous system for instance (Cole et al., 2005; Lee et al., 2009; Rezaval et al., 2014) the precise location of its activity could be determined. Knocking down the receptor in specific tissues or subsets of neurons using RNAi and then assessing the female's post-mating ovulation will help to determine the receptor's site of action. Another future direction should involve determining how ovulin's cleavage affects its interaction with the ovulin receptor. Two of ovulin's cleavage products produce a more robust egg-laying response than full length ovulin when ectopically expressed (Heifetz et al., 2005), so using receptor binding affinity assays, it would be interesting to observe whether the cleavage products were able to more strongly bind the receptor.

Finding the ovulin receptor could potentially open the door to better understanding how male SFPs induce a diverse array of physiological and behavioral post-mating responses in the female, leading to a more comprehensive insight into the interactions between males and females at the molecular level.

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APPENDIX

ECTOPIC EXPRESSION OF OVULIN IN THE FEMALE EYE

Introduction

In chapter 3, I discussed methods for identifying a potential receptor for ovulin, an SFP that increases ovulation within the first 24 hours after mating via octopamine signaling in the nervous system (Heifetz et al., 2000; Rubinstein and Wolfner, 2013). Ovulin's site of action is currently unknown and being able to identify the ovulin receptor would provide insight into how male proteins interact with signaling pathways in the female to elicit behavioral and physiological post-mating responses.

Ovulin is detected at the base of the ovaries as early as 3 minutes after the start of mating (ASM) (Lung and Wolfner, 1999) and by 10 minutes ASM, ~10% has entered the hemolymph (Monsma et al., 1990). Ovulin's effects on ovulation can be detected as early as 1.5 hours ASM (Heifetz et al., 2000). Therefore, the interaction between ovulin and its receptor is likely rapid and transient, which limits the availability of assays for detecting the interaction.

Using an evolutionary rate covariation (ERC) screen, we were able to narrow down receptor candidates based on their correlated rate of evolution with ovulin (see chapter 3). While a promising candidate was identified and is currently

being investigated, we sought to determine whether ovulin's site of action could be determined in order to further narrow in on a candidate receptor. I used a GAL4 driver to ectopically express ovulin in the female eye and assayed for its ability to increase ovulation. If ovulin's target is localized to the reproductive tract, it would be hypothesized that expressing ovulin in the eye would not be sufficient to increase ovulation.

Materials and methods

Flies

Flies were reared on standard yeast-glucose media on a 12:12 light/dark cycle. Driver stocks used were temperature sensitive *tubulin*-GAL80^{TS}; *tubulin*-GAL4 (TG80^{TS}; TG4) (McGuire et al., 2003), and *GMR*-GAL4 (Hay et al., 1994) (eye driver). In order to generate a *GMR*-GAL4; *tubulin*-GAL80^{TS} stock (GMRG4; TG80^{TS}), *GMR*-GAL4 flies were crossed to a *tubulin*-GAL80^{TS}/TM2 stock (w*; P{tubP-GAL80^{TS}2/TM2} (Bloomington Drosophila Stock Center, #7017). Non-balancer flies from the F1 generation were further crossed to CSX flies (w*; T(2;3)ap^{xa}, ap^{xa}/CyO; TM3, Sb^{1}) (Bloomington #2475). To confirm the genotype of the final stock, genomic DNA was extracted from ~10 flies and PCR was used to amplify DNA within the GAL4 and GAL80 genes using GoTaq DNA polymerase (Promega) and the following primers: GAL4-F (GGA TGC TCT TCA

TGG ATT TGA TTG G) and GAL4-R (CCA CAT CAT TAG CGT CGG TGA G), and GAL80-F (CGG TAC CAA GGG AGA TTT GA) and GAL80-R (CTT CGA AAT CGA AAC CTT GC).

In order to ectopically express ovulin in the female, the driver lines were crossed to two different UAS-ovulin lines: w; UAS-ovulin (Park and Wolfner, 1995) and UAS-ovulin generated in an attP2 strain (ref). For controls, the driver lines were either crossed to the background strain (attP2) or UAS-GFP (Yeh et al., 1995).

To generate ovulin-null males, flies carrying an ovulin mutation ($Acp26Aa^{1}$) (Herndon and Wolfner, 1995) were crossed to flies carrying a deficiency uncovering the ovulin locus, Df(2L)Exel6014 (Bloomington #7500). Ovulin-null males had the genotype w; $Acp26Aa^{1}$/Df(2L)Exel6014.

24 hour egg-laying assay

For the 24 hour egg-laying assay, 3-5 day old females were mated to 3-5 day old ovulin-null males in single pair matings on standard yeast glucose food that was evenly distributed with minimal imperfections to minimize substrate effects on egg-laying site preference. Matings that lasted under 15 minutes were discarded. After the completion of mating, males were removed from the vials and discarded while females remained in the vials for 24 hours after the start of mating (ASM).

Flies that expressed $TG80^{TS}$ were kept at a permissive temperature until 12 hours prior to the start of the assay, and then transferred to 29°C.

Twenty-four hours ASM, the females were removed from the vials and the number of eggs laid was determined using a dissecting microscope. For statistical analysis, pair-wise comparisons of experimental vs control eggs laid was performed in JMP using a students t-test.

Ovulation assay

For the 3 hour ovulation assay, 3-5 day old females were mated to 3-5 day old ovulin-null males in single pair matings on standard yeast glucose food that was evenly distributed with minimal imperfections to minimize substrate effects on egg-laying site preference. Matings that lasted under 15 minutes were discarded. After the completion of mating, males were removed from the vials and discarded while females remained in the vials for 3 hours after the start of mating (ASM). Flies that expressed $TG80^{TS}$ were kept at a permissive temperature until 12 hours prior to the start of the assay, and then transferred to 29°C. Within the first 3 hours ASM, ovulin's effects on egg-laying and ovulation have been initiated (Heifetz et al., 2000). After 3 hours ASM, females were removed and frozen in liquid nitrogen. They remained at -80°C until the dissections could be performed. Females were removed from the -80°C freezer and placed on ice prior to

dissection. Females were then placed under cold 1X PBS and an incision was made dorsally along the midline to expose the oviducts without disturbing the reproductive tract. The number of eggs found in the lateral oviducts, common oviducts, uterus and external opening was determined, in addition to the number of eggs laid on the food substrate during this 3 hour time period (Heifetz et al., 2000; Rubinstein and Wolfner, 2013). Pair-wise comparisons were performed in JMP using a students t-test.

Results

It is currently not known where ovulin acts to induce ovulation. Although most of the ovulin that is transferred to females during mating is localized to the base of the ovaries, ~10% of it enters the hemolymph (Lung and Wolfner, 1999; Monsma et al., 1990). It is possible that ovulin acts directly on the female reproductive tract, or it is possible that it acts elsewhere since it has access to other targets from the hemolymph, such as the nervous system or endocrine system. In order to investigate this question, I used a GAL4 driver, *GMR* (Hay et al., 1994), to ectopically express ovulin in the female eye and assessed whether the ovulin-induced increase in ovulation still occurred after mating her to an ovulin-null male. I first performed a 24 hour egg-laying assay and a 3 hour egg-laying assay as a proxy for ovulation rate and found that females expressing ovulin in the eye laid

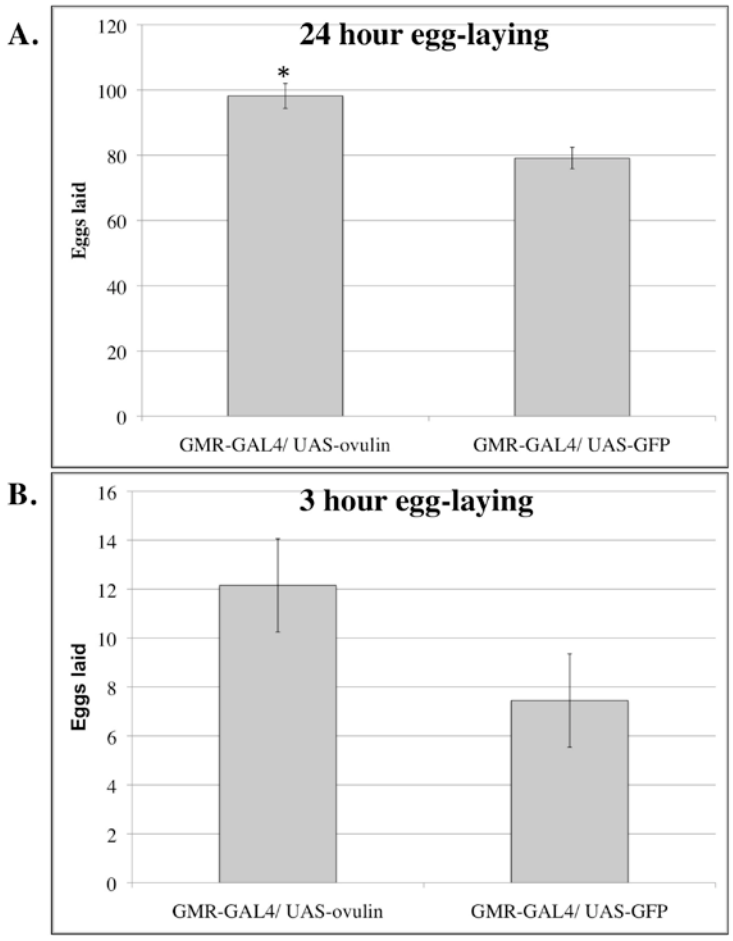

Figure 1. Females ectopically expressing ovulin in the eye laid more eggs than controls after mating to ovulin-null males. (A) Ovulin-expressing females laid significantly more eggs than controls at 24 hours ASM, p=0.0006 (n_{ovulin}=16, $n_{control}$=22). (B) Ovulin-expressing females did not lay significantly more eggs than controls at 3 hours ASM, p=0.0893 ((n_{ovulin}=20, $n_{control}$=20).

significantly more eggs than control females 24 hours ASM (p=0.0006) (Figure 1A) and more eggs than control females at 3 hours ASM, though not significant (p=0.0893) (Figure 1B).

One pitfall of using *GMR*-GAL4 to drive expression of ovulin in the female eye is that when the egg-laying and ovulation assays are performed, females are aged 3-5 days, which means that ovulin has been ectopically expressed for the females' entire lifespan. This raises the possibility that she has maintained an elevated rate of ovulation prior to performing the assay, which may confound the results. A better method would involve conditional expression of ovulin for more precise temporal control. In order to test this, I first attempted a 3 hour egg-laying assay using a ubiquitous temperature sensitive *tubulin*-GAL80^{TS}; *tubulin*-GAL4 (TG80^{TS}; TG4) driver (McGuire et al., 2003). Females were kept at a permissive temperature until 12 hours before the assay and were then shifted to 29°C. I found that females that ubiquitously expressed ovulin laid more eggs than controls (p=0.0492) (Figure 2A).

Ovulin stimulates the release of mature oocytes from the ovary by increasing contractions of the ovarian muscles while simultaneously relaxing the muscles of the oviduct through octopamine signaling in the nervous system (Heifetz et al., 2000; Middleton et al., 2006; Rubinstein and Wolfner, 2013). This phenotype can be measured by counting the number of eggs in the lateral oviducts 3 hours ASM,

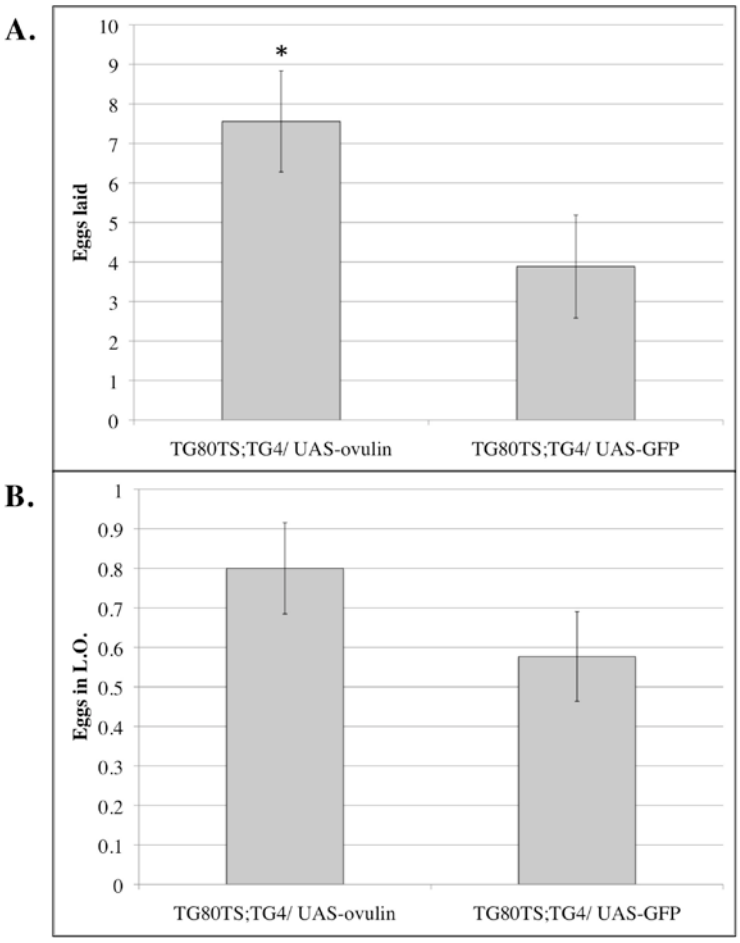

Figure 2. Ubiquitous expression of ovulin using temperature sensitive *tubulin*-GAL80^{TS}; *tubulin*-GAL4 in females increases egg-laying 3 hours ASM after mating to ovulin-null males. (A) The number of eggs laid by females expressing ovulin was significantly higher than controls at 3 hours ASM, p=0.0492 ((n_{ovulin}=27, $n_{control}$=26). (B) The number of eggs found in the lateral oviducts at 3 hours ASM was not statistically significant, p=0.1742 (n_{ovulin}=26, $n_{control}$=25).

a time at which ovulin has initiated the stimulation of the release of mature oocytes from the ovary but increased post-mating oogenesis has not yet commenced (Heifetz et al., 2000).

To determine whether ectopically expressing ovulin ubiquitously in the female would result in an ovulin-induced increase in ovulation, I once again mated $TG80^{TS}$;TG4/UAS-ovulin females to ovulin null males and counted the number of eggs in the lateral oviducts. There were more eggs found in the lateral oviducts of females ectopically expressing ovulin in than control females, though not significant ($p=0.1742$) (Figure 2B). These results indicate that ovulin might be able to increase ovulation even when it is ectopically expressed in the female, consistent with previous findings (Heifetz et al., 2005).

Once it was established that ectopically expressing ovulin using a temperature sensitive *tubulin*-$GAL80^{TS}$; *tubulin*-GAL4 driver could stimulate the ovulin-induced ovulation phenotype, I sought to determine whether a similar result would be observed with a *GMR*-GAL4; *tubulin*-$GAL80^{TS}$ driver, which would allow for spatial and temporal control of ovulin expression. In order to achieve this, I generated a driver line with temperature sensitive *tubulin*-GAL80 in addition to *GMR*-GAL4 (GMR-G4;$TG80^{TS}$). Females were kept at a permissive temperature until 12 hours before the assay and were then shifted to 29°C. I

performed a 3 hour egg-laying assay and found that ovulin-expressing females laid slightly more eggs

than controls, though not significant (Fig. 3). However, the assay was only performed once with a small sample size, so it should be repeated, in addition to an ovulation assay.

Discussion

I have shown data that suggest that ovulin can stimulate and increase in ovulation, even when expressed outside the female reproductive tract. These data indicate that ovulin's target exists outside the reproductive tract and is accessed from the hemolymph. However, further testing should be done using the GMR-G4;TG80^{TS} driver line. Only one 3 hour egg-laying assay was performed. Additional assays should include a 24 hour egg-laying assay and an ovulation assay. Also, ovulin expression should be confirmed either by Western blot or RT-PCR.

While the data suggest that ovulin's site of action is outside the reproductive tract, there are several considerations to be made. First, it is possible that even though ovulin expression is being driven in the eye, ovulin may still be entering the reproductive tract. The eye was chosen due to its distance from the reproductive tract, but this possibility cannot be eliminated. In fact, it was recently shown that

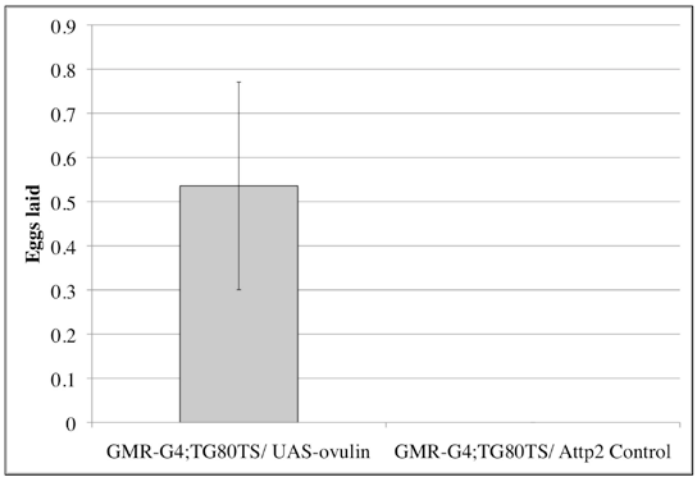

Figure 3. 3 hour egg-laying assay on females conditionally expressing ovulin in the eye using the GMR-G4; $TG80^{TS}$ driver. After mating to ovulin-null males, the difference in eggs laid was not significant between ovulin-expressing females and controls, $p=0.1095$ ($n_{ovulin}=28$, $n_{control}=29$).

when expression of a GFP-tagged sex peptide (SP) was driven by GMR-GAL4, it was able to localize to the oviducts and spermathecae (Tsuda et al., 2015). Western blot or RT-PCR on the female reproductive tract could be used to determine whether ovulin has entered it. Secondly, it was shown that *GMR*-GAL4 may drive expression elsewhere besides the eye, including the brain, trachea and leg discs (Li et al., 2012). The leakiness of the *GMR*-GAL4 driver may have led to the results discussed above. Although this assay cannot conclusively determine the site of ovulin action, it may provide insight into where to look for the ovulin receptor (see chapter 3).

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