The second sex

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NEUROGENETICS OF FEMALE REPRODUCTIVE BEHAVIOUR IN *DROSOPHILA MELANOGASTER*

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Abstract

Knowledge of the proximate mechanisms and ultimate functions of reproductive behaviours can contribute to a wider understanding of evolutionary processes such as sexual selection and sexual conflict. As sexual reproduction necessitates the interaction of at least two individuals, it is fundamentally a social collaboration. However, in most species research focuses mainly on male mating behaviour, resulting in an imbalanced comprehension of male and female reproduction. Arguably, the organism with the best-studied sex life is *Drosophila melanogaster*, but it too suffers from this male-heavy investigation bias. Moreover, what we do know about females pertains mostly to pre-copulatory mate choice and virginal sexual receptivity. To identify the gaps in our knowledge, we follow an adult *D. melanogaster* female through the important reproductive events following copulation. In the process, we review the molecular and neuronal mechanisms allowing females to integrate signals from both environmental and social sources to produce specific behavioural outputs. We attempt to connect the theories that pertain to the evolution of female reproductive behaviours with the molecular and neurobiological data, and suggest potential areas and experiments for future research to clarify existing ambiguities. We draw attention to the fact that the evolutionary and mechanistic basis of female reproductive behaviours, even in a species as extensively studied as *D. melanogaster*, remain poorly understood.

INTRODUCTION

The study of the genetics of the vinegar fly *Drosophila melanogaster* has taught us a lot about the mechanisms that control reproductive behaviours. Male reproductive behaviour in particular has been dissected using the fine scalpel of genetics for many decades. This approach has yielded tremendous insights into the cellular and physiological mechanisms underlying sensory signal transduction, as well as the neuronal connectivity and processing underlying male courtship, providing general lessons for understanding the mechanistic and evolutionary basis of behaviour (for recent reviews, see Billeter and Levine, 2013; Dickson, 2008; Pavlou and Goodwin, 2013; Siwicki and Kravitz, 2009; Yamamoto and Koganezawa, 2013). However, when it comes to understanding reproduction and mating systems, the complement of this story is missing as we have inadequate knowledge of how females receive and interpret the information communicated by males (Ferveur, 2010). This asymmetric level of understanding hampers the study of the fundamentally interactive processes of sexual communication and reproduction.

The importance of studying female reproductive behaviours is obvious. From an ultimate perspective, understanding female mate choice is important for our understanding of the evolutionary process. Females are the gatekeepers of gene flow not only within species (Bailey et al., 2011) but also between species as females from most species show strong behavioural isolation when courted by non-conspecifics (reviewed by Laturney and Moehring, 2012). Understanding the reproductive behaviours of *D. melanogaster* females thus not only sheds light on the genetic architecture of species-specific behaviour, the molecular basis of conspecific interactions, and the processes of selection within this species; but it also holds the key to evolutionary questions such as species formation and isolation by means of sexual selection. From a proximate perspective, one of the outstanding challenges of neuroscience is to understand how individuals identify others (e.g., member of the same species, sex, kin versus unrelated individuals, and familiar versus novel) and how to interact with them on the basis of their own identity (e.g., their genotype, sex, and experience; Insel, 2010). *Drosophila* female reproductive behaviour is a particularly ideal system in which to unravel such a quandary. The female brain must receive complex sensory signals about conspecifics, the physical environment, and her internal state; and process the information to control the differential treatment of individuals in a given social environment. What is perhaps less well appreciated is that females continue their choosy reproductive behaviour beyond mate choice into post-copulatory feeding habits and manipulation of sperm within her reproductive tract. The study of female behaviours thus has the potential

to tell us how complex cues from various sources are sensed, and how this information is integrated and processed, eventually leading to a variety of behavioural outputs. Taken together, investigating female post-copulatory reproductive behaviour offers the opportunity to not only address ultimate but also proximate inspired queries making this one of the most powerful and interesting species to work with.

Here we attempt to review what we know about the genetic, cellular, and chemical basis of female post-copulatory reproductive behaviours: how females transition into maternal behaviours (Section 1) and what control they have over progeny production (Section 2). As in-depth neurobiological studies of the female nervous system and the behaviours it supports are slowly beginning to emerge, we hope that this review, which contains more questions than hard facts, will isolate gaps in our knowledge that should be filled. We highlight evolutionary studies in female behaviour that have postulated many interesting features of female reproductive neurobiology, whose mechanistic basis is still unknown. By intersecting work generated from a more evolutionary angle with work aimed at a stronger mechanistic understanding of the neurogenetic and neurobiological basis of female reproductive behaviours, we hope to highlight potential new areas and spur greater dialog between evolution and neurosciences focused researchers.

1. The Post-mating Response

A mated female is fundamentally different from a virgin. Once mated, females exhibit a host of changes at almost all systemic levels. Compared to a virgin, a mated female has a different transcriptome, proteome, and pattern of neuronal activity. The consequences of these alterations can be observed via the post-mating response (PMR), a collection of behaviours that are consistently and uniquely produced by mated females. In the literature, the classic PMR focuses on two main behavioural changes, decreased sexual receptivity and increased egg laying (including ovulation), mainly due to the ease of scoring and the robust effect. More recently, additional faculties have been found to also adjust with this change in mating status such as feeding and food preferences, general activity level, and of course sperm storage/utilization. How a female behaves after mating can strongly influence offspring quantity and/or quality and, consequently, has strong fitness implications. Despite its clear importance, the mechanisms of female post-copulatory changes in physiology are incompletely understood. However, advances in genetics have enabled manipulation of gene sequence and expression, visualization of neuronal projections, and most recently exploitation of neuronal

activity. All these tools have allowed researchers to dive into the mysterious world of female post-copulatory behaviours.

1.1 The PMR and Physiological Decoupling: All or Nothing?

The collection of behaviours that are part of the PMR are triggered in concert. Very few examples exist where a single aspect of the PMR is induced in the absence of the others. Consequently, sexual receptivity and egg-related behaviours, the two best-studied PMR behaviours, have almost always been studied together. However, researchers have uncovered one important distinction. Although receptivity and egg laying cannot be easily disentangled, it seems that short- and long-term decreases in likelihood of remating are independent processes, may be governed by different SFPs, and therefore possibly different physiological processes and neuronal substrates within the female.

One of the original investigations into the mechanisms underlying the PMR uncovered two biologically distinct decreases in sexual receptivity following mating. Researchers employed males that transferred only seminal fluid (and not sperm) to their mates during copulation. Females mated to males were found to exhibit a short-term response in sexual receptivity but failed to remain unreceptive and were likely to remate ~24 h after copulation, which was significantly faster than females mated to controls (Manning, 1967). Based on this finding, female sexual receptivity was divided into two distinct biological events: the "copulation effect" for the short-term reduction, and the "sperm effect" for the long-term reduction as it requires the presence of sperm (Manning, 1967). Investigation into the molecular basis of these two responses uncovered a vast array of molecules transferred to females with varying outcomes on remating behaviour; namely, the identification of the famous sex peptide.

Sex peptide (SP) was first isolated from seminal fluid by Chen and colleagues (1988). These researchers injected different protein fractions purified from the male AGs into virgin female abdomens and showed that one fraction, containing a single peptide of 36 aa, was sufficient to induce rejection behaviour typical of mated females such as ovipositor extrusion and reduced receptivity for 24–72 h (Chen et al., 1988). Moreover, females mated to genetically modified males that produce and transfer sperm and SFPs but are SP deficient via either knock-out of the gene encoding SP (Liu et al., 2003) or knock-down by RNAi (Chapman et al., 2003b; Fricke et al., 2009; Isaac et al., 2010, Lee et al., 2009; Wigby et al., 2005) failed to produce the "sperm effect" and only a weak short-term "copulation effect".

Together, these experiments showed that SP is the molecular basis to prolonged reduction in sexual receptivity.

Interestingly, SP also regulates egg laying behaviour; and the physiological response of receptivity and ovipositioning seem to be tightly linked. Similar to receptivity, both manual injection of SP (Chen et al., 1988) as well as natural reception of SP via the male ejaculate (Avila et al., 2011; Chapman et al., 2003b; Fricke et al., 2009; Isaac et al., 2010, Lee et al., 2009; Wigby et al., 2005) cause an increase in ovulation and ovipositioning. As this single peptide was found to be responsible for the two classical PMR behaviours, suggesting a shared cellular substrate for the female response, much research was directed at determining if the two could be mechanistically disentangled.

Although SP is involved in changes in both behaviours, at some point the two must be physiologically decoupled. Support for the hypothesis that this split is most likely downstream of SP dependent signal reception, beyond the first order sensory neurons involved in SP signaling, comes from various findings. First, it is unlikely that SP initiates reduction in receptivity and increase in egg laying by interacting with different areas of the nervous system because ectopic expression in several different tissues of a membrane-bound form of SP, which can only act on the cells in which it is expressed, always either evoked both behaviours or no changes at all (Nakayama et al., 1997). Second, it is also unlikely that there are unique receptors for the two behaviours because injecting females with different fragments of SP evoked either both behaviours or no changes at all (Schmidt et al., 1993). Finally, the PMR is also influenced by DUP99B, a SFP that has the same aa sequence that is responsible for the effect of SP (Schmidt et al., 1993). Therefore, it is likely that SP and DUP99B induce a decrease in receptivity and increase in egg laying through the same signaling sequence (Saudan et al., 2002), implying again that the triggering of these two aspects of the PMR are not separable.

Other SFPs are thought to also act on both long-term receptivity and egg-production behaviours through their influence on the amount of sperm that is stored (and consequently less SP) and how much SP is released from sperm in storage. Although *Acp36DE*-null mutants males transfer normal amounts of sperm to females, females mated to mutants store significantly less sperm than females mated to wild type (Bloch Qazi et al., 2003; Neubaum et al., 1999) and have increased receptivity already 24 h after mating (Neubaum et al., 1999). Although it has been shown that Acp36DE enters the sperm storage organs (SSOs) before sperm does and is suggested to promote sperm accumulation within these structures, the mechanism of this process remains unknown (Bloch Qazi et al.,

2003). The most parsimonious explanation for the decreased sperm storage and the positively correlated influence on the sperm effect is that the reduction of sperm storage and consequently SP released over time results in a reduced sperm effect PMR. Similarly, Acps that are predicted to influence the association of SP to the sperm tails and its gradual release have also been shown to influence the sperm effect. An RNAi knock-down screen of candidate Acps-coding genes provided evidence that candidate genes *CG1652*, *CG1656*, *CG17575*, and*CG9997* were involved in the PMR. Females mated to modified males show a significant reduction in long-term PMR as they have increased remating and decrease egg laying at time points great than 24 hours (Ravi Ram and Wolfner, 2007). As these gene products have been shown to localize SP to the SSOs (Ravi Ram and Wolfner, 2007) it is most likely that these molecules influence the PMR through their action on SP release from sperm and not via a direct interaction with a dedicated female receptor for these peptides. Again, as these SFPs most likely influence the abundance of SP, it is not surprising that they influence both receptivity and egg laying simultaneously. Interestingly, not all Acps that influence sperm storage influence female behaviour.

Females mated to *Acp29AB*-mutant males have significantly less sperm within their SSOs 4 days after mating compared to females that mated with wild-type males; however, females of both groups showed similar long-term PMR (Wong et al., 2008). As Acp29AB is not found bound to sperm (Wong et al., 2008) and mutant males sperm is easily dislodged from female SSOs after females remate with a different male (Wong et al., 2008), the peptide may participate in efficient release of sperm from the storage during ovulation, rather than from defective storage itself. The unaffected female PMR is at first puzzling as other mutants that influence sperm storage do influence such behaviours (as Acp36DE mentioned above). The reported mean number of stored sperm at day 4 was ~300 sperms stored in females mated to controls and 200 in females mated to mutants (Wong et al., 2008). In another study that visualized SP through antibody staining of sperm found in the female storage organs, tails of sperm became "spotty" at day 2 and by day 5, tails look extremely sparse (Figure 1C and 1D in Peng et al., 2005a). Taken together, although there are significantly less sperm in storage on day 4 after mating to *Acp29AB* mutants, the consequential difference in level of SP in the female may be too small to have a physiological effect on the PMR.

From these studies, it is clear that SP is the biological agent within the ejaculate that directly causes the long-term reduction in receptivity and increase in egg laying associated with the female PMR. However, SP is not the end of the story. As we

have already seen, molecules that influence the ability of SP to localize to and be cleaved from sperm, proper storage of sperm within the female reproductive tract, and can also affect the female PMR.

1.2 Short-Term Decreased Receptivity

Although it is clear that the long-term decrease in receptivity is established though SP, the relationship between SP and the copulation effect, the decrease in receptivity seen within the first 24 h after mating, is not clear. Females mated to mutant males lacking SP showed receptivity similar to mated females after 4 h were intermediate between mate controls and virgins after 12 and 24 h, and were indistinguishable from virgins after 48 h (Liu and Kubli, 2003). Confirming these results, females mated to SP knock-out males show a decrease in receptivity at 4 (Peng et al., 2005a) and 6 h after copulation (Fricke et al., 2009). These results suggest that immediately following mating and lasting <48 h, factors associated with mating other than SP must be causing the short-term decrease in remating behaviour. However, if SP had no influence on short-term remating, the presence of SP within the ejaculate should not have any bearing on the response of females: females mated to SP+ or SP-null males should all have the same remating rate at any time point within the first 24 h, but this is not the case. The propensity of females mated to SP knock-out males to remate is significantly higher than females that mated with SP+ males suggesting that this peptide does have an effect within the first 6 h of mating (Avila et al., 2012; Chapman et al., 2003b; Fricke et al., 2009; Hasemeyer et al., 2009). It is clear that a portion of SP transferred to females during copulation is not bound to sperm and may be quicker to reach its target in the female thus impacting the short-term response (Peng et al., 2005a). Furthermore, ubiquitously expressing (Aigaki et al., 1991) or injecting SP (Chen et al., 1988) directly into the female abdomen causes females to decrease receptivity within a few hours providing evidence that if SP can reach its cellular target quickly within the female after a natural mating, it could contribute to the short-term effect on mating as well.

Regardless of the relationship between short-term response and SP, other components of the mating experience must contribute to the decrease in receptivity. Compared to the factors that influence the long-term PMR, much less is known about those that contribute within the first 24 h. One Acp has been shown to have influence over the propensity to remate within this window. Wild-type females that were mated to *PEBII* knock-down males were quicker to remate compared to those females mated to wild-type controls (Bretman et al., 2010). Interestingly, this gene also contributes to the formation of the mating plug, a gelatinous secretion deposited in the female reproduction tract during mating.

Thus, it is possible that problems in sperm storage (and ultimately SP storage) may be driving the increase in mating as the mating plug could facilitate such a process. However, females mated to these male showed no reduction in progeny indicating that sperm was stored normally. Therefore, another hypothesis is that the gene product could be exerting its effects on short-term female receptivity through the mating plug acting on stretch receptors in the posterior end of the uterus, making her unwilling to remate. Alternatively, PEBII could also act as a pheromone interacting with the central nervous system of the female to reduce remating via a plug-independent pathway. Future experiments involving the introduction of PEBII into the female via a mating plug independent method such as injection, and artificially activating the proposed stretch receptors at the posterior region of the female reproductive tract would clarify the relationship between PEBII and the copulation effect.

A number of studies have attempted to locate the specific molecule influencing the initial drop in sexual receptivity but continue to come up short. Due to sequence similarities between SP and DUP99B (Saudan et al., 2002) and its quick disassociation with the sperm a few hours after mating (Peng et al., 2005a), DUP99B became an excellent candidate for the short-term reduction in receptivity. To investigate this, researchers have used flies with a specific genetic manipulation involving the *paired* mutation, producing males that lack accessory glands and therefore also lack Acps. Conveniently, these males still develop morphologically normal testes, ejaculatory ducts, and ejaculatory bulb and therefore produce and transfer sperm and molecules made in the ejaculatory duct and bulb including DUP99B (referred to hereafter as Acp-null mutants; Xue and Noll, 2000). These males have been used to determine the role of DUP99B in short-term PMR. Females were mated to either mutants or wild-type males and then were presented with a novel wild-type male immediately after initial copulation event (Rexhepaj et al., 2003) or 12 h later (Xue and Noll, 2000). Females previously mated to mutants males all mated within 1 h compared to none that had been mated to wild-type males (Xue and Noll, 2000; Rexhepaj et al., 2003). Based on these results and confirmation that these mutants produce normal amounts of DUP99B, the copulation effect must be an Acp-mediated process and thus DUP99B does not reduce short-term receptivity. This result is surprising as injection of the DUP99B directly into the female abdomen evoked a PMR (Saudan et al., 2002).

A few possible explanations exist for this. First, although Acp-null males produce DUP99B, it could not be confirmed that these mutants transferred the molecule to females during copulation (Rexhepaj et al., 2003). Next, it may take more than 1 h but <12 h for DUP99B to exert its influence. Therefore, specific DUP99B-null

males (generated in Rexhepaj et al., 2003 and tested for receptivity 24 h after initial copulation) could be tested at different time points within the 12 h window. To be sure SP did not also influence receptivity, these males should be generated in an SP-mutant background. However, it should not be overlooked that this molecule may indeed not be involved in reduction of receptivity and could highlight the importance of methodology and the innate difference of an organic copulation event versus injection of the ejaculate directly into the female abdomen.

Whatever the relationship between DUP99B and receptivity, DUP99B may still be involved in the female PMR. Rejection behaviour (number of ovipositor extrusions within a 10 min period) was also recorded by Rexhepaj et al. (2003); and, although females mated to mutant or control males showed equal number of ovipositor extrusions, only those females that were mated to the Acp-null males remated (Rexhepaj et al., 2003). Therefore, counter intuitively, rejection behaviour and remating receptivity may be influenced by different male ejaculate components: the former being influenced by DUP99B (or other seminal products produced in the ejaculatory tract or bulb; Takemori and Yamamoto, 2009). It is also possible that DUP99B weakly influences all behaviours of the PMR, and the currently used behavioural tests do not have the same sensitivity for detecting significant effects of this peptide.

The relationship between DUP99B and the copulation effect remains unclear and the molecular components of the ejaculate that exert such a response remain unidentified despite intense investigation (Bellen and Kiger, 1987; Mueller et al., 2007; Ram and Wolfner, 2007; Yang et al., 2009). One reason for the lack of identification of SFPs associated with short-term decrease in receptivity might be linked with the way it is studied. For example Short-term receptivity may be more susceptible to social context, relative to other behaviours of the PMR. In nature, *Drosophila* mate in groups when aggregated on food patches (Markow and O'Grady, 2008; Spieth, 1974, 1993) causing females to be exposed to multiple males and females after their virginal mating. Experiments done in groups of females continuously exposed to males indeed show females remating multiple times within a mating arena containing food (Billeter et al., 2012; Krupp et al., 2008; Kuijper and Morrow, 2009), which differs from the traditional remating paradigm which isolates the female directly after mating. However, the social experimental design is also not completely natural: females and males are restricted to the enclosed arenas, which may artificially increase the remating rate (see convenience polyandry in next section). The point is: if short-term reduction in receptivity is more sensitive to social context than the long-term receptivity modulated by SP, then the behavioural assays that assess this behaviour should

reflect that. The "switch" from virgin-like to maternal state could also be under female control and forcing the female into isolation by removing her from all forms of social context (placing her alone in a vial) could induce the female to transition more quickly than a female in a social setting. Although Acps within the ejaculate may affect this process, the effect is lost due to isolation as male- and female-derived components would be working in the same direction. Future experiments should explore mating females to Acp-mutant males within different social contexts or different assays to explore this possibility.

1.3 Short-Term Ovulation and Egg laying

Short-term ovulation and ovipositioning (<24 h) are governed by male-derived molecules that may not require the presence of SP. Ovulation can be induced by the SFP ovulin (Acp26Aa) and *CG33943*, a protein-coding gene with no predictive domains, which stimulates ovulation 1 day after mating (Herndon & Wolfner, 1995). Ectopically expressing ovulin or either one of its C-terminal cleavage products causes eggs to be released by the ovary and enter the reproductive tract (Heifetz et al., 2005). Although the mechanism is unknown, ovulin and/or its cleavage products may elicit egg-release either by interaction with neuromuscular target in the lateral oviducts (Heifetz et al., 2000) or via the neurodendocrine system (Heifetz and Wolfner, 2004). Similarly, females mated to knocked-down *CG33943* males fail to induce the short-term mating response as they lay significantly less eggs within the first 24 h compared to female mated to control males (Ram and Wolfner, 2007).

1.4 Recent Additions to the PMR: Food Preference and Activity Patterns

There are other behaviours that should be considered as part of the PMR, not only because they are consistently characteristic of mated females but the change is also induced by SP. First, mated females have a striking change in eating behaviour. Exposure to SP causes females to increase food intake (Carvalho et al., 2006), and specifically yeast (Avila et al., 2012; Fleischmann et al., 2001; Ribeiro and Dickson, 2010; Vargas et al., 2010). The possible functions of this increased consumption of yeast may be related to the high post-mating rates of oogenesis and therefore required amino acids-rich diet (Drummond-Barbosa and Spradling, 2001). However, the depletion of protein resources is not the cause of the PMR preference shift because SP-induced yeast preference is still present in females that cannot produce eggs (Ribeiro and Dickson, 2010). Furthermore, SP has been shown to alter the expression levels of nutrient-sensing genes of the mTOR pathway in females 3 and 6 h after mating (Ribeiro and Dickson, 2010; Vargas et al., 2010). This pathway is thought to link ATP and aa levels within the

individual with food consumption behaviours (Itskov and Ribeiro, 2013). The SP signal within the ejaculate most likely modifies the internal nutrient-sensing pathways because experimental modification in expression of genes within this pathway caused an increase in yeast consumption (Ribeiro and Dickson, 2010; Vargas et al., 2010).

Mated females also display a reduction in general activity level (Tompkins et al., 1982). This reduction in movements may have evolved as a mechanism in females to reduce courtship by surrounding males as movement is attractive to males (Tompkins et al., 1982). A virgin female moving away from a courting male may actually encourage continued courtship rather than merely signal rejection. Furthermore, the architecture of the activity of females also changes depending on their mating status. Although both virgin and mated females show a spike in activity during light-transition periods (at dawn and at dusk) as well as low activity levels during dark phase, mated females show an increase in activity during light phase as compared to virgin females (Isaac et al., 2010). When females were mated to SP-deficient males, the activity of the female more closely resembled that of a virgin female with low amount of activity during the light phase compared to females that mated with control males (Isaac et al., 2010). These results were interpreted as virgins benefiting from low activity during the day to minimize risks to environmental conditions such as dehydration, whereas mated females benefit from high locomotor activity to satiate increased nutritional demands generated by increased egg production and the pursuit of appropriate egg laying site.

Although SP has received most attention and is associated with most aspects of behavioural change, multiple SFPs have been identified which influence the female PMR. With the vast array of molecular techniques available in the *Drosophila* tool kit and new experimental designs, identification of new SFPs that influence female PMR is still very likely. However, in order to achieve a full understanding of the mechanisms that support the transition from virginal to maternal behavioural patterns, we must determine the targets of the male-derived chemicals and the consequence of their interaction. In the next section, we dive deeper into the physiological post-copulatory changes in the female as we explore how the female central nervous system responds.

1.5 Neurobiology of Sperm/Seminal Fluid Effects

The majority of research attempting to understand the PMR has centered on the components of the male ejaculate and much less on the contribution of females to their own response to mating (as seen in sections 1.1, 1.2, and 1.3). This bias may

have been born from the ease of manipulating male ejaculate composition. Each seminal fluid component is a product of a single male gene and can be studied individually (Avila et al., 2011), but the female behavioural response probably includes an array of sensory neurons equipped with different receptors for different male seminal fluids (Hasemeyer et al., 2009; Rezaval et al., 2012; Yang et al., 2009), neuronal circuitry possibly involving neuromodulators (Avila et al., 2012; Heifetz and Wolfner, 2004), and eventually activation of specific behavioural patterns involving motor neurons, all of which is likely to involve the expression of multiple genes with varying effects. Unfortunately, the male-biased perspective has created an image of the female as a passive arena in which male-derived molecules and sperm combat for the grand prize of fertilization privileges. However, data is starting to accumulate indicating that females are not the submissive victims of male-generated peptides as once thought. Instead, they are more likely active participants who collect and integrate information from their environment, their mate, and their internal state to transform from virgin to preform maternal behavioural outputs. Therefore, the PMR does not need to be seen as a result of molecule oppression but can also be seen as a decision-making process by the female, where cost and benefits are weighted to optimize female fecundity in different conditions.

Much research has been done to understand sexual conflict; the tug of war that exists between the sexes fueled by different interests and ways in which they increase their fitness (Chapman, 2006). Although the details of the who-is-in-control-of-what debate remain unclear, one thing is certain: the female PMR requires the involvement of both males and females. Therefore, to understand the post-copulatory physiological changes in the female, we must determine not only the genes and corresponding gene products within the male ejaculate, but also the genes that produce the cellular components and neuronal circuitry within the female that the male-derived molecules interact with. Within the last decade, the molecular basis of the female PMR has slowly been unveiled with identification of genomic and proteomic changes that happen during and after copulation (Mack et al., 2006; McGraw et al., 2004; Prokupek et al., 2009; Swanson et al., 2004), the female genome and its influence on PMR such as female post-copulatory receptivity (Giardina et al., 2011) and offspring production (Chow et al., 2010). This change has fueled new discussions about the female PMR as an interaction between the sexes (Prokupek et al., 2009; Schnakenberg et al., 2012; Wolner, 2009). Here we review the relevant literature to identify the female cellular/neuronal substrate that interacts with the male ejaculate.

1.6 Sex Peptide Receptor

It is clear that SP and the closely related DUP99B (two members of the SP pheromone family; Ding et al., 2003) evoke at least a partial PMR in females; however, the question of how the signal produces behavioural changes in females still remains. Radiolabeled or alkaline phosphatase-labeled SP and synthetic DUP99B were injected into females to visualize their association with target female tissue. Both peptides interact with the same regions in the head: strong labeling of the antennal nerve, pharyngeal nerve, antennal mechanosensory center, the periphery of the suboesophageal ganglion, cervical connective, and weak labeling of the antennal lobes; thoracic ganglion; and the genital tract: the oviduct and the uterus (Ding et al., 2003; Giardina et al., 2011; McGraw et al., 2004; Ottiger et al., 2000; Saudan et al., 2002). However, this type of technique has some potential caveats. SP and DUP99B are normally transferred to the female during copulation within the ejaculate. This accumulation of sperm and SFPs in the female reproductive tract is a controlled process. For example, structural changes of the female reproductive tract and stages of sperm storage within the SSOs follow stereotypic systematic changes (Adams and Wolfner, 2007). Furthermore, both these molecules associate with the sperm and therefore require cleaving in order to be activated. The manual injection of molecules such as SP and DUP99B is not necessarily a similar delivery method and therefore the binding pattern of injected labeled molecules may not be comparable and thus will not reflect that of the post-copulated female. Interested in the binding pattern of SP within a mated female, Ding et al. (2003) sectioned females after mating at different time points and applied the alkaline phosphatase-labeled SP probe to compete with the natural SP acquired by females through mating. If no probe interacted with the tissue sections, then it was assumed that SP from the male was present and blocked the probe and therefore identifying tissues that interact with SP naturally in a mated female. In the uterus, a signal was found only after 7 h, where the pattern and signal intensity of the oviducts and central nervous system remained unaffected suggesting that SP did not interact with these tissues in vivo. Overall, these results show that SP and DUP99B have the potential to interact with many different tissues types throughout the body in order to evoke the PMR in females. However, more experiments investigating the timing of the physiological response of the female and the cellular components of the other male-derived molecules that are known to influence female post-copulatory mating behaviour are required. For instance, during the first 24 h after the virginal mating, females housed in groups composed of males of different genetic backgrounds remate more often than females in groups containing males from the same inbred strain (Billeter et al., 2012; Krupp et al., 2008). As SP seems to be at least one of the triggers for the short-term PMR (see section 1.2), it is possible that females could modify their

behaviour through modification of SP interactions. Therefore, it would be interesting to determine any changes in SP-binding patterns of a mated female in isolation and within a social context. Alternatively, one other SFP has been shown to influence the short-term decrease in post-copulatory receptivity (Bretman et al., 2010). To investigate if PEBII influences female post-copulatory receptivity through a plug-independent or -dependent pathway, this male-derived molecule could be visualized within the female reproductive tract to determine if it only is associated with the mating plug or if it also interacts with the female central nervous system.

A major turning point in the quest for the cellular target of SP was the identification of its receptor (Yapici et al., 2008). It was identified through an RNAi knockdown screen for neuronal genes required to produce a normal PMR. Females with central nervous system-specific knock-down of *CG16752* laid significantly less eggs after mating and remated significantly more 48 h later compared to controls (Yapici et al., 2008). Knock-down females directly injected with SP were significantly more receptive 5 h later compared to controls (Yapici et al., 2008). The gene, hereafter referred to as *sex peptide receptor* (*SPR*), is a G-protein-coupled receptor that is activated by both SP and DUP99B (Yapici et al., 2008). *SPR* is expressed in both the female reproductive tract (the common oviduct and the spermathecae) as well as in the central nervous system (predominately in the suboesophageal ganglion and the ventral nerve cord). Although *SPR* is not expressed in the male reproductive tract, it is expressed in the male central nervous system suggesting that SPR may interact with other ligands and may function in other processes than the female PMR (Yapici et al., 2008).

Although SPR has a relatively broad expression pattern, the PMR can be mapped to a subset of *SPR*+ neurons that co-express *fru*, a gene which is expressed in neurons that control sexual behaviour in both males and females, *pickpocket* (*ppk*) an ionic channel expressed in sensory neurons involved in taste and mechanosensation (Hasemeyer et al., 2009; Yang et al., 2009; Yapici et al., 2008). With use of neuronal activity manipulation tools, 6–8 peripheral nervous system neurons in the uterus that project to the brain were shown to convey the signal of SP for the PMR (Hasemeyer et al., 2009; Yang et al., 2009). When SP interacts with these neurons, neuronal activity is reduced and this change in activity is most likely the signal to higher order brain centers that the female has mated (Hasemeyer et al., 2009; Yang et al., 2009).

Investigation into another gene *doublesex, dsx, which* controls morphological sexual differentiation allowed for more unveiling of the circuitry supporting the PMR.

Similar to the experiments using *fru* and *ppk* expressing neurons, when *dsx*+ neurons were silenced, females also failed to produce the normal PMR (Rideout et al., 2010) indicating that *dsx* is involved in post-copulatory female reproductive behaviour. Indeed, the expression of *dsx* overlaps with some *fru* neurons in the CNS (Rezaval et al., 2012). *dsx* circuitry also supports upstream neuronal pathways projecting to higher brain areas (Rezaval et al., 2012). Unlike the SP-responsive neuronal cluster in the uterus that once associated with SP decreases activity, which induces the PMR, these neurons increase activity after mating to produce the PMR. These neurons originate in the abdominal ganglion (Abg) and project to the SOG and the reproductive tract most likely conveying information about the presence of SP or another SFP (Rezaval et al., 2012). Furthermore, *dsx* circuitry also supports downstream neural pathways projecting from the central nervous system to the reproductive tract (Rezaval et al., 2012). Of the roughly 27 neurons that express *dsx* in the Abg, it was determined using an intersectional method that about nine of these neurons co-express a *Tdc2*, marker for neurons that produce octopamine and project to the lateral and common oviduct, uterus, and the SSOs (Rezaval et al., 2014). Artificial activation of these nine neurons significantly reduced receptivity and increased ovipositor extrusion and egg laying—showing induction of the PMR in a virgin female (Rezával et al., 2014). When these neurons are silenced in mated females, reduction in receptivity and increase in egg laying are not observed, showing that these neurons are both necessary and sufficient for these aspects of the PMR (Rezával et al., 2014).

SPR has also been linked to the feeding change in mated females. Mated females have increased yeast preferences compared to virgin controls. However, mated SPR-null females act as virgins and do not show yeast preference supplying further evidence that food preference is part of the normal PMR. Restoring SPR specifically in ppk^{+} cells rescued the yeast preference in mated females (Ribeiro and Dickson, 2010). Although this preference switch results in consumption of more yeast, which is then utilized during egg production, the preference itself is not elicited by ovulation as females unable to make fully developed eggs still show the preference switch (Ribeiro and Dickson, 2010). Moreover, the preference switch also involves the TOR/S6K signaling pathway suggesting that feeding decisions involve internal sensing. As SP has been shown to alter the expression levels of genes of the mTOR pathway (Gioti et al., 2012), and functioning SPR is required for this behavioural change, neurons that express SPR may either modify expression levels of these genes or participate in a signaling pathway to modify the expression in other cells, which modifies the internal nutrient-sensing pathways and results in greater yeast consumption behaviour. Changes in feeding behaviour are paralleled by changes in digestive tract physiology (Cognigni et al., 2011). The

excrement of mated females is more concentrated and acidic, indicating that more proteinaceous nutrients are extracted from the food by mated females, relative to virgins. This is most likely due to the increased nutritional need as a mated female increases their activity (less sleep during the day; Isaac et al., 2010) and the metabolic requirement of egg production. However, this change in physiology is not a direct effect of diverting resources from the gut to the ovaries, as mutant females unable to produce eggs still demonstrate the post-copulatory change. With the use of SP-null males, it was determined that these changes are due to the SP that the females received within the ejaculate (Cognigni et al., 2011).

A recent report suggests that the SPR story may be more complicated than first thought. SPR-mutant females respond to SP when SP is directly expressed in neurons or when the blood–brain barrier is disrupted suggesting that although SPR is required for the SP response, it may not function as its receptor but as a mechanism to deliver the peptide to the nervous system (Haussmann et al. 2013). A confirmation of these results with visualization of the binding pattern of SP within SPR mutant females with either a working or disrupted blood–brain barrier would help to clarify these results.

The identification of the SPR and the neurons in the reproductive tract that express different combination of *fru*, *dsx*, and *ppk* responsible for eliciting the female PMR represents the first molecular entity of the neuronal circuitry underlying female post-mating behaviour. The next step in the signaling pathway of the PMR will be uncovered with the confirmation of their neuronal targets. From this starting point and armed with the genetic tools available in this species, mapping the rest of the circuitry that supports the PMR may not be too far away. Advancement in this field should be guided by experiments that make use of female genetic mutants (reviewed in Section 1.9) as well as the gynandromorph results. For example, Szabad et al. (1982) suggested that the area of the central nervous system that detected insemination was most likely in the head. Together with the findings that the neurons in the uterus responsible for the PMR project into the Abg and may target the suboesophageal ganglion (SOG; Hasemeyer et al., 2009; Rezaval et al., 2012) suggests that the SP signal is sensed in the uterus and relayed to neurons in the SOG that have their cell bodies in the head or relayed again to third-order neurons that do. One main goal of research in this area should be to uncover the uncoupling of the SP signal to identify the unique circuitry that supports the different behaviours of the PMR.

1.7 Unique circuitry

The majority of research on the circuitry supporting PMR has focused on sensory neurons responsive to male-derived SFPs and the findings demonstrate that most post-mating behaviours are initiated by SP and therefore share SP sensitive pathways, However, as these behaviours are indeed distinct from one another, it is reasonable to assume at some point each will be supported by their own unique circuitry. And indeed, indications of these unique pathways are apparent. For example, within the reproductive tract *ppk*, *dsx*, and *fru* are co-expressed in different combinations (Rezaval et al., 2012). This pattern of co-expression provides a tool to access different neuronal populations and assess these small populations for each PMR endophenotype. Interestingly, silencing *ppk* neurons inhibit egg deposition, presumably by impeding egg transport along the oviducts (Yang et al., 2009), yet females can still lay eggs when activity of fru^{+} neurons is blocked (Yang et al., 2009). This suggests that neurons with different genetic expression patterns can direct distinct PMRs and utilizing this methodology may be the key to teasing apart the incredibly interconnected circuitry of egg laying and sexual receptivity (discussed above).

Since almost all PRMs are elicited by SP, it follows that indeed those that are SP-dependent will also be SPR-dependent and remain at virgin-like levels in females that do not express this receptor. Recently, however, *SPR*-mutant females have been found to increase ovipositioning via SP (Haussmann et al., 2013). When researchers expressed membrane-bound SP (mSP) in dsx^{+} neurons within a SPR mutant background they found that females elevated their level of oviposition from a virgin baseline of ~5 eggs within 48 hours to ~20 and sexual receptivity remained (Haussmann et al., 2013). This demonstrates not only that the presence of mSP can increase egg laying via SP via a SPR-independent pathway, but also that again the circuitry supporting egg laying and sexual receptivity can eventually be untangled. However, the mechanism (such as another receptor for this molecule) has not yet been identified. A nice complement to these results would be temporal resolution to this effect. For example, placing mSP expression under the control of heat-shock in *dsx*+ neurons in SPR-mutant females and measuring oviposition rate.

Furthermore, there is some evidence that the circuitry underlying post-copulatory receptivity and egg laying behaviour are at least somewhat independent downstream of Acp-responsive neurons. Females mated to Acp-null males showed no decrease in post-copulatory receptivity at any time points (0.5, 1, 2, 3, 4, and 5 days; Rexhepaj et al., 2003; Xue and Noll, 2000) suggesting that both short- and long-term receptivity is an entirely Acp-mediated process. However, egg laying behaviour was consistently at intermediate levels compared to virgin controls

females mated to wild-type males (Rexhepaj et al., 2003; Xue and Noll, 2000) suggesting that egg laying is influenced by both Acp- and non-Acp-mediated processes. Although it has not been confirmed that the lack of developed AGs actually reduced the production of Acps, it is clear that specific PMRs can be initiated by different male-derived components, and are supported by their own unique neuronal circuitry.

1.8 Neurobiology of Egg Production

Another contribution from the gynandromorph studies was the identification of juvenile hormone (JH), the areas of the brain that control its production, and how this hormone and these neuronal populations influence post-mating behaviour. Szabad & Fajszi (1982) identified a subset of gynandromorphs that produced eggs (ovulate) but did not lay them (oviposit). These specific gynandromorphs were implanted with hormone-producing glands (CA–corpus cardiacum complexes and ring glands) of wild-type females or performed a topical treatment of JH analog and almost all the experimental flies began to lay eggs (Szabad & Fajszi, 1982). Although fine mapping of neuronal circuits is not possible with this technique, these findings fueled much interest in the effect of JH on female reproduction, particularly egg production and ovipositioning and opened the door for future studies with finer methods, eventually leading to a solid understanding of JH and egg development.

After mating, SP enters the female reproductive tract and eventually the hemolymph. As the *N*-terminal of SP can interact with the CA–corpora cardiaca complexes to stimulate JH in vitro, it is very likely that this process also occurs in vivo (Moshitzky et al., 1996). It is important to note here that DUP99B, which is most similar to SP at the C-terminus (functional domain that interacts with SPR) but differs at the *N*-terminus, does not elicit JH production. Since the injection of DUP99B into females does increase ovipositioning (Saudan et al. 2002), it must influence egg-related behaviours through another pathway. Egg production proceeds based on a balance of JH and 20-hydroxy-ecdysone (20E) by eliciting vitellogenic oocyte progression through stage 9, the putative control point of oogenesis in virgin females. The interaction of SP with the CA–corpora cardiaca complexes may represent the first step in the SP-dependent ovulation-specific pathway. Although SP has been shown to be more or less the general PMR regulator for all associated behaviours, the physiological pathways for each must split at some point. As application of JH analog does not increase ovipositioning or reduce receptivity, this may be that point (Kubli, 2003).

As JH is also important for male mating behaviour (Wilson et al. 2003), it is likely that this brain region and its ability to produce JH is not sexually dimorphic, but that the neuronal circuitry supporting either the amount or timing of JH is. One candidate region responsible for the sex differences with JH production is the pars interarticularis as it is known to have sexually dimorphic function (Martin et al., 1999), it has been shown to communicate with CA–corpus cardiacum complexes (Belgacem and Martin, 2002), and cauterizing this region stops mated females from ovipositing (Boulétreau-Merle, 1976). Alternatively, SPR could be more richly expressed in the CA–corpus cardiacum complexes in females, contributing to the increased JH synthesis relative to males. Although males also express SPR in a very similar pattern to females, males show less prominent binding in the entire thoracic ganglion including where the CA–corpus cardiacum form a complex (Ottiger et al., 2000). This hypothesis can be addressed by confirming the sexual dimorphism expression pattern of SPR in this region.

Ovulation is the process through which eggs are produced by the ovaries and released into the oviducts. Several studies have indicated that the neuromodulator tyramine and its derivative octopamine are involved in the regulation of this process (Cole et al., 2005; Lee et al., 2009; Middleton et al., 2006; Rubinstein and Wolfner, 2013; Tompkins and Hall, 1983). Octopamine is synthesized from tyrosine by actions of tyrosine decarboxylase (dTdc*1* and *dTdc2*) and tyramine beta-hydroxylase (Tbh). Mated females, mutant in *dTdc2* and consequently lacking both of these, were found to be sterile because although they can release eggs from the ovaries they cannot deposit them properly onto egg laying substrate (Cole et al., 2005; Monastirioti, 2003). Octopaminergic and tyraminergic neurons that project from the thoracic-abdominal ganglion innervate the reproductive tissues (Middleton et al., 2006) including the SSOs (Avila et al., 2012) as well as the common and lateral oviducts, adult brain, and the nerve cord (Cole et al., 2005). Octopamine is released through vesicles at buttons and most likely encourages ovulation by contracting the ovarian muscles and relaxing oviductal muscles (Rodríguez-Valentín et al., 2006). The receptor for octopamine that is critical for the increased ovulation after copulation is *oamb,* which has two isoforms (K3 and AS) that are produced by alternative splicing of the last exon. Both isoforms produce female sterility due to egg retention when mutated (Lee et al., 2003), both activate an increase in intracellular Ca^{2+} (Balfanz et al., 2005; Han et al., 1998; Jallon and Hotta, 1979; McGraw et al., 2008), and K3 stimulates a cAMP increase (Han et al., 1998). Although *oamb* is expressed in the brain, thoracic-abdominal ganglion and the reproductive tract, the critical site for ovulation is the oviduct epithelium (Lee et al., 2009). Although SP has been shown to increase and sustain long-term ovulation rates, the signaling pathway from SPR expressing sensory neurons to

these neuronal populations, or neuronal populations like these, has not be identified. Ovulin has also been shown to increase egg laying, within the first 24 hours after mating, and is likely through interaction with these neurons as it has been shown to relax oviduct muscles and indirectly cause an increase in octopaminergic synaptic sites most likely through directly or indirectly increasing neuronal activity (Rubinstein and Wolfner, 2013).

After ovulation, sperm is released from the SSOs and fertilization can take place. This highly coordinated process also involves the use of neurons expressing tyramine and octopamine as inhibiting both affected release from both organs. Interestingly, by selectively inhibiting octopamine-expressing neurons (by mutation of the gene responsible for the enzyme of chemical synthesis from tyramine to octopamine), researchers found that this uniquely inhibited release from the seminal receptacle (Avila et al., 2012) suggesting that different populations of neurons may control release from the two different sperm storage organs. As females first use sperm first from the seminal receptacle and then later from the spermathecae, these neurons may responsible for this pattern of SSO utilization. Future research should determine which neuronal populations they receive their information from or if these neurons can respond to JH, SP, or other SFPs. Therefore, it is possible that neurons that express *SPR* and *dTdc2* represent the beginning and end of the neuronal circuitry supporting the post-copulatory increase in egg production.

Much like the neuronal circuitry underlying egg production, ovulation, and fertilization, the circuitry for the rest of the PMR is slowly revealed bit by bit. Usually, the first clues to the neurons involved in a particular behaviour are through the use of single gene mutants that have very obvious mutant phenotypes. Although alone these studies cannot tell us much about the wiring of the PMR, they will help guide the future research.

1.9 Genetic Mutants and the Neurobiology of the PMR

As the cellular substrate of the PMR is only partially elucidated and the process in which information is integrated from different sensory systems remains elusive, genetic mutants that show abnormal PMR either after a natural mating or injection of SP can be used as tools to identify further subsections of the neuronal circuitry supporting it.

Females mutant for *dunce*, a gene that encodes a cAMP phosphodiesterase, have a higher remating propensity, do not lay eggs, and do not respond to SP (Chapman

et al., 1996). Furthermore, expressing the wild-type *dunce* allele prior to SP injection partially rescues the PMR suggesting that this gene is involved in the physiological response to SP but not the development of the neural circuitry that supports its response (Fleischmann et al., 2001). Because the SP signal must be integrated in *dnc*+ neurons, and the wild-type *dnc* allele is expressed in (but not exclusive to) the MB (Nighorn et al., 1991), researchers set out to determine if this brain structure was a vital hub in the neuronal circuitry supporting the SP signaling cascade. MB-ablated females injected with SP, however, showed the normal PMR of increase ovulation/oviposition, and decreased receptivity (Fleischmann et al., 2001). Therefore, the SP signal from the male ejaculate that eventually increases egg-related behaviours and decreases sexual receptivity does not require this brain structure. Combining these results suggests that (1) SP alleviates this suppression without the use of the MBs and (2) *dnc* is involved in the SP-dependent PMR in another part of the nervous system.

Mutations in other genes that produced females with a phenotype similar to that of *dnc* (decrease in oviposition but not ovulation or egg-retainers and increase in sexual receptivity) such as *egghead* (*egh*) have provided insight into PMR circuitry. Like *dnc*, females mutant for *egh* do not respond to SP (Soller et al., 2006). When *egh* is selectively rescued in a subset of *egh*-mutant neurons in the ventral nerve cord that co-express *apterous (Ap)*, mutant females were able to respond to SP as witnessed by decreased receptivity and increased oviposition after SP injection. These interneurons form a common fascicle and project from the ventral nerve chord to the central brain. The behavioural data was supported by anatomical findings, which showed that *egh* mutants exhibit innervation defects in ap^{+} neurons connecting to the central brain. Perhaps they are *dsx* neurons that have recently been shown to be critical for mediating PMRs. These *dsx* neurons lie in the abdominal ganglion (*Abg*), some of which target posterior regions of the brain (Rezával et al., 2012). As these neurons are afferent interneurons, they most likely receive information from sensory neurons (possibly the *dsx*+, *ppk*+, and *fru*+ neurons that express SPR in the reproductive tract) and bring information toward the central nervous system (Rezával et al., 2012).

Genes originally discovered through mutagenesis studies can be tested functionally to aid with fine grain mapping of the PMR. For instance, virgin females that overexpressed *sarah* (*sra*; previously called *nebula*), *norpA*, *broad*, *grapes*, or a targeted candidate gene (*CG11700*, *CG4612*, *CG30169*, and*CG3961*) within the nervous system with use of the Gal4-UAS system had increased ovulation rates compared to controls (Ejima et al., 2004) suggesting that not only the neurons that express these genes are involved with the PMR but that the gene products are also

functionally linked. Interestingly, *sra* also influences sexual receptivity as overexpression of this gene within the central nervous system of virgin females caused mated-like behaviour: low receptivity and rejection behaviour including ovipositor extrusions (Ejima et al., 2004). *sra* is expressed in the central nervous system of larvae and in the brain and nurse cells of the oocytes of adults (Ejima et al., 2004). The protein product of *sra* interacts with CanB2 and Pp2B-14D (the calcineurin regulator Sra plays an essential role in female meiosis in *Drosophila* (Takeo et al., 2006), and is involved in the egg development.

Finally, a long list of genes have been identified by microarray analyses that are either up- or down-regulated in mated/unmated flies or females that have been exposed to a specific Acps (Domanitskaya et al., 2007; Fedorka et al., 2007; Gioti et al., 2012; Innocenti and Morrow, 2009; Kapelnikov et al., 2008; Mack et al., 2006; McGraw et al., 2004, 2008; Peng et al., 2005b; Prokupek et al., 2009; Short and Lazzaro, 2013). However, the generation of lists like these does not provide much insight on how the post-copulatory changes are generated. Genes important for the construction of the neuronal circuitry, for example, are most likely expressed during development long before the female has any interactions with conspecific males. Similarly, other approaches such as association studies have provided evidence that there are female genotypic effects for female PMR (Clark et al., 1995; Clark et al., 1999; Fiumera et al., 2005, 2007; Civetta et al., 2008). Although these experiments do not show causational relationship between a candidate gene and the female PMR, they do suggest that genes within a specified region may be involved. Once these genes are identified and their role in PMR has been confirmed, they will be most helpful in the mapping PMR neuronal circuitry. For all the genes listed above, investigation into the expression pattern within the reproductive tract and CNS may help to determine how the gene is involved in the PMR and the neuronal circuitry underlying these behaviours in females.

1.10 SFPs and Natural Genetic Variation

Now that we understand which components of the ejaculate and the neuronal network in the females that support the PMR, we can use these to determine how much genetic variation exists within populations, how this variation produces variation at the cellular and/or behaviour level, and eventually if variation in female PMR contributes to the process of evolution, species formation, and species maintenance.

Once the genes that give rise to these SFPs were identified, the next step toward understanding the female PMR is to determine if natural genetic variation exists in the gene sequence, gene expression, or the amount of the gene product transferred

to females during copulation and the correlated behavioural response in females. If variation at any level influenced the PMR of the female, then it may hint to the underlying function of the peptide and interacting female components. Furthermore, if we can understand the variation, we can start to understand how evolution has shaped these molecules. Unfortunately, variation at these three levels has only been investigated for SP, future studies should be done to determine if the genes and/or expression vary for others and if this variation contributes to variation we see in the female response.

A few studies have investigated the genetic variation for SFPs. Fiumera et al. (2007) performed association test with the third chromosome substitution lines to determine if genetic variation in 13 previously identified male reproductive genes influenced female post-copulatory phenotypes. Of the genes investigated, a polymorphism in CG6168 was associated with female willingness to remate. Although this gene was already identified as a gene expressed in the male reproductive tract and its product transferred to females during copulation, its function to influence female PMR was unknown. On the other hand, two other genes previously identified as PMR genes also showed associations. Polymorphisms in esterase-6 were associated with changes in female egg laying behaviour. This relationship was previously found by Saad et al., (1994) that looked at variation in esterase-6 activity levels in 18-field-derived lines and found that they were negatively correlated with the number of eggs laid by female (Saad et al., 1994). Fiumera et al. (2007) also determined that variation in SP influences the variation that was seen in female willingness to remate. Although other labs have also found a relationship between SP gene expression and refractory period in female remating (Smith et al., 2009), others have not. With the use of chromosome extraction lines, Chow et al. (2010) identified multiple polymorphisms of SP, some of which affect the aa sequence and others that may alter the expression level of the peptide. Although they determined that levels of SP mRNA differed significantly between lines, they found no association between transcript abundance and PMR (egg laying rate and remating rate) and no association between transcript abundance and SP protein levels. Similarly, Smith et al. (2012) determined that there is no relationship between the variation in transcript abundance and number of eggs laid (Smith et al., 2012). Although it is clear that both the sequence of the gene and upstream regulatory elements vary (Chow et al., 2010; Cirera and Aguadé, 1997; Fiumera et al., 2007), and different lines produce different levels of protein product (Avila et al., 2011; Chow et al., 2010; Ram and Wolfner, 2007; Smith et al., 2009, 2012), it is unclear if variation at this level can give rise to the variation we see in female post-copulatory behaviour. It is possible that the lack of consistency that it found in previous research could be due to the indirect link of male gene expression and female behavioural response. Perhaps, instead of variation at the

genetic level, we should focus on the variation in the amount of gene product that is actually transferred to the female.

Although the amount of SP transferred to females during copulation may not be determined by the level of genetic expression of SP (Ram and Wolfner, 2007; Smith et al., 2012; Wigby et al., 2009; Wolfner, 2002) or in other words, more transcription does not mean more peptide donated to the female, the volume of SFPs found within the female reproductive tract after copulation may still have a male genetic basis. Lines selected for large and small AG size produced relatively more and less SP and transferred relatively more and less volume of SP to females during copulation, respectively. Armed with this tool, Wigby et al. (2009) investigated whether females that mate with males that donate significantly more SP produce a different PMR than those that mate with males that donate a wild-type amount. The authors performed a "multiple-mating competition assay" over a 10-day period. In this assay, 6 flies (2 females, 2 males, and 2 males from either the large AG selection line, small AG selection line, or control line) are housed together without interruption and number and paternity of progeny were counted. They determined that the males from the large AG sired significantly more offspring than males from the control or small AG lines. However, because the frequency of copulation events was not recorded, it is possible that the increase in offspring production was due to increased copulation events. The authors noted that there were no differences in premating competitive ability because each day for a 3-h period, assays were observed and mating pairs were noted. However, mating outside these limited windows was not included in the calculation and mating occurs at different frequencies during the day (Sakai and Ishida, 2001). As the raw data was not presented, it is difficult to know how many copulations were observed in total. Although the selection line for large AG did show a response, it is impossible to determine if the selection regime influenced other sexually selected traits like pheromone profile or courtship song production as these traits were not measured. Furthermore, although virgin large AG males contained greater amounts of SP within their AGs compared to control and small AG males, and they transferred more SP during their virginal mating, it was not determined if over a 10 day period females mated with large AG males consistently receive more SP than those mated to control and small AG males. Therefore, from this experiment, it cannot be said with certainty if variation in volume of SP transferred to females during copulation influences female PMR (Fiumera et al., 2007; Wigby et al., 2009; Wolfner, 2002).

The contents of the ejaculate transferred to females during copulation is not only influenced by the genotype of the male but also his social environment. For example, males can alter the amount of SFPs they transfer depending on the

number of "revile" males he is housed with before interacting with the female. Males exposed to more "rivals" donate more SFPs during copulation than males housed alone (Fiumera et al., 2007; Smith et al., 2012; Wigby et al., 2009), and the degree of female's PMR (specifically reduced receptivity and egg production) depends heavily on the male's prior experience to rival males (Bretman et al., 2009). Males can also alter the amount of sperm within the ejaculate based on rival males (Garbaczewska et al., 2012) or based on mating status of female (Sirot et al., 2011).

As the molecular and neurobiological basis of the post-copulatory physiological changes are determined, so too is the circuitry underlying decision-making behaviour. After mating, what a female does and the decisions she makes influences not only her fitness but also the resulting fitness of her mate. In the next section, we will review the types of choices a female faces after her first mating and the mechanisms that she may use to make such choices possible.

2. Female Post-copulatory Mate Choice: Cryptic vs Noncryptic

It is not always appreciated that sexual selection continues after copulation. What the female does after the virginal mating, and each subsequent mating, can impact the fertilization success of the first male she mated with. Post-copulatory mate choice (PCMC) is any decision regarding reproductive behaviour that is made after mating and includes not only decisions that can be clearly observed such as the degree to which a female is polyandrous (noncryptic PCMC) but also decisions that are made about the fate of the male sperm within the female reproductive tract that determines offspring production and paternity (cryptic PCMC). There is evidence in the literature that both noncryptic and cryptic PCMC have a genetic basis and, therefore, the genes involved and the neural network supporting these processes can be identified. Exploration into the molecular basis of PCMC could indicate how it evolves and influences the genetic make-up of *Drosophila* populations.

2.1 Noncryptic

It is generally accepted that *Drosophila* females mate several times during their lives (Markow, 2011). However, the notion that females mate multiple times within one reproductive episode (before the sperm from the previous male has been fully used) is often questioned or ignored by researchers. This is an oversight since female polyandry not only has been observed in both natural (Imhof, Harr, Brem, and Schlötterer, 1998; Markow, 2011) and laboratory settings (Billeter et al., 2012; Krupp et al., 2008; Kuijper and Morrow, 2009) but also profoundly alters offspring production and genetic make-up (Billeter et al., 2012; Lefevre and Jonsson, 1962) and therefore has strong evolutionary consequences. Several non-

mutually exclusive hypotheses have been suggested to explain female polyandry within an evolutionary framework of cost versus benefits (reviewed by Arnqvist and Nilsson, 2000; Birkhead and Pizzari, 2002; Brown et al., 2004; Byrne and Rice, 2005; Chapman et al. 2003a; Gowaty et al., 2010; Jennions and Petrie, 2000; Markow, 2011; Partridge et al., 1987; Salmon et al., 2001; Slatyer et al., 2011). With the identification of the numerous potential costs in terms of reproductive output incurred by females that multiply mate (reviewed below), it seemed unlikely that the behaviour could be profitable. However, genetic techniques have improved making more hypotheses available to be empirically tested. The cost–benefits analyses discussion is now in the laboratory in order to identify possible mechanisms with molecular biology techniques.

The cost of sex to females has been linked to the diversion of resources to increased egg-production and post-copulatory immune response. Copulation can be costly to the female due to the change in energy allocation from somatic maintenance to reproduction. The lifespan cost of producing offspring is supported by the finding that both females with inactive or absent ovaries (Flatt et al., 2008) and those unable to lay eggs due to removal of an appropriate substrate (Partridge et al., 1987) live longer than their fecund female controls. One possible mechanism is oxidative stress as it increases as a result of egg production following mating (Salmon et al., 2001). However, females that are mated but are unable to produce mature eggs due to the *ovo* mutation also suffer a cost of mating (decreased lifespan; Barnes et al., 2008) indicating there are egg-production-independent costs as well. The act of copulation itself has the potential to incur a cost to the female. Females can be physically (Kamimura, 2007) but also chemically damaged by the SFPs received during copulation. The ejaculate that males transfer to females along with sperm contains about 100 different molecules that vary in their detrimental effects to the female: some are protective and some toxic (Chapman et al., 1995; Mueller et al., 2007). SP is an important component of the ejaculate with regard to the female PMR (Section 1), but with use of genetic mutants, researchers were able to disentangle the Acp-related costs of copulation from the general cost of reproduction (such as increased egg-production). Females that were housed with SP-null males mated more frequently, but did not lay less eggs or die faster compared to control females (Wigby & Chapman, 2005); and twice mated *dunce*-mutant females (who do not show the normal female PMR to SP including increased egg production) have a shorter life span than once mated dunce females (Chapman et al., 1996). This supports the notion that SP is the molecule within the male ejaculate that incurs the cost of mating to females.

Another potential cost of mating in *D. melanogaster* is the increase in the female immune response: if females divert resources to the immune system after mating, they become unavailable for normal cell maintenance which could contribute to their decreased longevity. Although females up-regulate immune-related genes after mating (Innocenti and Morrow, 2009), the mechanism by which this cost is paid is still unknown. The presence of sperm, Acps, and nonsperm/Acp factors have all been linked to this effect suggesting that the ejaculate, microorganisms in the female reproductive tract, or the Acps that signal to the female to up-regulate could all contribute to the change in the transcriptome (McGraw et al., 2004). Although the first two hypotheses still require testing, the last proposition was confirmed. Females mated to SP-null males failed to show the typical change in expression of these genes (Gioti et al., 2012) and transgenic females that constitutively express SP (thus not needing to mate with males to acquire this peptide) also show high levels of antimicrobial peptide genes (Peng et al., 2005b). The identification of immune response genes that are differentially induced after infection in virgins and mated females may lead to the identification of the mechanisms of the tradeoff between mating and immune defense (Short and Lazzaro, 2013); however, a direct link has not been made (Morrow and Innocenti, 2012). A carefully designed experiment that mimics the genetic expression signature of mating specifically on immune-related genes to determine the impact on female longevity is still required to resolve unambiguously if the immune response is costly to the female.

Whatever the source of the cost of copulation and reproduction, one thing is for sure: sex is costly. The costs of mating accelerates quickly with each subsequent mating (Kuijper et al., 2006) and females are most fecund when given the minimal male exposure required to fertilize her eggs (Rice et al., 2006). Therefore, selection may act on the standing genetic variation within a population on remating rate and favor females that tend to mate less often, closer to this optimal female rate. Evidence that variation in remating behaviour is at least partially explained by genetic variation comes from selection studies. Both fast and slow remating rate can be selected for suggesting that the behaviour has a genetic basis (Gromko and Newport, 1988). Experimentally skewing the sex ratio of the population over several generations alters the selection pressures because females in high male:female ratio populations are more frequently courted and remate more often compared to equal and low male:female ratio populations (Wigby and Chapman, 2004). Although females showed no differences in survival when housed in the absence of males, females that were selected in high male:female ratio populations lived longer than females from the other populations when housed with wild-type males (Wigby and Chapman, 2004) suggesting that there is also a genetic basis of female resistance to the cost of mating. Although the propensity of female

remating behaviour has been shown to be a major factor contributing to the genetic variation in female resistance (Linder and Rice, 2005), the authors of this study did not identify remating rate as a causal variable for longevity. This could be due to the method of observing number of copulations: 10 observations of at least 20 min apart were made twice a week. This severely underestimates the actual frequency of mating for all flies within the experiment. Furthermore, as daily rhythms of mating behaviour is under genetic control (Billeter et al., 2012; Krupp et al., 2008; Sakai and Ishida, 2001; Tauber et al., 2003), this selection regime may have also influenced timing of mating, possibly causing failure to detect a difference between the two groups. Nevertheless, an important finding by the researchers was the ability to select for a more resistant female phenotype through higher intensities of sexual conflict. Taken together, these results indicate that there is genetic variation supporting female's willingness to remate and resistance to longevity-related costs of mating for females that multiply mate.

The cost of mating reported in most studies in a laboratory setting is a decrease of 4–8 days out of a roughly 30 day lifespan (Markow, 2011). Thus, mated females have a measurable decrease in lifespan. However, does this matter outside of the laboratory? Wild-caught females taken into the lab and allowed to lay eggs were found to produce offspring from 4 to 6 different males (Imhof et al., 1998) demonstrating that females remate multiply within one reproductive episode and hold the sperm of multiple males in their reproductive tract simultaneously. Female polyandry is thus not a lab artifact but a natural life history trait. This is not surprising as females from most species engage in copulation with several males; polyandry, rather than monoandry, is the observed norm (Holman and Kokko, 2013). So what is the missing piece? Why do the theories predict monogamy but we continue to observe polyandry? One possibility is that females from natural populations fail to pay the cost of mating. Although we could not find data on the life expectancy or fecundity of *D. melanogaster* females in natural conditions, in order to pay the cost of remating, females from a natural population must live more than 24 days and continue a consistent rate of offspring production. It is possible, but in our opinion highly unlikely. Therefore, polyandry may be selected for because females simply do not survive long enough to suffer the consequences. That is not to say that the findings from the lab are irrelevant; only the importance of this cost in wild situation may not contribute enormously to the evolution of female polyandry. This questions is further complicated by the observation that the lifespan of mated female caught directly in the wild is longer that that of virgin females caught directly from the wild indicating a cost of virginity rather than a cost of mating in a nonlab setting (Markow, 2011).

2.1.1 Female Polyandry is Advantageous

The cost of sex incurred by females and findings that female polyandry is under genetic control begs the question: why do females remate? Multiple reviews have been written (Arnqvist and Nilsson, 2000; Birkhead and Pizzari, 2002; Chapman et al., 2003a; Gowaty, 2012; Jennions and Petrie, 2000; Partridge et al., 1987; Singh et al., 2002; Slatyer et al., 2011; Yapici et al., 2008) and experiments conducted (Arnqvist and Kirkpatrick, 2005; Brown et al., 2004; Byrne and Rice, 2005; Fowler and Partridge, 1989; Gowaty et al., 2010; Markow, 2011; Salmon et al., 2001) in the attempt to understand just that. For years, female polyandry has puzzled biologists because the advantages gained by females are not obvious. Based on the asymmetrical investment into gamete formation, optimization of female reproductive output is achieved via maximizing resource accumulation, not copulation events. Without any clear benefits, why do females remate?

Even without immediate material benefits, remating can still be advantageous. Due to the lower cost of sperm production than egg production, males benefit from a higher frequency of matings compared to females (Bateman, 1948; but see Gowaty et al., 2012). Therefore, females are courted more often than that of their optimal remating frequency resulting in inevitable rejection behaviour toward the male. When rejection behaviour becomes more costly than mating, there would be an advantage for polyandrous females. Support for the hypothesis comes from research in other species such as the water strider. Rowe (1992) reasoned that the increase in female polyandry in a skewed sex ratio condition of 3 males to 1 female was due to the cost of rejection being outweighed by the cost of mating. The same phenomenon of increased remating occurs when populations of *D. melanogaster* are manipulated in a male-biased fashion (Wigby and Chapman, 2004). There is however no direct demonstration that the females do not benefit from remating in other ways than avoiding male harassment.

Polyandry may be advantageous through beneficial increases in offspring production and quality. Although females have a finite number of eggs they can produce and one insemination event usually supplies a sufficient amount of sperm, female remating increases offspring production as twice mated females produce slightly, but significantly, more offspring compared to once mated females (Lefevre and Jonsson, 1962). Subsequent matings may provide additional male-derived Acps required to sustain ovulation and oviposition machinery thus optimizing sperm utilization (Cameron et al., 2007). Although the Acps turn on machinery to increase offspring production (Xue and Noll, 2000), the function of polyandry to gain more of these male-derived molecules is still an untested hypothesis but findings suggest that it may be the case. Polyandrous females that either repeatedly mated with the

same male or allowed to mate with multiple males showed no differences in fitness-related measurement which suggests that females multiply mate in order to retain more SFPs and not to diversify her progeny (Brown et al., 2004; Jennions and Petrie, 2000; Mueller et al., 2007). A metaanalysis that was performed on 122 experimental studies and assessed the fitness effects of multiple matings on female insects found that lifetime offspring production increases as a consequence of mating multiple times, and these benefits far outweighed any costs to the female such as shorter lifespan (Arnqvist and Nilsson, 2000; Slatyer et al., 2011). Furthermore, female polyandry increases fecundity in closely related species *D. pseudoobscura* (Chapman et al., 1995; Gowaty et al., 2010; Slatyer et al., 2011) and does not cause female to live less long in nature (Gowaty et al., 2010). If nothing else, multiple mating may simply provide certainty of fertilization (Fisher et al., 2013; Jennions and Petrie, 2000). For example, when females were mated with recently twice mated males, sperm transfer was reduced and the resulting post-copulatory receptivity remained high (Lefevre and Jonsson, 1962). This suggests that inadequate ejaculate size can contribute to remating.

Alternatively, additional copulations may provide female not just more offspring but better offspring as well. With each new mate, the genetic diversity of the sperm increases within the female reproductive tract. Although these males may be comparable in terms of the male traits used during prezygotic mate choice as they all successfully obtained copulation, the sperm within and between ejaculates may vary for traits used during postzygotic mate choice. Evidence that males favored during pre-copulatory mate choice were not favored during fertilization suggests a multistage mate choice procedure by females (Pischedda and Rice, 2012). One possible function of the multistage mate choice system is to produce high-quality offspring: prezygotic selection allows for the most attractive males to mate, and post-copulatory selection mechanisms may provide a more reliable way to selecting the best or most compatible sperm (Jennions and Petrie, 2000). Although some studies fail to find differences between offspring fitness of polyandrous females and serial monogamous (for example, Brown et al., 2004) suggesting that mating with multiple males does not increase fitness of the mother, others are able to link increased female mating frequency with high lifetime reproductive success of their daughters (Priest et al., 2008). Therefore, it is possible that in order to fully identify the benefits of female polyandry, next generation fitness levels need to be assessed.

2.1.2 Genetic Basis of Variation in Female Polyandry

To determine if female polyandry has a genetic basis in *D. melanogaster*, researchers applied artificial selection techniques and biased offspring production to either fast-

or slow-to-remate females. Behavioural response to this selection pressure suggests that timing and propensity to remate is at least partially under genetic control (Pyle and Gromko, 1981). Furthermore, studies that made use of outbred strains with relatively high genetic variation showed the degree to which females remate had a genetic basis (Linder and Rice, 2005), and comparisons between inbred lab strains showed that there are line typical remating frequency and latency (Billeter et al., 2012; Lawniczak and Begun, 2005; Lüpold et al., 2013). A QTL analysis was completed and identified three regions of the genome, 57B, 87B-E, and 8D-9A, containing hundreds of confirmed and predicted genes, were associated with refractoriness to remating (Lawniczak and Begun, 2005). Future studies will be required to fine map these regions down to identify the genetic information that was driving the relationships in this study. Giardina et al. (2011) identified nonsynonymous polymorphisms in two genes (*CG9897* and *CG11797*) through association study and chromosome substitution lines: the variation within these genes contributes significantly to the variation in remating behaviour seen within the remating paradigm. *CG9897*, a predicted serine endopeptidase, is expressed in the SSOs (Prokupek et al., 2009) and *CG11797* (aka *Obp56a*) is an OBP, a protein that influences the processing of odors in olfactory sensillae. OBPs have previously been associated with mating behaviour such as the case with lush that binds to cVA and together interacts with OR67d (Ronderos and Smith, 2010). Neither of these studies directly show a causal relationship between identified genes and remating behaviour. Future studies that directly manipulate the sequence of these genes are required to determine the role they play in female polyandry.

Comparisons between inbred lines, QTL analysis, and mutagenesis studies indicate that female polyandry has a genetic component. Future research for this field should include identifying the genes within the regions of the QTLs and the regions significantly associated with remating and focus efforts onto how the gene functions within the female to contribute to her remating phenotype. Further investigation into the natural variation that exists at a population level within these genes at either the sequence or expression level and how that variation leads to changes in mating behaviour is also required.

2.1.3 Environmental Factor that Influences Female Polyandry

Female remating rate is influenced not only by the expression pattern of her genes or the organization of her nervous system, but also by the environment that she is in such as the number and genetic diversity of her social partners. To determine the effect of the social context, this classic paradigm including a single male and female had to be replaced with a new more social setup. Although high social densities were rarely shown to increase remating (Harshman et al., 1988), manipulating the

sex ratio (Wigby and Chapman, 2004) or genetic diversity of the males (Krupp et al., 2008; Billeter et al., 2012) can increase mating behaviour. This context-dependent increase in female polyandry may be caused by females perceiving social genetic diversity through olfaction as females carrying the *Orco* allele (impaired olfactory system) failed to show the effect (Billeter et al., 2012). It is possible that either the male CHC profile was modified as this male trait has been shown to be influenced by his social context (Kent et al., 2008; Krupp et al., 2008), or males are simply perceived to be more attractive in direct comparison to other males in the social context.

Other factors in the environment other than potential mates can also influence remating rate. Part of the PMR is a change in feeding behaviour: mated females consume significantly larger meals than virgin controls and this change is regulated through SP (Carvalho et al., 2006). Although the absence of food does not seem to deter virgin or sperm-depleted females to mate, recently mated females will not remate if food is absent (Harshman et al., 1988), and remate more often with more food (Trevitt et al., 1988) or higher quality food (Chapman and Partridge, 1996). Therefore, the nutritional status of the female (Fricke et al., 2010) and food availability in the environment determines how a female responds to the male ejaculate in terms of the normal PMR (Fricke et al., 2010). One proposed mechanism of nutritional-dependent remating is through the insulin/insulin-like growth factor-like signaling (IIS) pathway as it has been shown to be responsive to nutritional status (Ikeya et al., 2002). Ablation of the *Drosophila* insulin-like peptide (DILP)-producing median neurosecretory cells (MNCs), and knock-out of DILP genes (*dilp2, dilp3*, and *dilp5*) significantly reduced remating (Wigby et al., 2011). The MNCs that co-express all three DILP genes are located in pars intercerebralis (PI; Söderberg et al., 2012), a brain area that has been predicted to influence egg-production in mated females by communicating with CA–corpus cardiacum complexes causing the female to produce more juvenile hormone and ultimately producing more mature eggs (Kubli, 2003). As mated females have higher nutrient needs in order for optimal egg production, it is possible that the food-related receptivity is governed by the egg laying process and there is no direct link between receptivity and nutritional state. However, females with ovaries that produce underdeveloped eggs also show the same decrease in receptivity in response to SP (Barnes et al., 2007) suggesting that egg production is not the direct cause for the normal post-mating decrease in receptivity. Since the reduction in remating for MNC-ablated females was shown to be dependent on the receipt of SP, it is possible that SP interacts directly with or upstream of the PI which could signal the CA–corpus cardiacum complexes and also to a central controlling mating

behaviour. Research confirming that SP changes the neuronal activity of MNCs within the PI, and where these neurons project to is required.

2.1.4 Sexual Conflict and Sperm Competition in Polyandrous Females

We established above that *D. melanogaster* females should be considered polyandrous and therefore mate with more than one male within one reproductive episode. Although polyandry provides females with numerous benefits, it significantly reduces the number of offspring the first male she mates with will sire. This creates a conflict between females and males in ideal female remating rate. Sexual conflict theory (Chapman et al., 2003a) predicts that males should develop adaptations, over evolutionary time, to attempt to reduce female remating; and females should respond to these tactics in order to regain control over her own polyandrous behaviour (Moore and Pizzari 2005).

There are multiple examples of how males attempt to maximize their fitness by influencing some aspects of the female PMR such as SFPs and their influence on decreasing receptivity after mating, and thus reducing the chances of remating and increasing the chances of paternity. As we have already seen, SP (Chen et al. 1988) and DUP99B (Saudan et al. 2002) received by the female in the ejaculate and reduce female receptivity, and at least the sensory neurons responsible for the SP-dependent decrease in receptivity have been identified (Yapici et al., 2008; Hasemeyer et al., 2009; Yang et al., 2009; (Rezával et al., 2012). Additionally, SFPs within the male ejaculate contribute to the formation of the mating plug—a gelatinous secretion by the male temporarily closing the female reproductive tract. Although the plug is completely contained within the female, it could function to encourage sperm storage, impair copulation/sperm transfer by rival males, or decrease receptivity. PEB-me, PEBII, and the PEBIII proteins made in the ejaculatory bulb make up the posterior region of the mating plug, and proteins from the AG make up the anterior (including Acp36DE) region (Bretman et al., 2010; Lung and Wolfner, 2001). Although Acp36DE has been associated with sperm storage (Avila and Wolfner, 2009), females mated with PEBII knock-down males did not show any deficiencies in offspring production suggesting that the anterior and posterior regions are functionally different (Bretman et al., 2010). Females that mate with PEBII knock-down males formed abnormal mating plugs and remated significantly faster than females that mated with controls suggesting that the mating plug itself reduces post-mating receptivity or this gene has pleiotropic effects (also decreases remating through an unidentified plug-independent mechanism; Bretman et al., 2010).

Although chemically inducing low sexual receptivity in post-mated females is an advantageous tactic by males to increase chances of paternity, female polyandry still persists. And when females remate with a short remating latency, the ejaculates of different males interact with each other within the female reproductive tract, giving rise to male–male conflict and to the phenomenon of sperm competition. Studies have shown that the fertilization success of each successive male is not random. The fertilization set, the sperm stored within the SSO that compete for fertilization, does not contain equal number of sperm from each partner. In a twice mated female, the storage organs contain more of the second male's sperm, a phenomenon known as last-male sperm precedence (Manier et al., 2010). To study this, researchers mate a female, and remate her to a new phenotypically distinct male 1 to 4 day(s) later. The paternity of the resulting offspring after the second mating can be compared to determine the reproductive success from each male: either defensive ability (the proportion of offspring sired by the first male after the female has remated, aka P1) or offensive ability (the proportion of offspring sired by the second male, aka P2; Boorman and Parker 1976). However, as the number of copulations increase and the timing between copulations decrease, last male sperm precedence breaks down (Billeter et al., 2012), which suggests that the standardized assay provides a unique context rather than a generalized condition. Regardless, this paradigm has been useful to understand male and female contributions to competitive fertilization (reviewed by Wolfner 2002; Singh et al. 2002; Schnakenberg et al. 2012).

These examples of how SFPs decrease female receptivity or can influence the outcome of sperm competition is an excellent example of how both ultimate and proximate causes for behaviour can be studied in *D. melanogaster*. As polyandry is costly to males, sexual conflict theory indeed predicts that such mechanisms would develop over time, creating a selective advantage for those males that were better at either stopping their mate from remating and/or being successful in sperm competition. Moreover, sexual conflict theory has also helped guide research in this field. For example, if some SFPs indeed contribute to the outcome of sperm success, and the production of such entities are relatively metabolically costly, then increasing perceived competition should also increase SFP gene expression. However, males reared in groups of four produced significantly less Acp26Aa (ovulin) and Acp62f (Acp for sperm defensive behaviour), and no different levels of SP compared to those reared in isolation (Fedorka et al., 2011). This result indicates that ovulin, Acp62f, and SP are not differently expressed around rival males in a manner that would indicate they are used in sperm competition. Alternatively, the increased number of males in the social context may have decreased expression of ovulin in an attempt to reserve stores for future mating. If

the production of ovulin is costly, males may want to limit the production and transfer to females. As this Acp is associated with egg-production, one hypothesis is that the presence of rival males indicates to the focal male that females he encounters will most likely have previously mated or will mate again in the near future. Therefore gene expression and gene product transfer to females may tell very different stories.

Indeed, when researchers quantified the amount that males transferred to recently mated females they found they transferred 20% less ovulin compared to when mating with virgins (Sirot et al., 2011) suggesting that males can potentially hijack ovulin received by previous/future rivals. Furthermore, some studies show that transfer of Acp36DE by the first male facilitated storage and use of sperm from Acp36DE-null males (Chapman et al., 2000; and for a review, see Hodgson and Hosken, 2006). Taken together, these findings suggest that the Acps made by one male can contribute to the success of others and males can modulate the components of their ejaculates in order to not over invest in a single female. This modulation of ejaculate composition also suggests that males could also modulate levels of Acps that contribute to female sexual receptivity in contexts of high concentration of rivals as seen in levels of SP transferred to females SP transferred to females (Wigby et al., 2009).

2.2 Cryptic Choice

The battle of the ejaculates, known as *sperm competition*, is well studied, including its the genetic architecture (Fiumera et al., 2007), and the components of the male ejaculate has been extensively studied (for reviews, see Avila et al., 2011; Ravi Ram and Wolfner, 2007; Schnakenberg et al., 2012). However, females are not simply passive arenas in which males fight out sperm wars. Evidence is mounting that suggests females exert at least some control over the outcome of sperm competition making the result of fertility success an interaction of male and female contribution. Using the classic sperm competition paradigm, researchers have shown that offspring paternity is influenced by the mother's genotype (Clark et al., 1999; Chow et al., 2010; Giardina et al., 2011) as well as age (Mack et al., 2003) and reproductive tract morphology (Amitin and Pitnick, 2007; Miller and Pitnick, 2002). By mating two standard males to genetically different females, it was determined that the genotype of the female influences the outcome of sperm competition (Clark and Begun, 1998; Clark et al., 1999). QTL analysis identified regions of the genome that contribute to propensity to use first or second male sperm and 33F/34A and 67F-69A corresponding to nearly 300 genes (Lawniczak and Begun, 2005).

Another form of cryptic PCMC is the social context-dependent female fecundity found in the *Oregon-R* strain. When females mate with males of their own strain, in the presence of *Canton-S* males, females forgo their fecundity and produce little or no offspring (Billeter et al., 2012). Although these studies suggest that females contribute to the outcome of sperm competition and skew the paternity of her offspring, no candidate genes for this process or mechanism has been determined.

Many mechanisms by which females bias the paternity of her offspring have been proposed. Possible targets of cryptic female choice include changes in reproductive tract biochemistry, neurophysiology, and morphology that interact with ejaculate to bias the paternity (Lüpold et al., 2013) and interactions between male SFPs and sperm storage morphology (Schnakenberg et al., 2012) or female-derived components (Wolfner, 2009, 2011). Based on the evidence from previous studies, we review the possible mechanisms by which females exert preference or bias offspring production.

2.2.1 Modification of Acps

A substantial portion of Acps are predicted proteases, protease inhibitors, and lipases (Swanson, et al., 2001), which are hypothesized to cleave other SFPs to modify their biological activity (Wolfner, 2002). However, as SFPs are found in their modified state within the female reproductive tract, it has been suggested that female-derived molecules may also participate in their activity (Ravi Ram and Wolfner, 2007). Swanson et al., (2004) identified genes that are richly expressed in the female reproductive tract which are the most likely candidates to interact at the molecular level with the components of the male ejaculate, including Acps and sperm surface proteins. Although this screen produced 526 genes that could potentially influence the female PMR, the authors argue that the list of most likely candidates are those that show evidence of positive selection and based on characteristics of the gene product such as having a transmembrane domain or signal sequences, including *CG4928*, *CG10200*,*CG16707*, *CG7415*, and *CG3066* (Swanson et al., 2004). Mack et al. (2006) and McGraw et al. (2004) identified 1783 genes that were differentially expressed in mated females and virgins and 539 genes in the lower reproductive tract, respectively. The functions of these genes include transcriptional factors, signal transducers, enzymes, proteases, and protease inhibitors (Mack et al., 2006; McGraw et al., 2004). Therefore, female-derived molecules may act to protect sperm, destroy it, or a combination of the two (Wolfner, 2009). Prokupek et al. (2009) found that a quarter of genes expressed in the SSOs have a predicted serine protease function. This study also found that after mating, both ST and SR express JH hydrolases (*JHeh2* and *JHeh3*) genes that

encode for enzymes that inactive JH with a possible function to counter the effects of biosynthesis of JH stimulated by SP.
Although these studies provide indications that females-derived molecules interact with the ejaculate, no gene product has been experimentally shown to modify a male-derived product but the candidate gene lists produced previously is an excellent place to start. In the attempt to identify the functions of these molecules and their role in the female PMR, this list of peptides and female proteins has been prioritized with use of a new tool in bioinformatics (Findlay et al., 2014). Peptides and proteins that must interact to affect the PMR would evolve in unison across species. The Evolutionary Rate Covariation tool was used to identify interacting seminal fluid peptides and female proteins. This process lead to the identification of three new males proteins influencing the association of SP with sperm and three female proteins that may affect SP dissociation from stored sperm (Findlay et al., 2014).

2.2.2 Sperm Acquisition and Usage

Females may be able to bias the paternity of their offspring by controlling how sperm is stored during and after copulation and utilized during ovulation and fertilization. For instance, if females are capable of biasing the relative amount of sperm from each male that is stored in her SSOs, she would then be able to impact the resulting paternity of her offspring. A review by Schnakenberg et al. (2012) describes the female reproductive tract including the two SSOs: the paired mushroom-shaped sperm spermathecae (Sp) and the seminal receptacle (SR) with the latter as the primary source of sperm used for fertilization. It outlines the process of storage and presents the genes in both the male and female genome that have been demonstrated to influence sperm storage and sperm precedence. Although there is no clear indication that females actively manipulate sperm storage, evidence is starting to mount that it is at least partially controlled by females. One, an intact feminine central nervous system is required for proper sperm acquisition and storage (Arthur et al., 1998) suggesting that sperm storage is an active process. Two, females from different isogenic lines harbor heritable variation in female sperm storage behaviour (Lüpold et al., 2013). And three, a gene that is expressed in the spermathecal ducts called glucose dehydrogenase (*Gld*) is up-regulated in a mated female; and the gene product has been shown to be required for sperm storage in St (Iida and Cavener, 2004). Once sperm is successfully stored, it must be released from the SSOs to fertilize the eggs. Although the release of sperm from storage organs is coordinated by female-produced neuromodulators, tyramine, its derivative octopamine (Avila et al., 2012) and *Gld* (Iida and Cavener, 2004), it is unlikely that they can control the specific sperm that is being released. After remating, the sperm from multiple males

appears to be mixed (Manier et al., 2010) and thus the preferential use of one male's sperm over the others appears to be highly unlikely.

2.2.3 Sperm Ejection

As males transfer much more sperm that can be stored, consequently, the extra sperm is expelled from the female reproductive tract and is referred to as an *ejection* (Lüpold et al., 2013; Manier et al., 2010; Snook and Hosken, 2004). After remating, the ejection is composed of sperm from both the virginal and remating event (Manier et al., 2010). The timing of this ejection can influence the proportion of offspring sired by the first and second male (Manier et al., 2010). Females with longer ejection latencies produced more offspring from the second male compared to those females with shorter ejection latencies (Lüpold et al., 2013) suggesting that quick ejections may bias the offspring to the first male. Therefore, if the female actively controls the timing of this ejection, she could then indirectly manipulate amount of first male sperm within storage and thus the probability of offspring from each male. Furthermore, Manier et al. (2010) show that immediately after mating, the sperm from the first male was concentrated in the distal half of the SR and only slowly mixed over time. Therefore, ejections soon after remating should contain relatively more of the second male's sperm compared to ejections long after mating.

Another form of sperm release from the SSOs of the female occurs during remating. When remating occurs, some females release the sperm of first male into the uterus Snook and Hosken (2004). If females have control over this release, it is possible that by releasing more sperm from SSOs, they can influence how much of each male's sperm is stored after the second mating and ultimately biasing paternity of offspring.

2.2.4 Female Reproductive Organ Anatomy

Conformational changes take place in female reproductive tract during and after copulation (Adams and Wolfner, 2007), which require Acps, specifically Acp36DE (Avila and Wolfner, 2009), but not sperm. In a scan for genomic and proteomic changes, post-mating researchers identified genes involved in muscle activity, contractions, and muscle tissue development indicating that prepared females to manipulate sperm through sperm storage, displacement, or ejection (Mack et al., 2006). Consistent with these findings, Kapelnikov et al. (2008) showed that mating triggers changes in both the genetic expression and protein abundance that mediates the development of the reproductive tract to deal with the post-mating environment (Gioti et al., 2012).

The female reproductive tract is highly innervated by terminal branches of abdominal nerves (Heifetz and Wolfner, 2004). One function of these nerves could be to modulate the responsiveness of the musculature and epithelium. Heifetz and Wolfner (2004) examined the effects of mating on vesicle release in cells throughout the reproductive tract. Sperm, semen, and the mating itself induced changes in vesicle release within the reproductive tract. Initially, the peptidergic nerve termini innervating the lower reproductive tract including the uterus are found to increase vesicle release immediately following mating and by the higher reproductive tract including the common and lateral oviduct 3 hours after mating (Heifetz and Wolfner, 2004). These results provide evidence that reproductive tract may mediate the PMRs to the male ejaculate via neuromodulator release and active female control of these in a male trait-dependent manner would influence offspring production and support cryptic female choice.

2.2.5 Oogenesis Stage Manipulation

Once mated, females receive SP from males. In the CA complex, SP increases biosynthesis of JH, which causes eggs to move past the stage 9 block and increase uptake of yolk proteins. However, the presence of JH alone does not increase the transcription of these yolk proteins. Another molecule has shown to be involved in this process and has mated-status-related changes: 20-hydroxyecdysone (20E). As the hypothesized function of this molecule is to reduce ovulation rate, if females have active control over its expression, 20E may represent the female response to the male-derived SP and resulting JH synthesis (Soller et al., 1999) and thereby controlling egg production.

Findings of a strong genetic basis to ovulation and egg laying rate (Boulétreau-Merle et al., 1989; Lüpold et al., 2013) suggest that females may moderate progeny production. If this is possible, females may increase or decrease this process depending on her previous partner(s), potential partner(s) in the environment, or other physical variables such as food availability. Moreover, specific genes have been identified to be involved in egg-production rate through mutagenesis (*lozenge*; Fuyama, 1995) and expression manipulations (*sra*, *norpA*, *broad*, *grapes*, and candidate genes *CG11700*,*CG4612*, *CG30169*, and *CG3961*; Ejima et al., 2004). If females had control over the expression of those genes or the activity of the cells in which those genes are expressed, it could provide a mechanism for influencing her PMR in a context-dependent manner.

Although it is clear that females can influence offspring production, the degree to which this occurs and the mechanism in which she gains this control is still unknown. Females are active participants during the sperm storage and appear to

have at least some control over the fate of sperm within her reproductive tract. The identification of the genes involved in cryptic and noncryptic PCMC is the gateway into the exploration into the neuronal network that supports these decisions.

CONCLUSION

In this chapter, we set out to review post-copulatory female reproductive behaviour not only considering the ultimate and proximate perspective but also an integration of the two. We compiled the literature regarding the genetic, cellular, and chemical basis of how females make decisions that influence not only herself but also the genotype and of her offspring in hope of resolving the evolutionary processes that contribute to species-wide behaviour. One apparent theme that occurred but was not necessarily saliently highlighted was the influence of the environment on the fly, namely, the social environment and how it contributes to phenotypic plasticity. As we follow the adult fly through her reproductive decisions, the importance of her social life revealed itself.

Mate choice clearly requires the female to interact with a social group (even if the social group is a single male). Studying mate choice in a well-controlled environment and from a reductionist perspective may have biased researchers to observe single pairs in isolation, while overlooking important aspects of mating behaviours. With the identification of the genetic and neuronal basis of female pre-copulatory mate choice, including the detection and sensory basis of the male traits and the brain centers responsible for integration of this information, a full realization of the influence of the social environment on mate choice will eventually be achieved. Furthermore, after copulation, the physiological changes that facilitate the transition of the female into the PMR (Section 1), the reproductive decisions including polyandry (Section 2), and egg laying behaviour are all largely determined by the social and physical environment of the female.

In the natural environment, flies are almost always found in groups. The constant sociality of flies must lead us to think more about social influence on female behaviour as it is more far-reaching than once thought. The complexity of the social life of *D. melanogaster* goes beyond aggregation as the type and number of flies present on a given food patch has nontrivial effects on the genetics of individual group members. These effects are reviewed in detail by Schneider, Atallah, and Levine (2012). We however want to highlight that group composition affects gene

regulation, allowing the genotype of group members to affect each other's phenotype. The social experience influences the transcription of genes controlling circadian timing and pheromone production (Krupp et al., 2008) with correlated effects on rhythmic patterns of locomotor activity and pheromone displays phenotypes (Levine, 2002; Kent et al., 2008; Krupp et al., 2008,2013). In turn, these effects correlate with changes in reproduction: increasing the genetic diversity of the group also increases female mating frequency, which changes the genotype of offspring they produce (Billeter et al., 2012; Krupp et al., 2008). How information about the genotype of individual group members is transferred remains unknown. Chemosensory transmission is the prime candidate because an intact olfactory system is required for females to detect group composition (Billeter et al., 2012). However, most experiments reduce the social impact on fly genetic, physiological, and behavioural phenotypes. These lab assays may not reveal the full behavioural repertoire of flies in the wild. As we relay on the results from these studies to inform us of the proximate and ultimate processes of reproduction, we may be only getting part of the story.

A final take-home message from this chapter should also be that we really do not know much about female reproductive behaviour. Although environmental factors, genetic variants, and physiological changes have been identified, the present understanding of the neuronal circuitry that supports this behaviour and the evolutionary processes that shaped it is far from complete. This is not stated to be discouraging or disheartening; it is meant to promote attention to the areas that are lacking and help the advancement of the field. Although we site numerous papers with varying experimental designs, reporting results with varying effects, and concluding relationships with varying strengths, one thing is certain: we actually say so little. In the end, we reviewed not just what we know, but really what we do not know. And there is value in that.