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The second sex

Laturney, Meghan Elizabeth

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SYNTHESIS

Meghan Laturney

Reproductive behaviour is, fundamentally, a social affair requiring the interaction of two complementary forms. For sexually reproducing organisms that means males and females must copulate in order for their gametes to fuse, leading to offspring production. Although this collaboration overtly leads to mutual gain, it also leads to conflict. Males and females have different optimal mating strategies, which at times disadvantage the other sex in terms of reproductive success. The resulting reductions in fitness apply new selective pressures to reduce these costs and consequentially contribute to the development of adaptations to regain control over their own sex life (sexual conflict theory; Chapman et al., 2003a; Moore and Pizzari, 2005). Therefore, co-evolution continues as an arms race between the sexes over the control of reproduction and, over evolutionary time, this results in cycles of adaptation and counter-adaptation: adapt, select, respond, repeat (Moore and Pizzari, 2005).

Accordingly, the documented cases of male manipulation of female reproductive behaviour (Ablard et al., 2013; Chuang-Dobbs, 2001; Elias et al., 2014; Grafen and Ridley, 1983) and male gains at the cost of females are plentiful across taxa (Chapman et al., 1995; Kelly and Jennions, 2011; Malouines, 2016; Wedell, 2007; Peinert et al., 2016; Reinhardt et al., 2015; Tataric et al., 2014); however, the female equivalents are few and far between. The favouring of male-focused research, especially for reproduction-related topics, may have reflected the outlook of the human culture on sex. Social assumptions about appropriate female characteristics as coy and passive were prominent in early examinations of sexual selection theory (Bateman, 1948; Darwin, 1871) and continued to influence the perspective researchers took when interpreting knowledge in their field (Milam, 2010). Regardless of changing views (Gowaty, 1997) many areas of investigation still show signs of male-biased research (for a comment on this see Ah-King et al., 2014).

The focus on male tactics to gain control over female reproduction has led to the identification of keystone processes that impact the evolution of various species such as sperm competition, a form of post-copulatory sexual selection (Parker, 1970). However, these findings also implicitly legitimized the male focused research and even calling into question the importance of females in the process (Leonard and Córdoba-Aguilar, 2010). The neglect of female influence on her own reproduction, particularly in response to male domination, created a bias in the literature to view females as the victim. This bias materialized as it influenced experimental designs that negated the importance of female influence (see Appendix; **Chapter 4**) and therefore continued to fuel male-focused investigations, perpetuating the imbalance. More central to science, the dynamic association between the male and female, especially in terms of coevolution, necessitates a

parallel understanding of both sexes, making the omission of female control over reproduction a weakness in the field.

In response to this deficiency, investigations on female control have recently gained momentum as evident from the increased interest in female post-copulatory behaviours, and development of theories that proposed polyandry as a norm (for reviews see Gowaty, 2012; Jennions and Petrie, 2000; Parker and Birkhead, 2013; Slatyer et al., 2011). Ideally, to propel our understanding of female reproductive behaviour the field needs investigations pertaining not only to the ultimate causes that lead to female control but also the proximate mechanisms that support it. Such integrative feats are available only through a handful of model organisms, and *Drosophila melanogaster* being arguably the most ideal as knowledge of female reproductive traits would complement the large body of knowledge on male traits that have been documented to evolve via sexual conflict in this species.

Post-copulatory mechanisms of female control over reproduction allow for the optimization of female reproductive success. Females that could bias sperm storage and sperm usage, resulting in alternative patterns of paternity, would have a large selective advantage. In my review of the *Drosophila* female post-mating response (PMR; **Chapter 2**), I identified a few specific aspects of recently mated females that vary, and this variation may be the source of female influence on her own fitness. For example, although females in nature most likely eject sperm after each mating (Lüpold et al., 2013; Manier et al., 2010) and go on to remate at least a few times (Imhof et al., 1998), the timing of ejection, remating latency, the number partners she takes appear to be flexible (Billeter et al., 2012; Duménil et al., 2016; Lüpold et al., 2013). And it is in this plasticity that females shape their reproductive output (Chow et al., 2012; Lüpold et al., 2013) and, therefore, it is likely where their control exists. Although females may be evolutionary constrained to a series of post-copulatory actions necessary for offspring production, by modulating these behaviours females may be capable of tailoring paternity patterns in ways to optimize their reproductive success.

Females can influence various aspects of reproduction via timing of ejection

Copulation permits the transfer of the male-produced ejaculate to the female. Along with sperm, many other chemicals produced in the male reproductive tract are also passed along. A few hours after mating, besides the fraction of the sperm that the female has stored and the various compounds that remain bound to sperm

or to her cells or molecules, the unused portion of the ejaculate including the gelatinous mating plug is removed. Interestingly, the timing of this ejection can simultaneously influence many aspects of her reproduction, which I describe in this thesis.

As discussed in **Chapter 3**, males engage in chemical warfare in an attempt to reduce or eliminate the probability of polyandry, which would significantly reduce female's reproductive success. During copulation males pass along two anti-aphrodisiac pheromones: 7-Tricoscene (7-T) is transferred to the female cuticle, and cis-vaccenyl acetate (cVA) is delivered with the ejaculate to the female reproductive tract. The presence of these chemicals on/in the female, when found separately, do not reduce the amount of courtship elicited by males; however, together they significantly reduce her attractiveness. As cVA is contained within the reproductive tract of post-mated females, sperm ejection removes ~90% it fragmenting this synergistic relationship between the two chemicals and resulting in an increase in attractiveness. Therefore, male-manipulation to female attractiveness can be disrupted via ejection, a typical post-mating behaviour. However, I also found that the timing of sperm ejection is plastic, and more interesting, socially modulated as females that were held in groups advanced ejection latency 1 hour compared to females mated in pairs and isolated after copulation. As females are also found to mate faster and more often when held in social contexts that contain more flies and with more genetic diversity (Billeter et al., 2012), females may be able to modulate timing of ejection to influence attractiveness in order to augment reproductive strategies. In contexts which are favourable, such as a when genetically diverse males are present, females may shorten their ejection latency in order to attract potential mates and increase genetic diversity of offspring. However, if remating isn't likely or beneficial, such as when the female is isolated or with inbred males, females may lengthen their ejection latency in order to reduce unwanted sexual harassment or to ensure full usage of their already obtained ejaculate.

I also show that ejection is closely linked with remating behaviour. Ejection may be a prerequisite for remating, not only because it increases probability of courtship by potential mates, but also because it is likely that remating is impossible with the presence of the gelatinous mating plug that is removed via ejection. In support of this, I found that nearly half of the recently ejected females re-mated during a 30-minute observation period, where as all the non-ejected females abstained (**Chapter 3**). This finding also suggests a close temporal relationship between ejection and remating: females that are faster to eject may also be faster to re-mate.

As mating rate itself can influence the outcome of sperm competition in ways predicted to benefit the female, ejection latency may be the first step in a chain of events to modulate female reproductive success. For instance, remating benefits females as it increases the genetic diversity of her offspring but also can replenish her sperm stores and deliver more seminal fluid peptides that could influence her egg production (reviewed by Gowaty, 2012; Jennions and Petrie, 2000; Parker and Birkhead, 2013; Slatyer et al., 2011). However, due to last male sperm precedence, outcomes of sperm competition reduce the diversity among offspring by heavily biasing paternity of whoever was the last male a female mated with. This effectively reduces indirect benefits in terms of offspring genetic diversity that females may gain from mating with different males. In **Chapter 4**, I found that females might circumvent this problem by increasing mating rate (short mating latency and increased number of partners) to produce more equal proportions of offspring from her various mates. Overall, this increased the genetic diversity of her offspring compared to females of the different mating regimes- a benefit not likely to be attained without also modulating timing of ejection.

Finally, I found that the timing of ejection is also important to reproduction as it influences sperm storage. Previously, timing of ejection has also been found to influence progeny production in once-mated females (Lee et al., 2015), and patterns of paternity in twice-mated females (Lüpold et al., 2013). In **Chapter 5**, I performed a Gal4 screen for neurons that support sperm storage in *Drosophila* females. I drove the expression of temperature-sensitive machinery to acutely activate or silence neurons during the first hour of sperm storage. Although none of the control females ejected during this time period, I observed that some experimental females did and also showed deficiencies in sperm storage compared to non-ejected groups. This relationship between likelihood of ejecting and reduction in sperm storage suggests a link between short ejection latency and a decrease in sperm storage, consistent with previous findings (Lee et al., 2015; Lüpold et al., 2013). Overall, I found that the timing of sperm ejection has the potential to influence female reproduction. Its plasticity in timing and clear relationship with sperm storage suggests that females may control the fate of sperm to maximize the benefits of taking on multiple partners via this behaviour.

Although variation in ejection latency is related to degree of sperm displacement (second male sperm pushing resident sperm out of storage in a twice-mated female Lüpold et al., 2013), sperm ejection has other functions such as modulating attractiveness (**Chapter 3**) and indicating good egg laying sites (Duménil et al., 2016) making this behaviour constrained by other processes. Moreover, the ejaculate contains various non-sperm compounds, which likely need to be absorbed

or modified to increase female progeny production (Avila et al., 2011) so it may be beneficial for females to delay sperm ejection even if they would benefit from not storing the sperm. Ejection-independent mechanisms to control fecundity such as control over sperm storage would be a resolution to this plight.

Females can influence paternity and fecundity via sperm storage

Control of sperm's fate in the female reproductive tract is regarded as cryptic female choice (CFC): female control over paternity as a result of actions within the reproductive tract. Although CFC was not specifically examined in this thesis, many of the reproductive behaviours that I surveyed could contribute to it. Specifically, I noted a large proportion of polyandrous females that showed selective paternity as they failed to produce offspring from all males that they were observed to copulate with (**Chapter 4**). One explanation of this is female control over sperm storage allowing for female "choice" within the reproductive tract: to store or not to store. This finding and others like it (Billeter et al., 2012) indicate that in specific social contexts females may block accumulation of sperm into storage suggesting the existence of cryptic female choice mechanisms in *Drosophila*.

I extended our understanding of both sperm ejection and storage by also investigating the cellular substrates that support these behaviours. Such regulators of reproduction, however, become targets for male manipulations: if females have the capacity to control the number of offspring, or the paternity of her offspring, males that could gain access to this control would have a large selective advantage over the other mates. In doing so, males could alter offspring production in ways that maximize his reproductive success. From the findings from **Chapter 3** and **Chapter 4**, I hypothesize that there are neuronal populations that contribute to the production of these behaviours. Moreover, as they may be sources of sexual conflict, these circuits may be subject to modulation by males. Therefore, I also postulate they may be targets for male-derived mechanisms of control.

Neurons for female reproductive behaviour.

Behaviours, especially those associated with reproduction, must reflect the influence of genetic programs acting during development that have been selected over the course of evolution, while permitting plasticity in order to adjust to changing environments. For example, we have demonstrated that timing of ejection and remating have huge implications to patterns of progeny production

and paternity. How is such plasticity represented in the neural circuitry that supports these behaviours?

I performed a Gal4 screen to identify neurons that when artificially activated or silenced influenced sperm storage and/or ejection. By taking advantage of the expression pattern of genes necessary for the production of or that contribute to the variation in female reproductive behaviours, I was able to identify specific populations of neurons whose activity is associated with variation in these traits. Therefore, using the tools available within this species I went from behaviour, to gene, to neuron- a capability afforded by only a few resource-rich species.

Interestingly, in most cases I did not find such binary results. Instead, I found that the probability of spontaneous sperm ejection and the degree of sperm storage could be artificially modulated by manipulating sub-populations of neurons, suggesting that females' ability to modulate the timing of ejection or quantity of storage may be reflected in the neural circuitry that support it. One limitation of this type of screen is that it is not informative about the activity of these neurons in a normally behaving female. It is unclear if these neurons actually function to influence storage and ejection: I determined that they can, not that they do. Techniques that can quantify neuronal activity in a live behaving female could help to elucidate the role these neurons play during these behaviours, revealing if neuronal activity correlates with modulations in sperm handling. For example, social context has been shown to influence both the timing of sperm ejection (**Chapter 3**) and sperm storage (indirectly shown with fecundity, Billeter et al., 2012). Therefore, by measuring neuronal populations I reported here that increased the probability of ejection/storage across different social contexts (isolated vs grouped female, or varying the degree of relatedness/genetic diversity of males) one could determine if neuronal activity varies between conditions, and if this variation translates to modulation of behaviour.

The identification of mechanisms that support female control over reproduction, such as neuronal circuitry supporting the production and modulation of sperm storage and ejection, grants the opportunity to investigate the reach of sexual conflict (Chapman et al., 2003a). Based on sexual conflict theory, we would expect males to evolve mechanisms to increase storage and/or increase sperm displacement in a multiply-mated female possibly by directly influencing the activity of neurons that comprise such circuitry. Interestingly, I also found that the genes involved in the sex peptide dependent signaling are also involved in neurons that support sperm storage specific to the spermathecae (**Chapter 5**) and sperm ejection (**Chapter 6**). Moreover, I also found that sex peptide functions to delay

sperm ejection as females mated to males deficient in this seminal fluid peptide display significantly shorter ejection latencies compared to females mated to controls (**Chapter 5**), a predictable pattern given that sex peptide is suggested to evolve under sexual conflict (Chow et al., 2010) and lengthening ejection latency is associated with greater male reproductive success (Lee et al., 2015; Lüpold et al., 2013). Consistent with predictions of sexual conflict theory, I also found that mated *SPR* mutant females displayed longer ejection latencies compared to controls, suggesting that this receptor may function in neurons to speed up ejections to increase mating rate. Overall, the identification of the neuronal circuitry that supports both sperm storage and ejection exemplify the integrative strategy that marries proximate mechanism with ultimate cause.

Future investigations into the neuronal circuitry of sperm storage should determine if/how these populations of neurons communicate with each other to explain patterns that we found in shared and unique genetic and cellular manipulations. Thanks to the genetic toolbox of *Drosophila*, intersectional methods can be used to increase specificity of the manipulated cellular populations. For example, our screen made use of the Gal4-UAS system, which involved driving the expression of temperature sensitive tools to acutely manipulation neuronal activity. However, some of the populations we manipulated were large, such as *tsb-gal4*, which labels all neurons in the ventral nerve cord, limiting our understanding of how the central nervous system can produce behaviour. There are, however, several strategies that take advantage of overlapping expression patterns to reduce the number of neurons targeted by either thermogenetic or selective genetic silencing techniques (del Valle Rodríguez et al., 2012). Intersection methods allow researchers to refine large populations of neurons down to smaller subsets and even allow for the manipulation of single neurons. Moreover, these techniques can also determine if the various Gal4 drivers identified in our screens overlap, which may account for similar results obtained for different lines (for a review see (Sivanantharajah and Zhang, 2015)).

CONCLUSION

The sex life of *Drosophila melanogaster* is one of the most scientifically investigated in the world. Decades of poking and prodding, collecting and counting, and now even activating and visualizing has led to the understanding of reproductive behaviour on both an ultimate and proximate level. The fruit fly has been a staple in biology labs for so long and research using this organism makes use of so many readily

available genetic tools that it's easy to forget that this species evolved for millions of years outside of glass half pint milk bottles.

The most powerful aspect of laboratory experiments is the ability to control conditions in order to make precise measurements and resolves relationships between variables. But in doing so, we may have removed too much. The plasticity of female reproductive behaviour suggests that females likely integrate information from a variety of systems and processes which all contribute to her behavioural output. As the interest of female behaviour is on the rise, it is important to highlight the impact of social context, food availability, and appropriate egg-laying sites on reproductive decisions, which should all be accounted for in experimental design. The results reported in this thesis suggest that the control females have over their reproduction lies in the variability observed in their reproductive behaviours. Thus, a full understanding of this control requires the identification of variables that can account for the variation and the neuronal circuitry that can support this plasticity. However, many standard protocols employed in research are designed to reduce variation in order to expose associations between researchers' manipulations and the flies' biological response. If we keep searching for female control in paradigms engineered to identify male variation or that limit the biological relevance we may be completely missing the point. Or in the very least, if we want to understand female influence, choice and/or control- at least give her some room to do so.

