Review

Neurobiology of Egg-Laying Behavior in *Drosophila*: Neural Control of the Female Reproductive System

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Abstract: Egg-laying is one of the key aspects of female reproductive behavior in insects. Egg-laying has been studied since the dawn of *Drosophila melanogaster* as a model organism. The female's internal state, hormones, and external factors, including nutrition, light, and social environment, affect egg-laying output. However, only recently has light been shed on certain neurobiological features of egg-laying behavior in both the central and peripheral nervous systems. The central nervous system regulates egg-laying behavior and decision-making, and many studies use *fruitless* and *doublesex*, two genes in the sex determination pathway, to identify reproductively important neurons. Neuronal groups like aDNs and pC1clusters modulate egg-laying behavior in the brain, and other neurons like oviINs and oviDNs affect oviposition specifically. In the ventral nerve cord, the abdominal neuromere houses neurons that send information to and from the reproductive tract, including sex peptide abdominal ganglion neurons (SAG) and octopaminergic motor neurons. The reproductive tract itself houses sensory neurons that respond to different aspects of egg maturation and mating, including the male accessory gland products and mechanical stimuli. I conclude this review by summarizing the importance of egg-laying neuronal control in various evolutionary phenomena like cryptic female choice and highlight two *Drosophila* species and provide intriguing avenues for the future of the field.

Keywords: reproduction; egg-laying; cryptic female choice; *Drosophila*; sensory neurons

Introduction:

Reproduction is a costly phenomenon that shapes the life history of a species through phenomena like decreased survival and a lower number of future reproductive opportunities (Harshman & Zera, 2007; G. C. Williams, 1966). Animals have limited energy resources, which results in trade-offs between different physiological functions, behavior, and reproduction (Harshman & Zera, 2007). Specifically, the reproductive costs to the female can be grouped into resource-allocation costs, for instance, production of the yolk protein, and non-resource-based costs, which include hormonal changes to the female body (T. D. Williams, 2005). Egg production and oviposition are major contributors to this costly phenomenon in females (Azevedo et al., 1997; Rosenheim et al., 2000). Therefore, egg-laying behavior needs to be closely regulated by the female's physiology and neuronal systems. Hormones and peptides have a long-term effect and change the physiology of the female animal. For example, insulin-like peptides in *Drosophila* play a key role in oogenesis by regulating stem cell division in response to nutrition intake (LaFever & Drummond-Barbosa, 2005), resulting in a more long-term effect in the female body. In contrast, neurons provide a more immediate response. For example, recent studies have discovered specific neuronal candidates affecting oviposition in female *Drosophila* and artificial activation of some of these neurons results in the animal exhibiting oviposition sequence (F. Wang, Wang, Forknall, Patrick, et al., 2020).

Egg-laying behavior needs to provide enough flexibility for the female to produce offspring while encountering variable external environments (Kienzle et al., 2020; Reguera, 2002; Roy et al., 2018; Van Wielendaele et al., 2013) and physiological changes (Roy et al., 2018; J. Xu et al., 2010). For instance, unlike *Drosophila melanogaster*, *D. suzukii* prefers healthy unwounded fruit as an egg-laying

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substrate. Kienzle et al. (2020) showed that *D. suzukii* will lay its eggs on wounded fruit when the healthy fruit becomes less available. Conversely, change in external environmental factors affects the internal physiology of the female. In mosquitos, the expression of microRNA-309 is dependent on the blood meal, and disruption of microRNA-309 results in failure of ovarian development (Y. Zhang et al., 2016). These regulations on egg-laying, whether promoted by hormones or neurons, correlate directly with the female's fitness and ensure that the female's genes reach the next generation.

Since the early days of *D. melanogaster* as one of the model organisms for genetics, egg-laying behavior has been a phenotype that caught the interest of biologists. Egg-laying has been used as a phenotype to study hybridization (Engels & Preston, 1979; Price et al., 2001; Sturtevant, 1920), as an indicator of fecundity (H. Shapiro, 1932; Tantawy & Vetukhiv, 1960), and for quantitative genetics (McMillan et al., 1970). Egg-laying has been studied in connection with female life-span (Partridge et al., 1986, 1987) later came into light as a proxy for cryptic female choice (Frazee and Masly, 2015; Frazee et al., 2021; LeVasseur-Viens et al., 2015) and decision-making (Yang et al., 2008). Studying the egg-laying of *Drosophila* using genetic tools has always been a subject of interest for researchers, and many studies have focused on the production of the egg itself (Hall & Greenspan, 1979; Wieschaus, 1980). Early studies on female *Drosophila* focused on reproductive behavior holistically (Manning, 1967) or used decapitated females to assess the neuronal control of egg-laying between closely-related *Drosophila* species (Grossfield & Sakri, 1972). In recent years, the neurobiology of egg-laying and how females control their reproductive output has received more attention in insects, including oriental tobacco budworm (C. Wang et al., 2020) and bean bug (Hasebe & Shiga, 2021). More in-depth studies have been conducted on specific neurons affecting fruit-fly egg-laying behavior (Rezával et al., 2012, 2014; Shao et al., 2019; F. Wang, Wang, Forknall, Patrick, et al., 2020; K. Wang et al., 2021; Yang et al., 2008).

Egg-laying involves different neuronal components (Figure 1) that overlap with other aspects of female reproductive behavior and motivation (Ishimoto & Kamikouchi, 2021). Ovipositor extension can be part of mating, rejection, and egg-laying behavior. During mating, the female extends her ovipositor horizontally, whereas, during egg-laying, the female extends her ovipositor toward the substrate and bends her abdomen (K. Kimura et al., 2015). This stereotypical pattern lasts around six seconds and is called the "ovipositor motor program" (Yang et al., 2008). Internally, ovarioles need to be stimulated to produce more oocytes, the mature egg needs to move from the ovariole to the uterus via the oviduct, and the female needs to decide on a good location to lay her eggs; all of these components involve neuronal control (Rodríguez-Valentín et al., 2006; White et al., 2021; Yang et al., 2008).

Studying reproduction without also considering hormonal control is impossible. There are studies in many groups of dipterans that hormonal control plays a significant role in egg-laying. Hormonal control has been studied extensively in *Aedes aegypti* since the 1970's (Gwadz & Spielman, 1973; Wheelock et al., 1988). For instance, blocking the decrease in juvenile hormone (JH) levels after a blood meal results in abnormal egg development and decreased egg hatching (A. B. Shapiro et al., 1986). JH (J. P. Shapiro & Hagedorn, 1982), and ovarian ecdysogenic hormone (OEH) (Matsumoto et al., 1989) are two of the neurosecretory hormones that modulate follicle maturation and vitellogenesis in *A. aegypti* (Nijhout, 1998). In the case of OEH, *D. melanogaster* sister species offer a curious case: they lack the gene *neuroparsin,* which is the homolog of OEH and widely conserved among many insects (Veenstra, 2010). Instead of OEH specifying egg maturation, in *D. melanogaster*, JH modulates egg maturation (Bilen et al., 2013). After eclosion, the corpus allatum produces JH that stimulates the yolk protein production in the fat body (Jowett & Postlethwait, 1980) (Figure 1 b). The fat body and follicle cells synthesize yolk proteins that are vital for the development of the oocyte (Mirth et al., 2019). With the sexual maturation of the female fruit fly, JH levels fall. Yolk production starts again with the stimulation provided by sex peptide (SP), an accessory gland protein that is transferred from the male to the female reproductive tract during mating (Soller et al., 1997). It has been shown that hormones can change the firing properties and ion currents of neurons in mice (DeFazio & Moenter, 2002). However, the effect of hormones and SP on *Drosophila*'s neurons is understudied. Experiments with JH suggest that this hormone plays an active role in dimorphic behavior between males and

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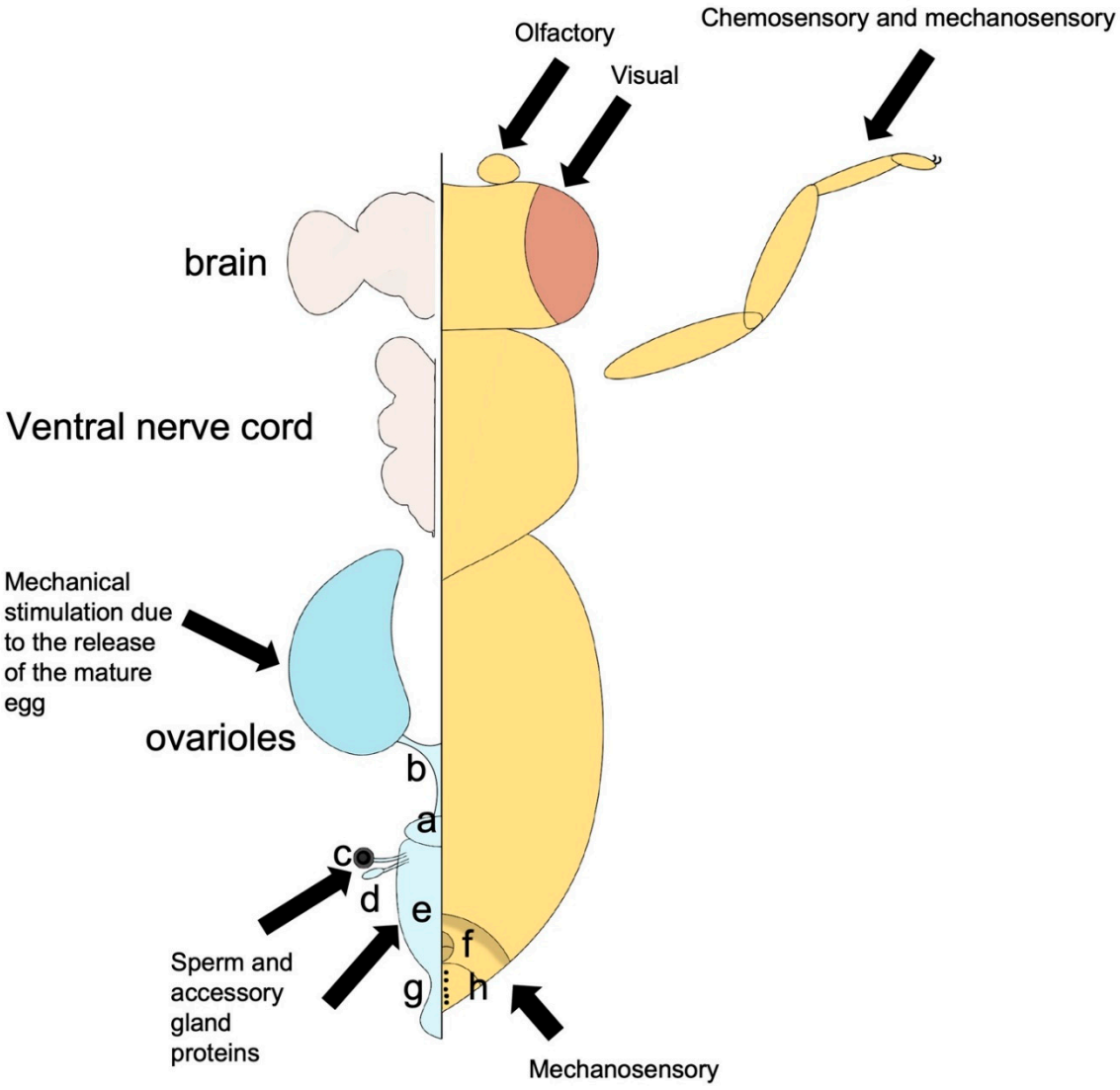
females. For instance, inhibiting JH activity by feeding the flies Fluvastatin, a drug that inhibits JH, results in the feminization of the walking behavior of male flies (Belgacem & Martin, 2002). The intertwined nature of hormonal and neuronal control calls for more in-depth studies of hormone titer's effect in female *Drosophila*'s neurons.



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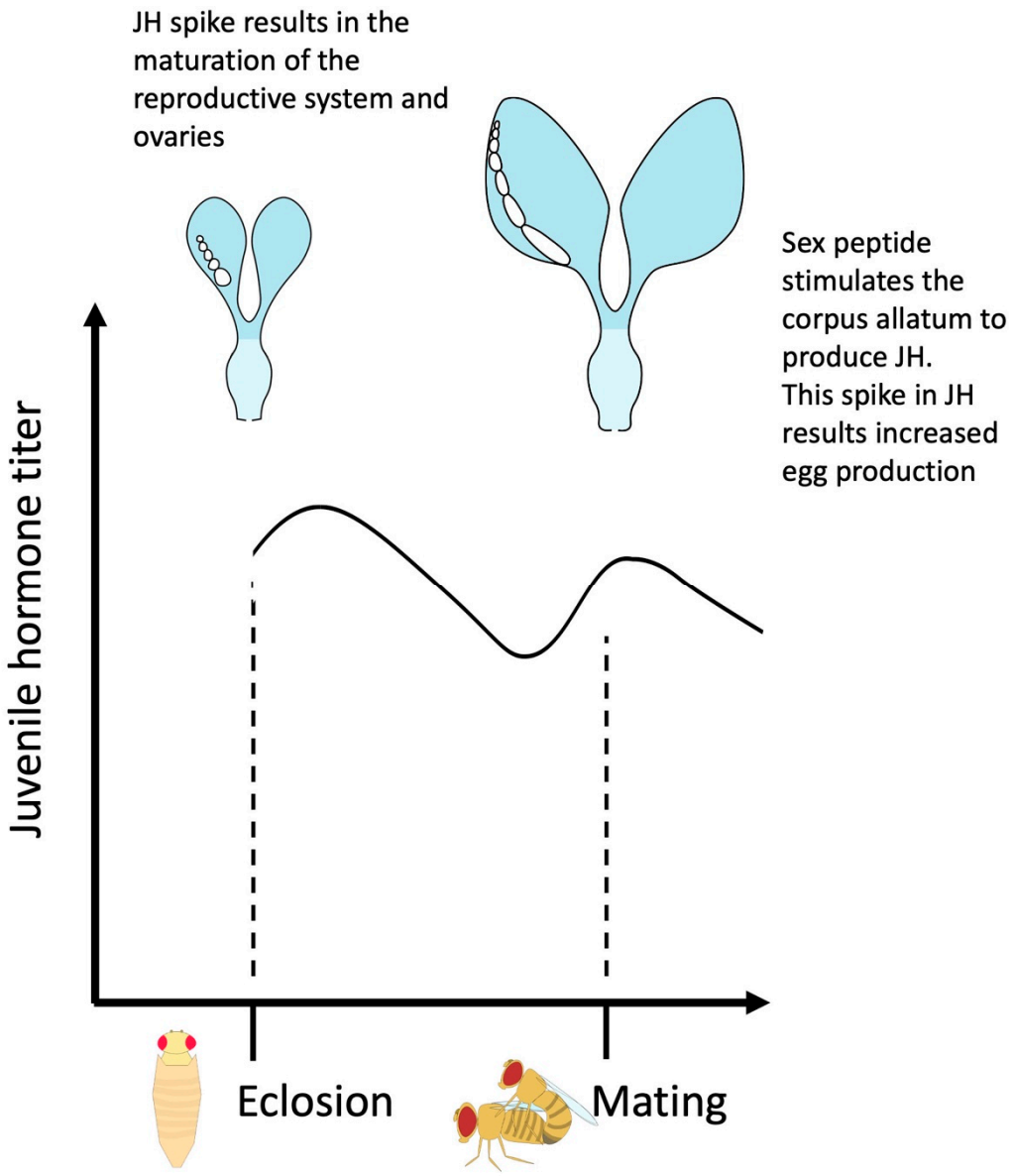

(**b**)

Figure 1. (**a**) The female reproductive structures and sensory input. a. Seminal receptacle, b. oviducts, c. spermatheca, d. Female accessory glands, e. Uterus, g. The internal part of the ovipositor, f. Anal plate, h. Ovipositor. Arrows indicate locations where females receive sensory inputs that direct egg-laying behavior. (**b**). The Juvenile hormone (JH) titer throughout the adult female *D. melanogaster*'s life. After eclosion, the JH spike results in the maturation of the female reproductive tract.

Some aspects of the environment modulate *D. melanogaster*'s fecundity. For example, the nutritional environment affects the egg-laying output and site selection. Lifetime egg-laying of female *D. melanogaster* increases with an increase in the in food quality (Tracey Chapman & Linda Partridge, 1996). The egg-laying behavior is also affected by the site-selection decision-making process. *Drosophila* females lay fewer eggs when no appropriate sites are available (Eisses, 1997). The presence of chemical cues in the food affects the egg-laying output. For instance, Geosmin-sensing neurons express Odorant receptor 56a (Or56a) and target a brain region called DA2 glomerulus to inhibit oviposition and feeding (Stensmyr et al., 2012). The presence of external and internal pathogens modulates egg-laying as well. Flies avoid laying eggs on contaminated food by an olfactory circuit in the brain that represses the egg-laying circuit (Stensmyr et al., 2012). Further, bacterial infection reduces the rate of egg-laying in female *Drosophila* through the NF-kB signaling pathway (Kurz et al., 2017). For example, Schwenke and Lazzaro (2017) have shown that JH affects both increased egg

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laying and decreased infection resistance, which results in immunosuppression in mated females (Schwenke & Lazzaro, 2017), and thus illustrates a trade-off between a physiological function (immune response) and reproduction.

The light environment is another factor that affects egg-laying decisions. Markow (1975) showed that genetic variation exists for light dependent behaviors and established that some fly strains lay more eggs in the dark while others lay more eggs in light treatment. Female flies display UV aversion for egg-laying site selection. They sense this specific light environment using their R7 photoreceptors (Zhu et al., 2014). *Gr66a*-expressing neurons on the leg induce aversion, while *Gr66a*-expressing neurons in the pharynges induce attraction to the chemical lobeline as an egg-laying site (Joseph & Heberlein, 2012). *Gr66a*-expressing bitter-tasting neurons that are sensitive to H_2O_2 also promote UV avoidance in the egg-laying site selection (Guntur et al., 2017).

In addition, the social environment affects the fruit fly's behavior. Mating is known to increase aggression in females (Bath et al., 2017), and field studies of oriental fruit flies have shown that females increase their aggression to defend their egg-laying sites (Shelly, 1999). However, increased aggression in the social environment does not directly affect egg-laying (Bath et al., 2017, 2018). A recent study found that non-egg excrements of other females, combined with the female's social exposure before the mating, can modulate the number of fertilized eggs by the female (Fowler et al., 2022). The presence of a male-produced pheromone affects female egg-laying site selection by activation of *Or7a*+ neurons. Additionally, *Or7a* mutant and *Or7a* ablated flies lay a higher number of eggs (Lin et al., 2015). Chemical cues by other mated females also guide the female egg-laying site selection (Duménil et al., 2016). Olfactory cues are one of the key players in *D. suzukii*. Orco-expressing sensory neurons are necessary for the egg-laying site selection of this species (Karageorgi et al., 2017).

Until this point, I covered external factors and hormones that affect egg-laying. From here, I will focus on the neurons in the brain and the abdominal neuromere (abdominal ganglion), where a significant part of processing sensory clues and regulating egg-laying behavior occurs. I will discuss the sensory neurons, specifically on the abdomen, and focus on neurons that detect important signals during and after mating that have been shown to affect egg-laying. Lastly, I explore the field's future and areas that show potential for expansion. Neurobiology of egg laying in *Drosophila* offers exciting avenues to study evolutionary forces like cryptic female choice using genetic tools already available in this classic model.

Major sex determination genes as indicators of central neurons involved in female reproduction

Doublesex (*dsx*) and *fruitless* (*fru*) are two major sex determination genes (Billeter et al., 2006) (Figure 2). *Dsx* is involved in male and female developmental differentiation and regulates sex-specific behavior in both sexes (Waterbury et al., 1999). *Fru* is a major player in male courtship behavior and is involved in the development of male-specific muscle of Lawrence (Billeter et al., 2006). Neurons expressing either or both of these genes are usually important for specifying reproductive behavior (K. Kimura et al., 2008; Sanders & Arbeitman, 2008; K. Wang et al., 2021; Waterbury et al., 1999; Yamamoto & Koganezawa, 2013). For instance, the elimination of synaptic transmission with tetanus toxin in *dsx*-expressing neurons results in no egg laying in female *D. melanogaster* (Rideout et al., 2010). Further dissection of this phenotype shows that sperm were transferred normally to these females, and inseminated females produced mature oocytes. This result suggests that infertility is due to mature oocytes not being moved into the uterus and being fertilized (Rideout et al., 2010), indicating an essential role for *dsx* neurons in egg laying.

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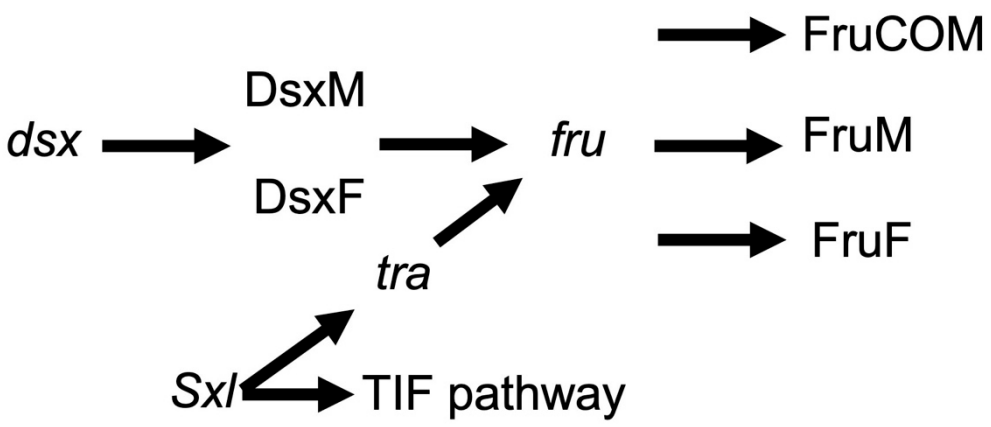

Figure 2. Sex determination cascade in *Drosophila*. The genes *fruitless* (*fru*) and *doublesex* (*dsx*) are critical players in sex determination, and both are expressed in various neurons involved in reproductive behavior. *Sex-lethal* (*Sxl*) regulates the expression of *transformer* (*tra*) and controls aspects of female neuronal development through the *tra*-insufficient feminization (TIF) pathway. *fru* is in turn regulated by *tra*. Expression of *tra* in females modulates *fru* expression and results in a small peptide called FruF with no known function. However, *fru* in the males gives rise to FruM and gives rise to male-specific characteristics.

The gene *dsx* encodes an mRNA that is differentially-spliced to produce sex-specific protein isoforms, one of which is the Dsx^F protein in female *Drosophila*. Dsx^F protein is crucial for sexual differentiation including production of female pheromones and yolk proteins (Baker & Wolfner, 1988; Waterbury et al., 1999). *Dsx*-expressing neurons have a wide distribution in the female central nervous system (CNS). In adult females, *dsx* is expressed in 700 CNS neurons and there are 30-40 *dsx*-expressing neurons in each brain hemisphere of female *Drosophila*, specifically around 16 cells in the pC1 and pC2 regions (Sanders & Arbeitman, 2008). As discussed below, the pC1 region houses neurons that modulate egg-laying. *Dsx*-expressing neurons that affect egg-laying are not limited to the brain. *Dsx*-expressing neurons in the ventral nerve cord are sufficient to elicit an ovipositor extrusion and egg ejection behavior (K. Kimura et al., 2015). Other genes affecting *dsx* expression might have an effect on egg-laying as a result. For instance, deletion of *mir-iab-4/8* and *homothorax* (*hth*) mutants result in increased egg-laying and decreased receptivity, thus showing that *mir-iab-4/8* and *hth* regulate *dsx* expression levels during development (Garaulet et al., 2021). Other studies have shown that co-expression of the gene *dissatisfaction* (*dsf*) and *dsx* in abdominal neuromere interneurons modulates vaginal plate opening, ovipositor extrusion and movements of uterus walls (Duckhorn et al., 2022). Taken together, these studies suggest a critical role for *dsx*-expressing neurons in female reproductive behavior, including egg-laying.

The other major sex-determination gene *fru* has sex-specific splice isoforms as well as common isoforms. *Fru* is a complex locus that has at least four promoters (P1-4), and only transcripts produced from P1 are sex-specific. Female-specific splice of Fru (Fru^F) has no known function (Demir & Dickson, 2005). It has been shown that the male-specific P1 *fru* promoter is active in some neurons in the female and affects egg-laying behavior (Kvitsiani & Dickson, 2006). Recent studies have shown that the non-sex-specific Fru proteins (known as FruCOM) are expressed throughout the developing nervous system (Sato & Yamamoto, 2022). Other studies have shown that there are five *fru*-expressing mAL interneurons present in the female brain (Goto et al., 2011; K.-I. Kimura et al., 2005). Many of the recently described neurons in the egg-laying circuit are *fru*-expressing, including oviDNs (F. Wang, Wang, Forknall, Patrick, et al., 2020). These results suggest that even though there is no known function for Fru^F protein in the female *Drosophila*, neurons that have an active *fru* regulatory region are involved in egg-laying and other reproductive behaviors.

Fru and *dsx* are not the only genes that regulate sexual differentiation and behavior (Figure 2). Other genes that have been shown to affect female behavior include *Sex-lethal* (*Sxl*)(Evans & Cline, 2013; Sawala & Gould, 2017), *retained* (*retn*)(Shirangi et al., 2006), and *transformer* (*tra*) (Castellanos et

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al., 2013) and may prove valuable in further determining the pathways involved in female egg-laying. *Sxl* has been shown to have targets other than transformer and through a branch of downs stream genes, collectively called *tra*-insufficient feminization (TIF) pathway, controls the development of *fru+* neurons that regulate passage of the mature egg to the oviduct (Evans & Cline, 2013).

Central nervous system: The Brain

The *Drosophila* brain is one of the well-studied animal brains in terms of circuits and neuronal connectivity (Dorkenwald et al., 2022; Schlegel et al., 2017; C. S. Xu et al., 2020). The adult fly brain has around 135,000 neurons (Bates et al., 2019) and shows a strong sexual dimorphism both in the number of neurons and the presence and absence of them (Cachero et al., 2010). Anatomically, the *Drosophila* brain has multiple regions, some of which have a more prominent role in the modulation of reproductive behavior.

The female brain has several distinct groups of neurons that are important in egg-laying (Figure 3). For example, pC1 neurons are a group of neurons in the brain that express *dsx* (Zhou et al., 2014). P1 neurons are a male-specific subset of pC1 neurons that express both *fru* and *dsx* genes (K. Kimura et al., 2008). P1 neurons are located near the mushroom body and are composed of 20 interneurons. In females, most P1 neurons die as the result of the female-specific *dsx* transcript (Sanders & Arbeitman, 2008). It has been suggested that pC1 neurons receive information of the female reproductive state and regulate the output accordingly (F. Wang, Wang, Forknall, Patrick, et al., 2020) (Figure 1 a, b). Sex peptide abdominal ganglion (SAG) neurons are one type of these neurons that send information on the female reproductive state to the brain (Feng et al., 2014). In a recent study, Wang et al. (2020) have shown that out of five morphologically different pC1 neurons (pC1a-e) in each hemisphere, three (pC1a-c) have an amplitude of synapses with SAG neurons and the other two have synapses to a lesser degree. Out of the five pC1 neurons, photoactivation of SAG neurons induces the strongest activation in pC1a neurons. The neurons in the pC1 cluster have synapses with each other. Experiments have shown that virgin females with ablated pC1 neurons lay more eggs (F. Wang, Wang, Forknall, Parekh, et al., 2020). These findings, along with the fact that cholinergic pC1 neurons have only a few synapses with oviposition descending neurons, suggest that pC1 neurons suppress egg laying in virgin females by an indirect connection. (F. Wang, Wang, Forknall, Patrick, et al., 2020).

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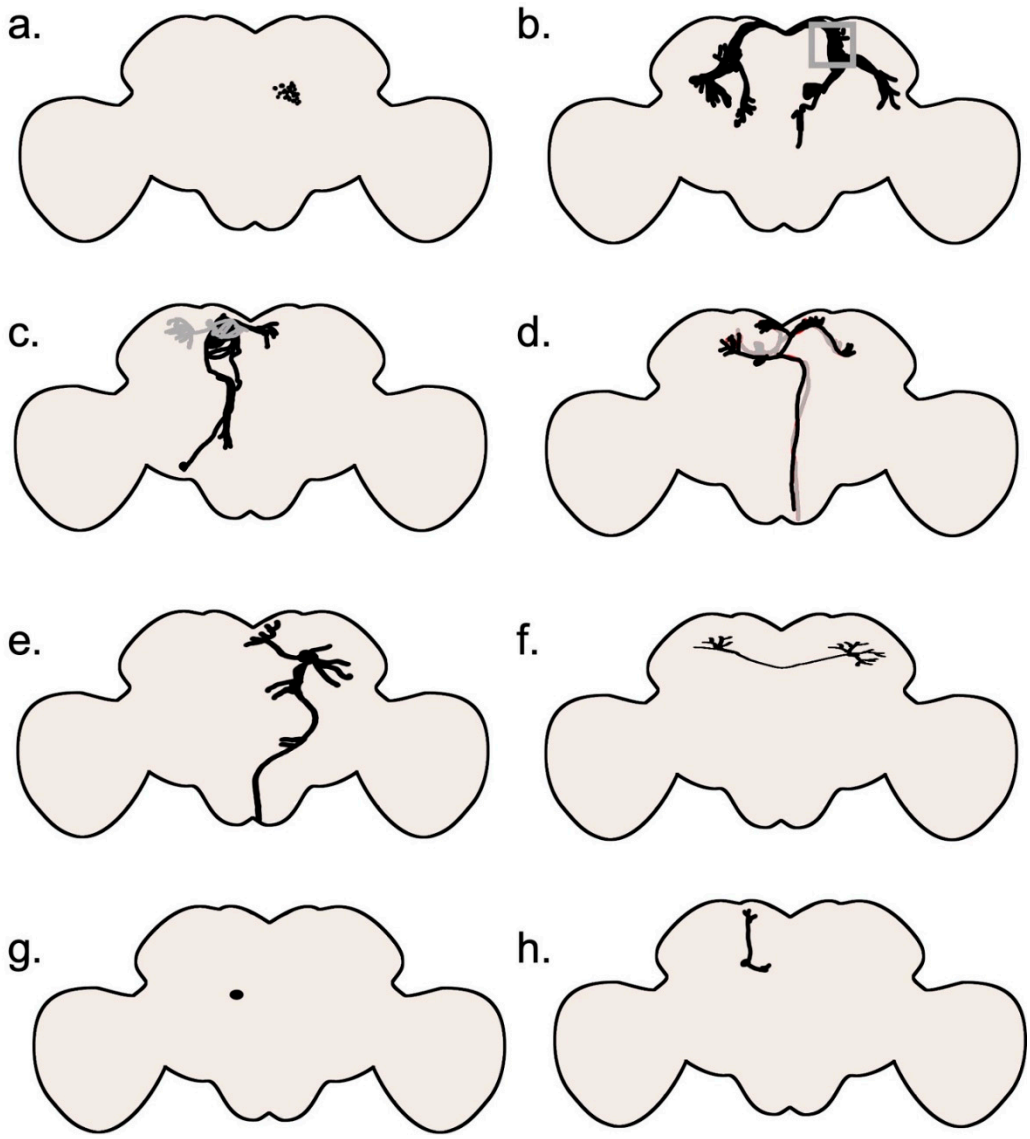

Figure 3. Schematic fly brain and location of neurons known to affect egg laying. Only cells from one hemisphere of the brain are depicted. a. pC1 neuron cell bodies. b. pC1 neurons shown with their arborizations; the Lateral Junction, a site of convergence for the majority of *Dsx+* neuron projections, is highlighted with red box. c. oviIN (shown in black) and oviEN (shown in grey) neurons. d. oviDNa (shown in black) and oviDNb (shown in grey) neurons. e. pMN2 neuron. f. pC2l neuron. g. DNp13 (pMN1) neuron cell body. h. dorsal paired medial (DPM) neuron.

Another subset of pC1 neurons, pC1d and pC1e have a role in receptivity and affect aggression in combination with alPg neurons (Deutsch et al., 2020). SAG neurons connect directly to pC1 neurons; pC1 neurons, in turn, are connected to oviposition inhibitory neurons (oviINs) (K. Wang et al., 2021) (Figure 3 c). There is one oviIN per hemisphere and silencing their activity results in higher egg-laying in virgins, whereas increasing their activity in mated females reduces their egg-laying (K. Wang et al., 2021). The activity of oviposition descending neurons (oviDNs) induces egg deposition behavior (F. Wang, Wang, Forknall, Patrick, et al., 2020). The cell body of these *fru+ dsx-* cholinergic neurons resides in the brain, and they enervate the A ganglion in the ventral nerve cord (Figure 4 c). The two types of oviDNs, ovDNa and oviDNb, have similar arborization in the brain, but differ in their axonal branching in the abdominal ganglion (F. Wang, Wang, Forknall, Patrick, et al., 2020) (Figure 3 d). The GABAergic oviposition excitatory neurons (oviENs) send excitatory signals to oviDNs playing a key role in the regulation of egg laying in the process (F. Wang, Wang, Forknall, Patrick, et al., 2020) (Figure 3 a). For example, it is known that vaginal plate opening is an important

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response to courtship and is controlled by a pair of female-specific descending neurons (vpoDNs) (K. Wang et al., 2021). However, the specific role of these neurons in vaginal plate opening during egg laying is unknown. These neurons get excitatory input from auditory neurons (vpoENs) and pC1 neurons (K. Wang et al., 2021).

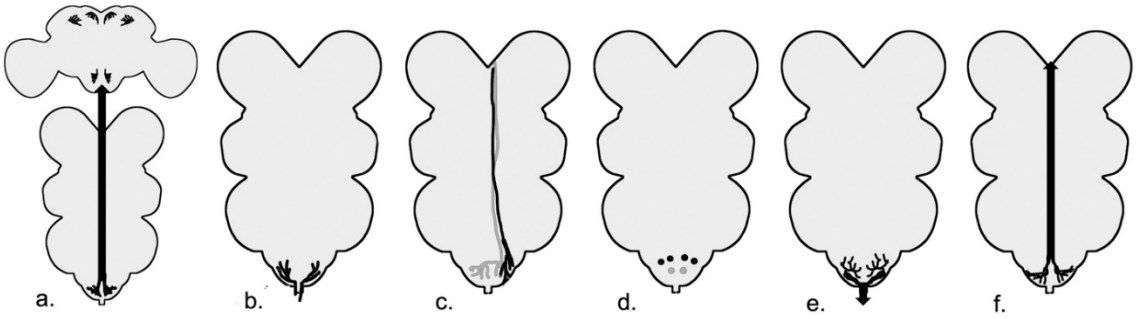

Figure 4. The schematic diagram neurons arborizing or residing in the abdominal neuromere (ANm). a. The SAG neuron in the ANm and their arborization in the brain. b. SPSN arborizations in the ANm. c. Right oviDNa (shown in black) and oviDNb (shown in grey) arborizations descending from the right hemisphere of the brain. d. MIP expressing vAL (*dsx*+) neurons (black) and vAM neurons (grey). e. CMU neurons. d. LSANs.

The *dsx*-expressing anterior dorsal neuron (aDN) cluster has two glutaminergic (Milyaev et al., 2012) neurons per hemisphere. The aDN has dimorphic dendritic arborizations between the sexes. Male aDN receives visual information, whereas the female receives multiple sensory signals, with the main senses being olfactory, thermosensory, and hygrosensory. Interestingly, oviposition excitatory neurons (oviENs) have the most synaptic input, including axo-axonic connections to the aDNs. Oviposition inhibitory neurons (oviINs) also form connections with axons of aDNs. Optogenetic activation of olfactory neurons has been shown to increase the calcium signal in the female aDN neurons. Further, this calcium signal is more intense in mated females than in virgin females. Axons of aDN neurons are restricted to the superior medial protocerebrum (SMP) and specifically have a direct output to oviDNa. Another one of the postsynaptic targets is a neuron called SMP156, which suggests that SMP156 might be a neuron joining together different parts of the egg-laying circuit (Nojima et al., 2021). The discovery of aDNs not only provides evidence for the use of sexually divergent sensory input and behavior output by the same neurons in males and females, but also sheds light on another piece of the egg-laying circuit puzzle.

Other noteworthy neurons include pC2l and pMN2 neurons (K. Kimura et al., 2015) (Figure 3 e, f). pMN2 are female-specific *dsx*-expressing neurons that are responsible for extrusion of the ovipositor during oviposition, while pC2I neurons are responsible for extrusion of the ovipositor during mating (K. Kimura et al., 2015). Kimura et al., (2015) suggest that pMN2 neurons direct egg laying and activate the VNC motor program. Other studies suggest that oviposition command was mis-assigned to pMN2 (Wang et al., 2021 supplement). DNp13 (pMN1) are *dsx*+ neurons that control the ovipositor extrusion in mated females and form synaptic connections with SAG neurons and pC1 neurons. Silencing the activity of DNp13 neurons does affect the number of eggs laid (Mezzera et al., 2020) (Figure 3 a). The activity of two dorsal paired medial (DPM) neurons is important for maintaining the egg-laying substrate choice, and expression of the gene *amnesiac* is necessary for the correct function of these two neurons (Wu et al., 2015) (Figure 3 h).

Another class of key neurons is myo-inhibitory peptide (MIP) expressing neurons. There are an average of 24 *Mip*-expressing neurons in each hemisphere(Shao et al., 2019). Previously, it has been shown that some MIP neurons affect satiety and affect body weight (Min et al., 2016). There are *fru*+ MIP neurons in the female brain. However, these neurons show no effect on female receptivity or remating (Jang et al., 2017). Interestingly, Shao et al. (2019) showed that acute photoactivation of brain *Mip*-expressing neurons can result in complete suppression of egg-laying. These results suggest an

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important role for *Mip*-expressing neurons in the egg-laying circuit, but await further characterization of their connection to other key neurons in the circuit.

The abdominal neuromere in the ventral nerve cord:

In adult *Drosophila,* the neuromeres of the ventral nerve cord (VNC) are merged to form segmental thoracic neuromeres and a smaller fused neuromere for the abdominal sections (Court et al., 2020). The *Drosophila* VNC has around 20,000 neurons (Bates et al., 2019) and the abdominal neuromere (ANm) or abdominal ganglion (AG) is the most posterior segment of *Drosophila*'s VNC (Court et al., 2020) (Figure 4). Unlike its name, this fusion of abdominal neuromeres resides completely in the thorax and connects to the abdomen through the main nerve called the abdominal nerve trunk (AbNT). There are four other bilaterally-paired nerves coming out of ANm or its adjacent regions (AbN1-4)(Court et al., 2020), but for the purpose of this review, I will focus only on ANm and AbNT. Most of the cell bodies of neurons reside on the outer cortex of ANm and project toward the center (Court et al., 2020). Some subsets of ANm neurons have been studied regarding egg-laying behavior and regulation. Studies have shown that the activation of *dsx-GAL4+* in the ventral nerve cord results in oviposition extension and egg ejection in some, but not all, decapitated female flies (K. Kimura et al., 2015). There are roughly 300 *dsx+* neurons in the ANm (K. Kimura et al., 2015).

SP abdominal ganglion (SAG) neurons (Figure 4 a), as the name suggests, are *dsx+* and *Fru-*female specific cholinergic neurons that reside within ANm and receive information from sex peptide sensory neurons (SPSNs) (Feng et al., 2014) (Figure 4 b). There are two SAG neurons, one on each side of ANm, and they send their projections bilaterally to the dorsal protocerebrum and ipsilaterally to the periesophageal regions in the brain (Feng et al., 2014). SAG neurons have excitatory synapses with neuropeptide allatostatin-C (AstC)-producing neurons in the brain; mating silences the SAG-Ast-C axis and results in the production of JH and vitellogenesis (C. Zhang et al., 2022). Reduction of the activity of SAG neurons reduces female receptivity and increases the number of eggs laid by the female fly (Feng et al., 2014) and increases the appetite for salt and yeast (Walker et al., 2015). The SPSN-SAG axis modulates post-mating decrease in sleep (Garbe et al., 2016). Studies suggest that SAGs are not the only pathway regulating female behavior downstream of SPSNs (Feng et al., 2014). There are four *dsx/ETFLP250* neurons in the abdominal neuromere with the opposite effect of SAG neurons that send their axons to SOG as well (K. Kimura et al., 2015; Rezával et al., 2012).

Octopaminergic neurons regulate female reproduction in many insects (White et al., 2021). In the locust, *Locusta migratoria*, digging and egg retention are under the control of coordinated central pattern generators (CPGs) in the abdominal ganglion. Octopamine modulates egg laying by inhibiting digging behavior and relaxing the oviducts (R. Wong & Lange, 2014). Ovipositor valve contractions are the result of rhythmic activity of the ovipositor muscle, which in turn is controlled by the initiator and oscillator interneurons in the terminal abdominal ganglion (Ogawa et al., 2011). Even though CPG's are not commonly studied in terms of female *Drosophila* reproduction, studies on other species provide insight on how fruit fly's egg-laying is modulated. In *D. melanogaster*, oviduct contraction is the product of a set of neurons in the abdominal neuromere that is both glutamate and octopamine-releasing. Octopaminergic neurons are inhibitory, whereas glutaminergic neurons excite the muscles (Rodríguez-Valentín et al., 2006). The ring-like muscles rhythmically contract and relax to push the egg forward in the oviduct (Hudson et al., 2008) and subset of *Insulin-like peptide 7* (*Ilp7*) expressing neurons are glutaminergic motor neurons that enervate the oviduct and are necessary for egg-laying (Castellanos et al., 2013). Studies have shown that female flies that lack octopamine retain their developed eggs (Monastirioti et al., 1996), while additional studies have shown that the octopamine neurons enervate ovaries and oviducts and trigger ovulation (Monastirioti, 2003). A pair of motor neurons enervating uterus were recently discovered. The pair of circular muscle of the uterus (CMU) neurons residing in ANm are necessary for uterus constriction during egg-laying sequence and egg expulsion and silencing the CMU neurons reduces the egg-laying (Cury & Axel, 2023) (Figure 4 e). Whether CMU neurons also incorporate octopamine as a neurotransmitter has yet to be identified.

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SPSNs come into contact with other neurons in the ANm. The ventral abdominal lateral (vAL) (*dsx*+) neurons and ventral abdominal medial (vAM neurons) are MIP-expressing interneurons in the abdominal neuromere that relay the signal from SPSNs to neurons that send their signal to the brain (SAG neurons in case of vAM) (Jang et al., 2017) (Figure 4 d). A pair of ascending neurons in ANm, called LSANs, convey the information of the act of copulation sensed by *Piezo*-expressing neurons in the abdomen to the brain (Shao et al., 2019). Shao et al. (2019) further showed that artificial activation of LSANs suppresses egg-laying (Figure 4 e). While the connection of some neurons in the ANm, for instance SAG, is well characterized, potential synapses between other neurons involved in the egg-laying circuit and the nature of these connections are waiting to be discovered (Figure 5).

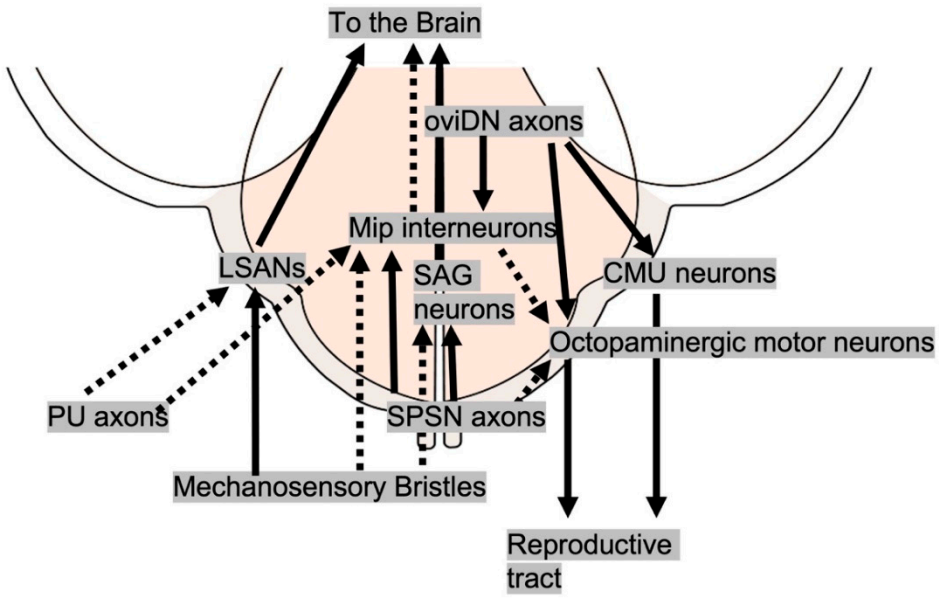

Figure 5. Interactions between ANm neurons that affect egg-laying output and behavior. The black arrows indicate a known connection, and the dashed arrows show a potential connection.

Sensory neurons that influence egg-laying:

Drosophila possesses numerous sensory neurons throughout its body, including sensory organs located on the head and legs. The relationship between sensory organs on the head or legs and egg-laying has been extensively discussed in a review by Ishimoto and Kamikouchi in 2021. For this review, I will focus specifically on sensory cues received by the sensory organs located in or on the abdomen (Figure 6).

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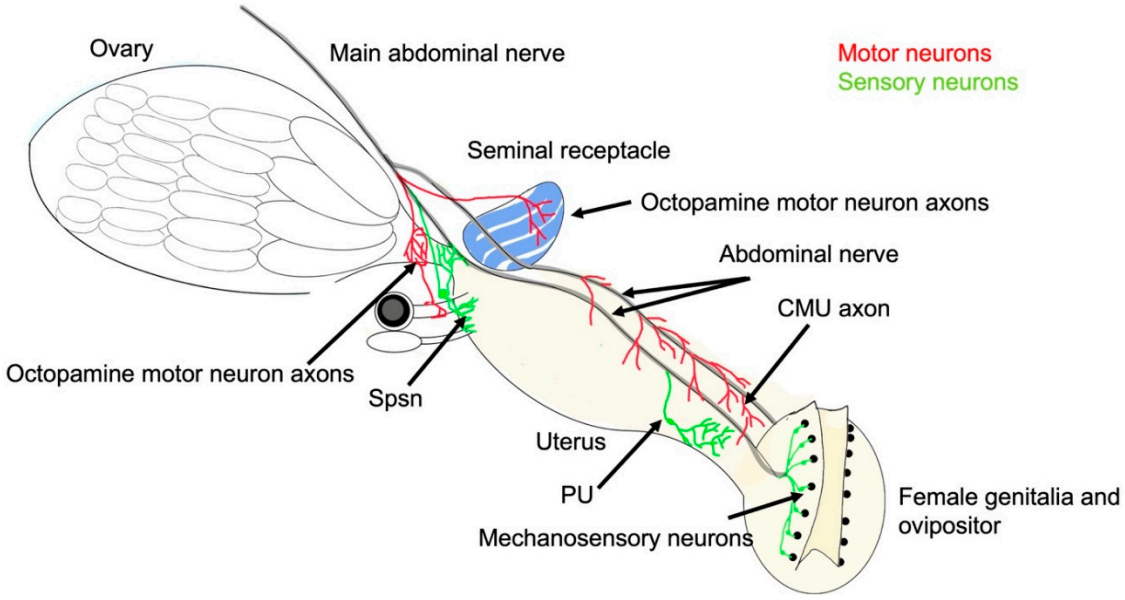

Figure 6. Diagram of the major components of the female *D. melanogaster* female reproductive tract. Motor and sensory neurons in the abdomen affect egg-laying behavior. Sensory neurons are depicted in green, and motor neuron axons are shown in red. Spsns are the primary neurons known to specify the egg-laying behavior through SP pathways. Octopaminergic neurons comprise the majority of motor neurons and modulate egg and sperm release through muscle movement.

Sensory cues triggered by mating:

The process of mating changes female fruit flies' behavior. It reduces receptivity and increases ovulation, egg-laying (Avila et al., 2011), and food intake (Ribeiro & Dickson, 2010). These changes in female behavior are modulated by components of mating, including pheromonal cues (Billeter & Wolfner, 2018), seminal fluid proteins (SFPs) (Haussmann et al., 2013; Ribeiro & Dickson, 2010), and the physical act of mating itself (Shao et al., 2019). Mating and its components modulate vesicle release at the nerve terminals of the reproductive tract (Heifetz & Wolfner, 2004). The act of mating and some components of the seminal fluid change the status of the lower reproductive tract, while SPFs modulate the vesicles released from neurons, enervating the seminal receptacle, while sperm alters the neurons enervating the upper reproductive tract (Heifetz & Wolfner, 2004). Furthermore, SFPs are responsible for the conformational change of the lower reproductive tract (Adams & Wolfner, 2007). In addition, mating affects gene expression in different tissues. For example, gene expression changes in the female *D. melanogaster's* head after mating are strongly correlated with metabolic pathways (Newell et al., 2020).

SP is one of the SFPs secreted by male accessory glands and, in its mature form, is composed of 36 amino acids (Kubli, 2003). SP is sensed by *pickpocket* (*ppk*)+ and *fru*+ neurons in the female reproductive tract, which are called sex peptide sensory neurons (SPSNs)(Yapici et al., 2008). Silencing SPSNs in virgin female flies results in increased egg laying that is comparable to mated females (Häsemeyer et al., 2009; Rezával et al., 2012). SPSNs express a G protein-coupled receptor called the sex peptide receptor (SPR). SPR is also expressed by spermatheca secretory cells and aids in sperm release (Avila et al., 2015). Knockdown of the SPR gene with RNA interference reduces egg laying significantly (Yapici et al., 2008). SP is not only detected by neurons and cells in the reproductive tract, but also passes through hemolymph and is sensed by neurons in the CNS and VNC (Yapici et al., 2008); specifically *fru*+ neurons in the brain (Chow et al., 2010). SP stimulates JH synthesis in the corpus allatum resulting in the development of oocytes (Moshitzky et al., 1996).

SPR expressed in the neurons has a similar structure to myo-inhibitory peptide (MIP) receptors. SP is more stable than MIPs and can survive the harsh environment of the female reproductive tract (Isaac et al., 2014). There are 5 MIPs in *Drosophila,* and they have been known to act as neuropeptides (Carlsson et al., 2010). One early study showed that pan-neuronal knockdown of MIP does not affect

receptivity and remating, but at least in one fly line, there was a significant decrease of the egg-laying (Kim et al., 2010). As discussed in the previous sections, more recent studies have shown that MIP-expressing neurons in the CNS are essential contributors to female receptivity and copulation effect (Jang et al., 2017; Shao et al., 2019) However, the role of expression of MIP itself .

Other seminal fluid proteins affect the female egg-laying as well. Acp26Aa, also known as ovulin, has a small increasing effect on egg-laying (Herndon & Wolfner, 1995). Specifically, it helps release the mature oocyte from the ovary after mating (Heifetz et al., 2000) by increasing the activity of octopamine-releasing neurons (Rubinstein & Wolfner, 2013). Studies show that ovulin is a protein that interacts with itself. However, the pathway by which this molecule interacts with octopaminergic neurons, whether as a monomer or dimer, has not yet been identified (A. Wong et al., 2006). In a systematic study of gustatory receptors (Grs), Park and Kwon (2011) found the expression of 21 Grs in the abdomen that send their projections to the ANm. Most of these Grs are expressed in multidendritic neurons on the abdominal wall, while 4 are expressed in the reproductive tract. *Gr28b*.b, *Gr28b*.c, *Gr32a* (involved in pheromone detection), and *Gr64c* (a sugar receptor) are expressed in the female's upper reproductive tract (Park & Kwon, 2011) and can be candidates in modulation of ovulation. Other studies have shown that uterine *Gr32a*-expressing and *ppk*+ neurons, increase aggressive behavior after mating. Similarly, activation of *Gr32a*-expression in virgin females reduced their sexual receptivity (Liu et al., 2020). These results suggest that *Gr32a*-expressing neurons might be potential candidates that affect egg-laying behavior as well. In males, *Gr32a*-expressing neurons detect pheromones and enforce reproductive isolation (Fan et al., 2013) and affect aggressive behavior (Andrews et al., 2014).

Sensory cues triggered by ovulation and egg delivery:

Ovulation and release of the egg are done by both virgin and mated females. As mentioned above, the release of the oocyte into the uterus and uterine contractions are modulated by octopamine. However, the movement of oocytes into the uterus triggers mechanosensory neurons and provides feedback to the insect (Chiang et al., 1991). The *ppk*- and *dsx*+ neurons not only sense sex peptide, but also can act as stretch receptors (Rezával et al., 2012). Silencing of a group of two to three *ppk* and *CG3542* expressing neurons residing on the upper oviduct reduces egg-laying but does not affect the mating receptivity (Lee et al., 2016), thus hinting that these neurons might be involved in stretch sensation in the oviduct. Other studies suggest that *fru*+ neurons are developmentally controlled by TIF pathway and might also be involved in the release of egg from the ovary (Evans & Cline, 2013). Cury and Axel (2023) have identified a pair of sensory neurons in the posterior uterus, called PU neurons. PU neurons sense the movement of the egg to the vagina and ovipositor and are necessary for transition into egg burrowing behavior (Cury & Axel, 2023).Taken together, studies suggest that defects in the neuronal control of mature egg release from the ovary can reduce the number of eggs significantly, and awaits further exploration.

Mechanosensory cues from mating direct egg-laying behavior:

Mechanosensation provides valuable external information and internal feedback to egg-laying females. The mechanical stimulus on the *D. melanogaster* body is sensed by two types of devices: ciliated type I neurons, which include mechanosensory bristles, and multidendritic neurons (Hehlert et al., 2021). Female *Drosophila* genitalia have many mechanosensory bristles arising from the genital disc during development (McQueen et al., 2022). It has been shown that the genes *invected* and *engrailed* are necessary for the development of normal bristle phenotype of the female genitalia, and mutants of these two genes lack the bristle phenotype (Figure 3 of Casares et al., 1997). Specification of these genitalia bristles is under the genetic control of *Hairless*, and a mutation in this gene results in female genitalia with almost no bristles (Figure 3 of Bang et al., 1991). A system that affects the development and enervation of the bristle is the *achaete-scute* system (García-Bellido & Santamaria, 1978; Simpson, 1990) and its adjacent genes like *asense* (Domínguez & Campuzano, 1993) *Daughterless, Senseless* (Jafar-Nejad, 2006) and *musashi* (Nakamura et al., 1994). However, their effects on the female genitalia bristles haves not been characterized extensively. The mechanosensation modulated by the

Piezo mechanoreceptor affects the reproductive behavior of *D. melanogaster* and optogenetic activation of *Piezo*-expressing *ppk-* neurons in the female abdomen increases neuronally in LSANs, in turn affect egg-laying (Shao et al., 2019). The bristles directly on the ovipositor have also been studied in *D. suzukii* and resemble mechanosensory neurons in *D. melanogaster* and express mechanosensory channels, including *Piezo* (Crava et al., 2020). A recent study shows that the bristles on female *D. melanogaster* genitalia express the *nompC* (Cury & Axel, 2023) as well as *Piezo*. Cury and Axel (2023) show that the mechanosensory bristle on the posterior end of the abdomen are necessary for assessing the firmness of the substrate for egg-laying.

Mechanosensation of the genitalia affects the female's egg-laying in other insect species and can provide basis for further discovery in *D. melanogaster*. For example, in female crickets, *Gryllus bimaculatus* the mechanosensory hairs on the vulva send the signal to the terminal abdominal ganglion and result in a change in the motor pattern of the vulva after the egg is laid (Ogawa et al., 2011). Elimination of sensory feedback from these mechanoreceptors resulted in the cricket staying in the egg-deposition pattern (Ogawa et al., 2011). Similar studies have not been conducted in *D. melanogaster* and are a puzzle piece of many unknown aspects of the effect of mechanosensation on the egg-laying behavior of *Drosophila.*

Why is neuronal control of egg-laying important in light of evolution?

The neuronal basis of female *Drosophila* reproductive behavior is less explored compared to some male reproductive behaviors such as courtship. The male reproductive phenotype has been studied extensively with regard to evolutionary pressures. Female reproductive phenotype and, by extension, egg-laying behavior is under the control of natural and sexual selection as well and have direct consequences on not only the individual's fitness, but also its mating partner. However, males and females have different evolutionary interests, and sexual conflict happens when the optimal outcome of male-female interaction is different for one sex compared to the other (Chapman et al., 2003; Parker, 1979). In *Drosophila*, sexual conflict can occur due to SFPs and their effect on egg-laying. Females can respond to this conflict by controlling the activity and longevity of SFPs (Sirot et al., 2015) or their sensitivity to them. For example, SP can result in increased mating costs in females by increasing egg production (Wigby & Chapman, 2005). Wensing and Fricke (2018) showed that the egg-laying response to SP varies across wild populations of *D. melanogaster*. Therefore, response to SP and the subsequent egg-laying phenotype can be used to measure conflict in *Drosophila*.

Eberhard brought about the study of cryptic female choice, a process that allows the female to alter a male's chances to father her offspring (Eberhard, 1996). However, experimental evidence for and against cryptic female choice is difficult to obtain due to its complex nature and overlap with other evolutionary processes like sexual conflict. Egg-laying may be used as an indicator of cryptic female choice. Shining light on the neural control of egg-laying can elucidate the neuronal basis of cryptic female choice. The female nervous system plays an important role in sperm storage in *D. melanogaster*. For example, Arthur et al. (1998) have shown that females with a masculinized nervous system or isolated abdomens store much less sperm, suggesting evidence for female control at the sperm storage level. Other studies have shown that females egg-laying output is affected by the morphology of a novel trait on male genitalia suggesting a sensory component in the females that assesses mate quality and modulates the egg-laying output based on this signal (Frazee et al., 2021; Frazee & Masly, 2015).

D. melanogaster as a species provides an excellent resource for studying female behavior, and the genus *Drosophila* offers a wide diversity of reproductive traits that are ideal for studying the evolution of reproductive behaviors (Anholt et al., 2020). This field can benefit from more in-depth studies on neuronal control within *D. melanogaster* sister species and other members of this genus. Two ideal candidates to study comparative female reproductive behavior are *D. suzukii* and *D. sechellia*. These two species present two cases of evolutionary adaptations on egg laying. *D. suzukii* shows a shift of preference in egg-laying substrate compared to *D. melanogaster*. *D. suzukii* prefers ripe fruit, which provides a firm egg-laying substrate compared to rotten fruit. This indicates a shift in the mechanosensory and chemosensory preferences (Durkin et al., 2021; Karageorgi et al., 2017), and it

is coupled with a change in the ovipositor morphology (Atallah et al., 2014). In contrast, *D. sechellia* has a specialized food source and habitat, the *Morinda citrifolia* shrub, which repels other members of the genus *Drosophila* (Grunwald Kadow & Gompel, 2020). *D. sechellia* has the largest eggs size amount the among the *D. Melanogaster* sister species. Its number of ovarioles and rate of egg production has also been reduced when compared to its sister species, each with separate genetic mechanisms (R'Kha et al., 1997). In laboratory cultures *D. sechellia* egg-laying can be stimulated by adding *Morinda* to their food. The neuronal basis of *D. sechellia*'s attraction to morinda has been well studied (Auer et al., 2020; Dekker et al., 2006). However, the underlying neuronal control of egg-laying in *D. sechellia* is unknown. Due to *D. sechellia* long retention of the fertilized egg in the uterus (Markow et al., 2009), researchers have struggled with incorporating modern genetic tools such as CRISPR-Cas9 in studying this species but recent studies have successfully incorporated these methods in *D. sechellia* (Auer et al., 2020).

In conclusion, egg-lying behavior in the fruit fly offers new and exciting avenues to study evolution, reproduction, and decision-making. Special attention needs to be paid to sensory neurons residing in and on the female's abdomen that sense mating and environmental signals and affect egg-laying. With the newer tools developed for this model organism and their decreased cost, the topic of neuronal control of egg-laying behavior promises new discoveries.

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