FEMALE X MALE INTERACTIONS THAT SHAPE REPRODUCTIVE SUCCESS IN *DROSOPHILA MELANOGASTER*

A Dissertation

Presented to the Faculty of the Graduate School

Of Cornell University

In Partial Fulfillment for the Requirements for the Degree of

Doctor of Philosophy

By

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May 2020

FEMALE X MALE INTERACTIONS THAT SHAPE REPRODUCTIVE SUCCESS IN *DROSOPHILA MELANOGASTER*

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Cornell University 2020

The meeting and fusion of gametes and the production of offspring are the main goals of sexually reproducing organisms. In internal fertilizers, fertilization success is enhanced by a suite of physiological and (at least in insects) behavioral changes that mated females undergo after copulation and exposure to the male's ejaculate. However, conflicts within and between the sexes and context-dependent mating strategies maintain genetic variation in some molecules involved in reproduction. As a result, some combinations of female and male genotypes have a higher reproductive success than others; a phenomenon that could contribute to reproductive isolation as well as to idiopathic infertility in humans. Studies using *Drosophila melanogaster* elucidated several aspects of female x male genetic interactions that influence reproductive success. For example, natural variation linked with male seminal fluid proteins correlates with a male's performance in sperm competition and his ability to induce egg production in his mate. However, the female's genetic and molecular contributions to these interactions remain underexplored. I present four projects that have contributed to our understanding of the female's side of female x male interactions. First, I employed natural variation in *D. melanogaster* in combination with transcriptome measurements to identify genes in females whose transcript levels are either altered by mating in general or are altered by mating in a female x male genotype-dependent manner. These

experiments indicated that the transcript levels of immune response genes and genes with neuronal functions are especially sensitive to female x male genotype interactions. Second, my collaborator and I showed that these same groups of genes are also sensitive to interactions between the female's microbiome and her mating status, indicating that not only genotype, but also environment (in this case the microbiome) can influence female x male interactions. Further, using transcriptome data, I detected male RNAs that were transferred to the female during mating. Finally, in a separate project, using sperm competition assays and tissue-specific candidate gene knockdown, my colleagues and I identified genes and neurons in females that influenced the paternity success of the first vs. second male a female mated with. Collectively, the results presented in this thesis encourage further investigation of RNAs in the male's ejaculate and suggest that the female's immune system and nervous system act as an important interface between the female and her mate.

BIOGRAPHICAL SKETCH

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ACKNOWLEDGEMENTS

I thank my advisors, Dr. Mariana Wolfner and Dr. Andrew Clark, for giving me the chance to work on a variety of projects while pursuing a Ph.D., for guiding me through these projects and for stimulating me to learn many new skills. I further thank my committee members, Dr. Brian Lazzaro and Dr. Daniel Barbash, for giving constructive feedback on my experiments. I thank present and past members of the Wolfner and Clark labs for the helpful scientific discussions we have had and the friendly atmosphere they created in the labs. I thank the sources of funding that supported me throughout my Ph.D: an NIH grant awarded to Dr. Mariana Wolfner and Dr. Andrew Clark (R01-HD059060), and fellowships awarded to me by the Belgian American Educational Foundation and the Cornell Graduate school. Finally, I thank my family, friends and Mike for their love and support.

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CHAPTER 1:

INTRODUCTION

The fusion of sperm and egg and the production of offspring are the fundamental, primary goals of sexual reproduction. However, how likely it is for this fusion to occur, how often, which sperm are used and whether the resulting embryo will develop without deficiencies, depends on much more than just sperm and egg. In internally fertilizing species, mating gives rise to a multitude of female post-mating responses that change the female's protein and RNA levels, physiology, and in insects, behavior, with the goal to enhance reproductive success (Avila et al. 2011; McGraw et al. 2015). Generally, we would expect sexual selection to lead to an increase in the frequency of alleles that optimize the reproductive output of females and males. However, conflicts between females and males, among males, and context-dependent mating strategies maintain genetic variation in molecular components that are involved in female x male interactions (Mank 2017; Neff and Svensson 2013; Clark 2002). As a result, post-mating responses and fusion of egg and sperm vary in their success depending on the genotypes of female and male, a process that has been proposed to also contribute to reproductive isolation (Kirkpatrick et al. 2002) and human infertility (Bonnici et al. 2017; Bosler et al. 2014; Avila et al. 2011; McGraw et al. 2015).

Drosophila melanogaster has proven to be an invaluable model for the study of reproductive biology, delivering insights into the composition and function of the male ejaculate and female post-mating changes induced by the ejaculate (Avila et al. 2011). Further, studies in *D. melanogaster* have delivered insights into the evolutionary

dynamics of genes contributing to the ejaculate, and have shown that there is genetic variation in many ejaculate components (Swanson et al. 2001). Combined with variation in female molecular components on the receiving end, female x male genotype interactions arise that can influence post-mating pre-zygotic processes, which in turn increase or decrease reproductive success. These female x male interactions have been described predominantly at the level of phenotypes (e.g. egg production) and have been associated with genetic variation in male contributors (Zhang et al. 2013; Chow et al. 2010; Fiumera et al. 2006; Arbuthnott et al. 2014; Pischedda et al. 2012; Hollis et al. 2019; Civetta and Clark 2000; Civetta and Ranz 2019). Variation in female tissues, molecular and genetic components directly involved in and downstream of these interactions is an area of active research. The four data chapters presented in this thesis describe work that was done to further our understanding of the female side of female x male interactions on the level of gene expression, genes and tissues, in *D. melanogaster*. Below, I first review what is known about the male and female contributions and responses to mating. Then, I discuss what is known so far about variation in the genotype of female or male that can influence the strength of female post-mating responses and reproductive success, and where the gaps are in our current knowledge. I will focus on studies performed using *D. melanogaster*, but will refer to studies performed using other species when relevant.

The male's assets: sperm, vesicles, proteins and RNAs

The male's ejaculate is the main source of molecules that elicit post-mating responses in females. Proteins in the ejaculate have long been the main focus of

research (Avila et al. 2011; Poiani 2006), but there is growing evidence that RNAs are a functional part of the ejaculate as well (Bono et al. 2011; Alfonso-Parra et al. 2016; Hosken and Hodgson 2014). Studies in other species than *D. melanogaster* have shown that seminal fluid also contains small molecules involved in endocrine signaling (e.g. in mosquitoes (Gabrieli et al. 2014; Pondeville et al. 2008; Clifton et al. 2014), sugars and lipids, immune cells and microbes (Poiani 2006). Sperm and small extracellular vesicles could act as vehicles for the transfer of some of these macromolecules in the seminal fluid (Wilson et al. 2017).

Seminal fluid proteins

SFPs are perhaps the best studied constituents of the *D. melanogaster* ejaculate, next to the sperm proteome (Wasbrough et al. 2010). While sperm are produced in the testes, SFPs are produced in separate, specialized secretory tissues of the male reproductive tract. In *D. melanogaster*, the main producer of SFPs is the paired accessory gland (Kalb et al. 1993). This gland consists of two cell types: small main cells, which produce the majority of SFPs, and around 40 large secondary cells located at the tip of the gland (Bertram 1994).

SFPs have been discovered in ejaculates from a wide variety of species (e.g. *D. melanogaster* (Findlay et al. 2008), mosquitoes (Degner et al. 2019; Boes et al. 2014), honeybee (Baer et al. 2009), chicken (Labas et al. 2015), mouse (Dean et al. 2011) and human (Jodar, Sendler, and Krawetz 2016)). *D. melanogaster* males with ablated accessory gland main cells fail to induce post-mating responses in their mates (Kalb et al. 1993). This observation, together with the widespread prevalence of SFPs and the

fact that many SFPs evolve quickly (Wilburn and Swanson 2016), indicates that they are crucial ejaculate components. Functional studies have uncovered individual proteins in the seminal fluid that are responsible for specific post-mating responses in the female (reviewed in Avila et al. 2011; Chapman 2008), and some of these will be discussed when we review female post-mating responses.

RNA molecules

RNA molecules remain more enigmatic and understudied factors of the ejaculate, especially in insects. The transfer of a handful of RNA molecules encoding seminal fluid proteins has been observed in mosquitoes (Alfonso-Parra et al. 2016) and in heterospecific matings between *D. arizonae* and *D. mojavensis* (Bono et al. 2011). (Fischer et al. 2012) recorded the presence of mRNAs encoding transporters and translation machinery inside *D. melanogaster* sperm and showed that these mRNAs are transferred into oocytes after fertilization. However, RNAs in *D. melanogaster* ejaculates have not been fully characterized.

Ejaculate RNAs have been better studied in mammals. In mammalian ejaculates, RNAs are present in sperm and in extracellular vesicles secreted by somatic tissues of the reproductive tract. Since sperm are transcriptionally silenced, they are thought to acquire RNAs through fusion with extracellular vesicles (Chen et al. 2016). There is evidence that ejaculate RNAs in mammals are functional. For example, prostasomes, small vesicles secreted by the prostate, fuse with sperm and stimulate the acrosome reaction *in vitro* (Palmerini et al. 2003). In mice, extracellular vesicles transport small RNAs from the epididymis to sperm and the presence of these RNAs in sperm

facilitates embryo implantation (Sharma et al. 2018; Conine et al. 2018). Additional studies in mice suggest that miRNAs in sperm influence offspring phenotypes, and this has been proposed to be a form of epigenetic inheritance (reviewed in Chen et al. 2016). Small extracellular vesicles isolated from human seminal fluid were shown to contain a range of small RNAs, among which were miRNAs with the ability to regulate immune genes - but this hypothesis was not tested (Vojtech et al. 2014). Clearly, there is potential for RNA molecules to be important in reproductive success, and *D. melanogaster* may until now have been underused to study this aspect of ejaculates.

Extracellular vesicles

Extracellular vesicles could traffic RNA, proteins or other macromolecules to the female and are becoming a focus of research in *D. melanogaster* (Wilson et al. 2017). Small extracellular vesicles are generated within multivesicular bodies in cells and are released by fusion of the multivesicular body with the cell membrane (Edgar 2016). Extracellular vesicles carry macromolecules from one cell to another to mediate intercellular communication (Edgar 2016). This is important in the immune response (e.g. Tassetto et al. 2017), but, as described in the previous paragraph, these vesicles also play a role in reproduction.

In *D. melanogaster*, a class of small extracellular vesicles called exosomes have been studied. Exosomes are defined by their size, which is around 40-100 um in diameter (Corrigan et al. 2014). Exosomes are secreted by secondary cells in the male accessory gland (Corrigan et al. 2014), but it is unknown what the contents of these exosomes are, or whether other tissues of the male reproductive tract contribute

vesicles to the ejaculate. Fluorescent marker based assays have shown that secondary cell-derived exosomes associate with epithelia and fuse with sperm inside the female reproductive tract (Corrigan et al. 2014). Exosomes were also shown to influence a subset of post-mating responses, as will be discussed below (Corrigan et al. 2014; Leiblich et al. 2012, Hopkins et al. 2019). The fusion of male vesicles with female somatic cells of the reproductive tract has been proposed as a possible route for molecular communication between males and females, but has not been investigated experimentally.

In conclusion, males transfer sperm, vesicles and macromolecules to females and these are known to or have the potential to induce a wide range of post-mating responses.

The female's responses: altered physiology and behavior, accompanied by benefits and costs

The receipt of an ejaculate induces a wealth of responses in females, switching their physiology, and in insects their behavior, from a virgin to a mated female state (Avila et al. 2011; Carmel et al. 2016). Here, we give an overview of female post-mating responses and the female and male contributions necessary to elicit these responses in *D. melanogaster*.

Oocyte production and metabolism

Three responses are important to boost egg laying in mated *D. melanogaster* females. First, oogenesis and yolk protein production are stimulated by endocrine signals from juvenile hormone, ecdysone and ecdysis-triggering hormone (Meiselman et al. 2017; reviewed in Santos et al. 2019). Second, ovulation is stimulated through changes in the reproductive tract musculature and neuronal signaling, particularly of the neurotransmitter octopamine (Middleton et al. 2006; Monastirioti 2003; Lim et al. 2014; Rubinstein and Wolfner 2013; Kapelnikov et al. 2008). Third, a metabolic shift occurs. Mated females undergo changes in feeding behavior (Uchizono et al. 2017; Carvalho et al. 2006) and midgut morphology and physiology (Reiff et al. 2015; Cognigni et al. 2011; Apger-McGlaughon and Wolfner 2013). These changes are thought to occur to sustain the high energetic demands of oocyte and yolk production (Cognigni et al. 2011; Reiff et al. 2015). At least three male SFPs stimulate oocyte production in *D. melanogaster*. Dup99B and ovulin mediate a short-term boost in oocyte production (Saudan et al. 2002; Herndon and Wolfner 1995), while Sex Peptide mediates a long-term increase (Chapman et al. 2003; Liu and Kubli 2003) and mediates the metabolic switch (White et al. *submitted*). Sex Peptide achieves this by binding to a receptor in the female (Sex Peptide Receptor), modulating the activity of female *ppk*$^{+}$ neurons (Yapici et al. 2008). Concurrently, Sex Peptide also stimulates the production of juvenile hormone (Moshitzky et al. 1996). Juvenile hormone in turn also stimulates female gut growth (Reiff et al. 2015).

Sperm storage

To fertilize oocytes, sperm must be stored and maintained, depending on the species for hours up to days or even years (Orr and Zuk 2012; Suarez 2016). *D. melanogaster* females have two specialized types of sperm storage organ, the seminal receptacle and paired spermathecae. It is thought that females mediate sperm storage into these organs via muscle contractions (Middleton et al. 2006; Avila et al. 2012). Two SFPs, Acp29AB and Acp36DE are required for sperm storage (Wong et al. 2008; Neubaum and Wolfner 1999; Avila and Wolfner 2009). If Acp36DE is omitted from the ejaculate, the reproductive tract musculature fails to undergo necessary conformational changes, resulting in failed sperm storage (Neubaum and Wolfner 1999; Avila and Wolfner 2009). SFPs also contribute to sperm storage by forming a mating plug, which prevents sperm from flowing out of the reproductive tract until sperm storage is complete (Avila et al. 2015). Once sperm storage is complete, the female ejects the mating plug and any unstored sperm through the action of *Dh44*$^{+}$ neurons (Lee et al. 2015) (But what triggers sperm ejection is unknown.) Sperm then have to be released from storage in a controlled way so that passing oocytes can be fertilized. The dynamics of this process are not fully understood, but we know that it is influenced by Sex Peptide (Avila et al. 2010) and by female signaling of the neurotransmitters octopamine and tyramine (Avila et al. 2012).

Refractory behavior to remating

Mating lowers a female fly's receptivity towards other males. This post-mating response is induced by Sex Peptide (Chapman et al. 2003; Liu and Kubli 2003), through

its action on SPR and *ppk*$^{+}$ neurons (Häsemeyer et al. 2009), and requires secondary cell-derived exosomes (Corrigan et al. 2014; Leiblich et al. 2012; Hopkins et al. 2019). In addition, pheromones in the seminal fluid and on the male's cuticle are transferred to females during mating, and make mated females less attractive to males (Laturney and Billeter 2016).

Changes in the immune response

Mating also modulates the female immune system in *D. melanogaster*. First, copulatory wounding occurs during mating in *D. melanogaster* (and in many other insects; Reinhardt et al. 2015; Mattei et al. 2015; Kamimura and Mitsumoto 2012). This inflicts a cost onto females, but is potentially beneficial for males. SFPs can be found in the female's circulatory system during and after mating in *D. melanogaster* (Lung and Wolfner 1999). Outside of the reproductive tract, they have the potential to elicit post-mating responses all throughout the female. SFPs could have different methods to traverse the lining of the female reproductive tract, but a wound would provide an easy way into the hemolymph.

Second, sperm and certain SFPs, among which is Sex Peptide, elicit an upregulation of antimicrobial peptides in the epithelium of the female reproductive tract (Kapelnikov et al. 2008; Mack et al. 2006). This could be a beneficial response, if it is protective against STDs, and this hypothesis is consistent with the presence of antimicrobial components in the ejaculate (Lung et al. 2001). But the up-regulation of immune transcripts could also be a side effect of the ejaculate, in which the female immune system responds to unknown male proteins (Morrow and Innocenti 2012;

McGraw et al. 2004). This can even go a step further, since the ectopic expression of certain SFPs in virgin females is “toxic” and impairs viability (Mueller et al. 2007). It remains an outstanding question whether the local immune response in the female reproductive tract can compromise sperm health in *D. melanogaster* and whether this could even be a way for females to select against certain sperm. If the immune response can affect sperm, a balancing act would exist for certain SPFs, weighing influence on the female’s behavior and physiology against a too strong upregulation of the female’s immune system. At least in some vertebrates, experiments suggested that an immune response in the reproductive tract can contribute to post-mating pre-zygotic reproductive isolation (reviewed by Wigby et al. 2019).

In *D. melanogaster*, despite a local upregulation of the immune response in the female reproductive tract, there is a global downregulation of the effectiveness of the immune system, making mated females less likely than virgin females to survive infections (Short et al. 2012). This response is also due to the action of Sex Peptide, more specifically due to the Sex Peptide-induced upregulation of juvenile hormone levels in mated females (Schwenke and Lazzaro 2017). This is hypothesized to be caused by a systemic trade-off between reproduction and immunity, processes with high energetic demands (Schwenke et al. 2016).

Mating-induced gene expression changes

Many of the post-mating responses described above are reflected in mating-induced transcriptional changes in the female. In *D. melanogaster*, gene expression is altered in response to mating in the sperm storage organs (Prokupek et al. 2009), lower

reproductive tract (Mack et al. 2006), oviduct (Kapelnikov et al. 2008), head (Dalton et al. 2010), gut (White et al. submitted) and throughout the body (McGraw et al. 2008; McGraw et al. 2004; Lawniczak and Begun 2004), with changes recorded from 0 hours up to 72 hours after mating. However, across different tissues, gene expression changes peak around 6 hours after mating (McGraw et al. 2008; Mack et al. 2006). Mating influences the transcript abundance of genes involved in the immune response, detoxification (CytP450), odorant binding, eggshell and yolk formation, proteolysis, metabolism, ion transport, muscle development, juvenile hormone metabolism and, specifically in the sperm storage organs, nervous system development (Mack et al. 2006; Kapelnikov et al. 2008; Prokupek et al. 2009; McGraw et al. 2008; Dalton et al. 2010; Lawniczak and Begun 2004). Sperm and accessory gland proteins have distinct effects on the expression of these genes (McGraw et al. 2008; McGraw et al. 2004). Interesting dynamics were also observed when measuring changes in female gene expression in response to particular SFPs (McGraw et al. 2008). Two SFPs, Acp36DE and ovulin, had barely any effect on the female's transcriptome (McGraw et al. 2008). On the other hand, Acp62F and Acp29AB influenced the abundance of over 100 transcripts in the female, including transcripts involved in eggshell formation (Acp62F and Acp29AB) and the immune response (Acp62F) (McGraw et al. 2008). Interestingly, the abundance of certain transcripts involved in muscle contraction was also regulated by SFPs, but in opposite directions depending on the specific SFP (McGraw et al. 2008). This is likely because these SFPs exert different functions inside the female. This observation demonstrates the modularity of the ejaculate and suggests that

reproductive success is influenced by the function of and balance between these different modules.

Insights from studies of female post-mating responses

Taken together, studies on phenotypic and transcriptomic post-mating responses demonstrate that the male's ejaculate has far-reaching effects on females. But they also hint at a complex molecular crosstalk and evolutionary dynamics. Molecular crosstalk occurs at the level of protein interactions (e.g. Sex Peptide and its receptor), transcriptonal responses and modulation of neuronal signaling. If females and males carry different alleles of genes encoding molecules involved in this crosstalk, this clearly has the potential to influence the strength of their interaction and downstream signaling. We would predict that polymorphisms exist in the female and male molecules involved in reproduction, since many of the post-mating responses described above can be a cause of conflict between female and male (Sirot et al. 2014). For example, males benefit from stimulating oogenesis, sperm storage and refractory behavior, but females could benefit from exerting control over the time of egg laying or over which sperm are stored, and whether they remate or not (Sirot et al. 2014). Moreover, the post-mating responses described above have been studied in a setting in which one female mates with one male. However, as in many other species, *D. melanogaster* females mate multiply, giving rise to sperm competition inside the female reproductive tract (Parker 1970). Whether a male is first or last to mate dictates whether he has to resist sperm displacement ("defense") or succeed in displacing sperm ("offense"). Depending on this context, different ejaculate components are important, and this could lead to

antagonistic evolutionary forces working on the ejaculate (Clark 2002). This is also exemplified by McGraw et al. 's (2008) observation that different SFPs can have opposite effects on female gene expression. Because of these evolutionary forces, we would expect to encounter genetic variation in reproductive molecules, which would in turn influence female x male interactions and reproductive success. In the next section, I will discuss experiments that have shown that this is in fact the case.

Natural variation in females and males influences reproductive success, and this is correlated with genetic variation in male ejaculate components.

Even though mating induces robust responses in females, the strength with which these responses are induced varies. Experiments using *D. melanogaster* isogenic or chromosome substitution lines and experimental evolution assays have demonstrated that genetic interactions between females and males influence how many eggs a female produces or how receptive she is to (re)mating (Zhang et al. 2013; Chow et al. 2010; Fiumera et al. 2006; Arbuthnott et al. 2014; Pischedda et al. 2012; Hollis et al. 2019; Civetta and Clark 2000). Reinhart et al. (2015) even described males as "generalists", if they had a consistent performance across all of the female lines they mated with, and "specialists", if they performed best with a particular female line. Such female x male interactions are also evident in the context of sperm competition. Typically, in *D. melanogaster*, the last male to mate will sire most of a female's offspring. However, P_1, the proportion of offspring sired by the first male to mate, varies significantly depending on genetic variation in the female and the competing males (Zhang et al. 2013; Reinhart et al. 2015; Bjork et al. 2007; Chow et al. 2010, 2013;

Clark 1999; Arbuthnott et al. 2014; Clark et al. 1995; Fiumera et al. 2005; Lüpold et al. 2013).

Genetic variation in male ejaculate components contributes to female x male interactions.

Association studies identified significant correlations between variation in post-mating responses or sperm competition, and polymorphisms in or closeby known SFPs (Reinhart et al. 2015; Zhang et al. 2013; Chow et al. 2010; Clark et al. 1995; Fiumera et al. 2005). Interestingly, different genetic markers correlated with either defense or offense capacity in sperm competition experiments (Reinhart et al. 2015). This fits with the observation that males who lack certain ejaculate components can actually have a higher P_1 in sperm competition experiments. This has been observed for males with ablated secondary cells (Gligorov et al. 2013), males that do not transfer secondary cell exosomes (Hopkins et al. 2019) and null mutants for Sex Peptide (Avila et al. 2010) and Acp62F (Mueller et al. 2008). This again demonstrates that the benefits of and selective pressures on certain ejaculate components can be context-dependent.

Genetic variation in SFP loci could affect the expression level or protein sequence of SFPs, and this can in turn impact the triggering of responses in females. For example, the amount of SFP transferred could impact how strongly the female nervous system is stimulated by mating. (Although Smith et al. (2012) did not observe differences in female egg laying depending on variation in the transcript abundance of *Sex Peptide* in their mates.) If different alleles of certain SFPs are segregating in or among populations, there could be variation in how well those SFPs are processed in

the female reproductive tract. For example, the SFP seminase cleaves another SFP, Semp1, which in turn cleaves and activates ovulin and Acp36DE inside the female reproductive tract (Ravi Ram et al. 2006; LaFlamme et al. 2012). Two amino acid residues in Semp1 are required for its cleavage and activation by seminase (Laflamme et al. 2014). Thus, variation in the amino acid sequence of this SFP could impact its processing. Laflamme et al. (2014) also identified a naturally occurring null mutant of *Semp1* in a DGRP line, and males from this line were unable to activate ovulin. Alternatively, there could be variation in how efficiently SFPs process or bind to female target molecules. As described above, the processing of ovulin and Acp36DE requires two male proteases in the seminal fluid, but those are not sufficient. Ovulin's and Acp36DE's processing is incomplete in ejaculates collected directly from the male -as opposed to ejaculates dissected out of the female's reproductive tract-, indicating the need for female contributions (Laflamme et al. 2014).

Divergence of male ejaculate components can contribute to reproductive isolation.

Divergence of reproductive molecules, due to drift or sexual selection, has also been proposed to contribute to incompatibilities between females and males of different populations or subspecies, resulting in initiation or reinforcement of speciation (Kirkpatrick et al. 2002). These incompatibilities can act on pre-copulatory processes. For example, incipient speciation has been described between Zimbabwe strains and cosmopolitan strains of *D. melanogaster*, since Zimbabwe females are less likely to mate with cosmopolitan males, due to the males' cuticular hydrocarbon profiles (Grillet et al. 2012). But post-copulatory, pre-zygotic processes can also contribute to

reproductive isolation. This is clear specifically when a female mates with both a male of her own species and a male of a different species. In this case, the conspecific male will sire most of the female's offspring, regardless of the order of mating ("conspecific sperm precedence") (Howard et al. 1998, Price 1997). Several SFPs in *Drosophila* were shown to be required for a male to be successful when competing against a male of his own species and when competing against a male of a different species (Castillo and Moyle 2014; Civetta et al. 2002; Civetta and Finn 2014). While SFPs are important, the female likely contributes as well. Conspecific sperm precedence has been studied using GFP- and RFP-labeled sperm in *D. simulans* and *D. mauritiana.* These experiments showed that *D. simulans* females rapidly eject heterospecific *D. mauritiana* sperm, and that there is a bias in sperm storage organ and sperm use when a *D. simulans* female has mated with both a *D. mauritiana* and a *D. simulans* male (Manier et al. 2013). This could be caused by incompatible interactions between females and males.

Female genetic contributions to female x male interactions: what is known and what remains cryptic.

Even though female genotype and its interaction with male genotype clearly impact reproductive success (Lüpold et al. 2013; Clark 1999, Lüpold et al. 2019), we still know very little about direct interactions between female and male molecules and what the effect of natural variation is on those interactions.

A notable exception is Sex Peptide in *D. melanogaster*. For Sex Peptide to elicit post-mating responses, action is required of both male and female so-called "Sex Peptide network proteins." These proteins assist in the transfer of Sex Peptide to the

female and in its binding to stored sperm and action in the female (Findlay et al. 2014; LaFlamme et al. 2012; Ram and Wolfner 2009; Singh et al. 2018). As mentioned above, Sex Peptide stimulates responses in mated females among others by binding to the female Sex Peptide Receptor (SPR), influencing signaling of *ppk*$^{+}$ neurons (Yapici et al. 2008; Häsemeyer et al. 2009). Polymorphisms exist in the genomic loci encoding Sex Peptide and SPR and interactions between these polymorphisms correlate with female x male interactions influencing P_1 and female remating behavior (Chow et al. 2010). Outside of the Sex Peptide network, we know that several (predicted) proteases, protease inhibitors, transporters and signal transducers expressed in the female reproductive tract evolve quickly, suggesting that these could interact with male ejaculate components (Prokupek et al. 2008; Swanson et al. 2004). Further, Giardina et al. (2011) identified nonsynonymous polymorphisms in a protease and an odorant binding protein in females, which correlated with the female's receptivity to remate, but whether there was a causal effect was not tested.

In addition to gaps in our knowledge regarding direct interactions between female and male molecules, we have a fairly limited understanding of variation in female responses to male ejaculate components on a sub-organismal level (i.e. on the level of tissues and transcripts or proteins rather than phenotypes like receptivity, P_1 or egg laying).

Progress in this field has been made by (Chow et al. 2013). Chow et al. (2013) performed sperm competition assays using females from the *Drosophila Genetic Reference Panel* (Mackay et al. 2012) and standard males. In these assays, P_1 differed

significantly across female genotypes and correlated with genetic variation in or near many genes expressed in the nervous system (Chow et al. 2013). These associations suggest that the female nervous system is an important player in female x male interactions that influence reproductive success. This is not a surprise, since we know that the nervous system is required for processes such as sperm storage and ejection (Arthur et al. 1998; Avila et al. 2012; Lee et al. 2015), but Chow et al. (2013) were the first to show that variation in neuronal genes is significantly associated with variation in reproductive success. Moreover, Chow et al. (2013) established that three of the identified neuronal genes influenced male reproductive success (measured as P_1) when knocked down in female *ppk*$^+$ neurons. This suggests that differences in a male's ability to influence these neurons in females, and/or differences in how sensitive these neurons are to mating, contribute to female x male interactions, and thus can influence how good of a "match" female and male are.

Another study of variation in female responses on a sub-organismal level was done by McGraw et al. (2009). McGraw et al. (2009) measured mating-induced changes in transcription in whole females mated to males from either one of two lab strains, at 1-3 hours after mating. Unexpectedly, even though male genotype influenced egg laying, negligible differences in transcript abundance were found depending on the genotype of the male (McGraw et al. 2009). McGraw et al. (2009) suggested this could indicate that transcriptional changes are of minor importance early after mating, or that limited divergence of the two male strains contributed to the absence of effects.

To conclude, it has been difficult to identify female molecules that interact directly with male molecules in the reproductive tract or elsewhere in the female. In addition, we have a limited characterization so far of the molecular changes that occur in females in response to mating with males of different genotypes. We propose that studying the effects of female x male interactions on downstream processes such as variation in neuronal signaling or transcription, is worthwhile: These types of studies will indicate which molecular processes in females are particularly sensitive to the male's genotype, and this can give clues as to where to look for female components that are upstream and could directly interact with male ejaculate components.

Progress in understanding the female side of female x male interactions that influence reproductive success.

The second chapter of this thesis describes variation in the female's transcriptome after mating, depending on female and male genotype. I assayed female transcript levels six hours after mating in genetically diverged *D. melanogaster* strains from the Global Diversity Lines (Grenier et al. 2015). Contrary to what was found by McGraw et al. (2009), I identified specific sets of genes (immune genes and genes with roles in sensory perception) that were influenced by female x male genotype interactions, indicating that the female's transcriptome is sensitive to the male's genotype (Delbare et al. 2017). This is different from McGraw et al. (2009), but the fly strains used here were more diverged than those used by McGraw et al. (2009). Variation in post-mating transcript levels did not correlate with variation in egg laying or

receptivity, perhaps due to the time point that was studied, or the fact that whole females were sequenced instead of specific tissues.

In the third chapter of this thesis, my colleagues and I followed up on the study by Chow et al. (2013). Chow et al. (2013) tested only four out of around 30 candidate genes identified using a GWAS. In chapter 2, we describe the knockdown of 27 candidate genes, either ubiquitously or in (subsets of) the female nervous system (Chen, Delbare, White et al. 2019). The knockdown of eight genes influenced P_1 in sperm competition assays. Two of these genes encode molecules that could potentially be secreted into the female reproductive tract, allowing interactions with male molecules. Based on their known functions, other candidates could influence the sensitivity and signaling potential of female *ppk*$^+$ or *tdc2*$^+$ (octopaminergic) neurons. Thus, this study revealed neurons and genes that can be further investigated to understand mechanisms underlying female x male interactions.

Chapter 4 focuses again on transcript levels in mated females, but this time in the lower reproductive tract, and within 15 minutes after mating (Delbare et al. *in prep*). Similar to earlier studies (e.g. Mack et al. 2006), I observed many small magnitude (less than 2-fold) changes in transcript abundance after mating. These reflected changes in genes involved in neuronal and muscle differentiation and a response to copulatory wounding. Surprisingly, I also observed an upregulation of transcripts encoding SFPs. Using SNPs that differed between the female and male lines, I identified several transcripts that are likely transferred from the male to the female during mating. I further

investigated if male secondary cell-derived exosomes were responsible for changes in the female's transcriptome, or for the transfer of RNA molecules, but found no significant effects at this time point.

Chapter 5 stands out from the other data chapters in that it does not focus on female x male genetic contributions to reproductive success. Instead, it investigates whether female and male microbiomes are important for reproduction. Previous studies have shown that germ-free females lay fewer eggs (Elgart et al. 2016; Gould et al. 2018), that egg laying can be influenced by the male's microbiome (Morimoto et al. 2017), and that the absence of a microbiome influences the flies' transcriptome (Broderick et al. 2014). However, it was not known whether interactions between the female's microbiome and her mating status (mated or virgin) influence gene expression changes. In addition, it was not known whether interactions between the female's microbiome and the male's microbiome influence female gene expression. My collaborator and I measured egg laying and transcriptome changes in females with or without a microbiome, 6 hours after mating with a male with or without a microbiome. We established that interactions exist between the female's microbiome and her mating status: transcripts involved in reproduction and transcripts linked with neuronal functions were differentially abundant depending on whether females had a microbiome, but only in mated females, not in virgin females. In addition, immunity genes showed specific responses to either the microbiome, mating, or a combination of those two factors. Interactions between the female's microbiome and the microbiome of her mate influenced the transcriptome of germ-free females. Using an egg laying assay, we

further observed a lower egg laying not only in germ-free females, but also in regular females that had mated to a germ-free male, suggesting that the microbiome can somehow influence the male's ejaculate and its capacity to induce post-mating responses.

Chapter 6 contains a brief discussion of the data chapters and future directions. The appendix describes the design and initial analysis of experiments to examine the role of female genetic constituents in con- vs. hetero-specific sperm precedence.

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CHAPTER 2:

ROLES OF FEMALE AND MALE GENOTYPE IN POST-MATING RESPONSES IN *DROSOPHILA MELANOGASTER*[1]

Abstract

Mating induces a multitude of changes in female behavior, physiology and gene expression. Interactions between female and male genotype lead to variation in post-mating phenotypes and reproductive success. So far, few female molecules responsible for these interactions have been identified. Here, we used *Drosophila melanogaster* from five geographically dispersed populations to investigate such female x male genotypic interactions at the female transcriptomic and phenotypic levels. Females from each line were singly-mated to males from the same five lines, for a total of 25 combinations. Reproductive output and refractoriness to re-mating were assayed in females from the 25 mating combinations. Female x male genotypic interactions resulted in significant differences in these post-mating phenotypes. To assess whether female x male genotypic interactions affect the female post-mating transcriptome, next-generation RNA sequencing was performed on virgin and mated females at 5 to 6 hours post-mating. Seventy-seven genes showed strong variation in mating-induced expression changes in a female x male genotype-dependent manner. These genes

[1] This chapter was published in Journal of Heredity as Delbare, S.Y.N., Chow, C.Y., Wolfner, M.F., Clark, A.G. (2017). Roles of female and male genotype in post-mating responses in *Drosophila melanogaster. Journal of Heredity* 108(7): 740-753, and is reprinted here with permission. Delbare S.Y.N wrote the manuscript, conducted egg laying and receptivity assays and analyzed egg laying, receptivity and RNA-seq data. Chow C.Y. collected samples for the RNA-seq experiment and conducted preliminary analyses. Wolfner M.F. and Clark A.G. provided the initial ideas and design, provided advice throughout the project and provided part of the funding.

were enriched for immune response and odorant-binding functions, and for expression exclusively in the head. Strikingly, variation in post-mating transcript levels of a gene encoding a spermathecal endopeptidase was correlated with short-term egg production. The transcriptional variation found in specific functional classes of genes might be a read-out of female x male compatibility at a molecular level. Understanding the roles these genes play in the female post-mating response will be crucial to better understand the evolution of post-mating responses and related conflicts between the sexes.

Introduction

In sexually reproducing organisms, reproduction is the result of complex interactions between females and males at the organismal, cellular, and molecular levels. In addition, reproductive success varies depending on the genotypes of the female and her mate. Female and male genotypic effects are often non-additive in their impact on mating. Genotypic interactions between females and males can affect pre- and post-copulatory traits that in turn influence reproductive success. For example, interactions between female and male genotype were found to affect female mating rate, fecundity, refractoriness to re-mating (i.e. the likelihood that a previously mated female will re-mate) and sperm competition outcome (Andrés and Arnqvist, 2001; Chow et al., 2010, 2013; Clark et al., 1999; Clark and Begun, 1998; Giardina et al., 2011; Lawniczak and Begun, 2005; Nilsson et al., 2003; Reinhart et al., 2015). Furthermore, female x male genotypic interactions can mediate gametic incompatibility between species (Phadnis and Orr, 2009; Satyaki et al., 2014; Tang and Presgraves, 2015).

Allelic variation in genes important for reproduction largely underlies these female x male genotypic interactions.

Often genes involved in reproductive processes show accelerated rates of evolution; this is thought to be triggered by pressures arising from sexual selection and sexual conflict (Panhuis and Swanson, 2006; Swanson et al., 2001, 2004; Swanson and Vacquier, 2002). Post-copulatory sexual selection potentially mediates co-evolution between females and males from the same population, and this selective force acts to optimize reproductive success. On the other hand, reproductive genes can also be impacted by sexual conflict, as female and male reproductive interests do not always align (Birkhead and Pizzari, 2002). For example, female refractoriness to re-mating is beneficial for the first male to mate. However, it is not necessarily advantageous for the female, as females might benefit from mating with and acquiring sperm from different males. Sexually antagonistic selection can prompt an arms race between females and males, leading each sex to move towards their own reproductive optimum (Sirot et al., 2015). Female x male co-evolution within populations can promote inter-population divergence of molecules required for reproduction. Divergence of reproductive molecules is hypothesized to lead to “miscommunication” between females and males from isolated populations, eventually resulting in reduced reproductive output and the generation of reproductive barriers that may ultimately lead to speciation (Kirkpatrick and Ravigné, 2002; Panhuis et al., 2001; Ritchie, 2007).

In *D. melanogaster*, male-derived molecules have been identified that govern female x male genotypic interactions that affect reproductive phenotypes. After mating, females undergo behavioral, physiological, morphological and gene expression

changes, that are collectively termed “post-mating responses” (Apger-McGlaughon and Wolfner, 2013; Avila et al., 2011; Heifetz et al., 2014; Kapelnikov et al., 2008; M. K. N. Lawniczak and Begun, 2004; Mack et al., 2006; Mattei et al., 2015; McGraw et al., 2004, 2008; Reiff et al., 2015). Female post-mating responses are mediated in part by male-derived seminal fluid proteins (Avila et al., 2011). For example, females mated to transgenic males that lack specific seminal fluid proteins, show differences in post-mating transcript abundances, as compared to wildtype males with a full complement of seminal fluid proteins (Domanitskaya et al., 2007; Gioti et al., 2012; McGraw et al., 2004, 2008). Additionally, polymorphisms in genes encoding seminal fluid proteins impact female post-mating responses and the male’s reproductive success (Clark et al., 2000; Fiumera et al., 2005, 2006; Greenspan and Clark, 2011; Hughes, 1997; Lüpold et al., 2012a; Prout and Clark, 1996; Zhang et al., 2013). Thus, male seminal fluid proteins represent a major molecular component in the reproductive interactions that affect post-mating phenotypes in *D. melanogaster*.

With the exception of the female receptor for the seminal fluid protein Sex Peptide (Yapici et al. 2008; Chow et al. 2010), the female proteins that are involved in these interactions remain poorly understood. One study aimed to address this gap by quantifying female transcriptional responses after mating with a male from the same isogenic strain vs. a male from a different strain (McGraw et al., 2009). No female transcripts responded significantly differently to mating depending on male genotype, however there was limited divergence between the two strains that were used (McGraw et al., 2009). Still, identifying female genes involved in female x male interactions is essential to understanding the molecular and physiological mechanisms behind

variation in post-mating responses. Furthermore, establishing the female genetic basis that underlies female x male interactions is necessary to shed light on the biological processes that play a role in the evolution of post-mating responses. Particularly interesting are those that are affected by sexual conflict, as they potentially advance reproductive isolation and speciation.

In this study, we aimed to identify female genes involved in female x male genotypic interactions, by measuring post-mating transcriptional changes in females mated to males from diverged populations. We exploited natural genetic variation by using five lines drawn from the Global Diversity Lines, a panel of 84 *D. melanogaster* inbred lines collected from five geographically dispersed populations (Beijing, Ithaca, Netherlands, Tasmania and Zimbabwe) (Grenier et al., 2015). Using females and males from five Global Diversity Lines, we used a 5x5 mating scheme to produce 25 different mating combinations. We measured post-mating gene expression changes in whole females using RNAseq and evaluated the effects of female genotype, male genotype, and female x male genotypic interactions on post-mating transcriptional variation. To assess whether variation in post-mating transcription affected reproductive success, we also measured physiological post-mating responses (fecundity and hatchability) and a behavioral post-mating response (female refractoriness to re-mating) for the 25 mating combinations. We found evidence for extensive variation due to female x male genotypic interactions in all post-mating responses that we investigated. In particular, female x male genotypic interactions influenced classes of genes that might be predictive of female x male compatibility at the molecular level.

Materials and Methods

Lines of Drosophila melanogaster and husbandry

Five *Drosophila melanogaster* inbred lines were used. These lines are derived from five geographically dispersed populations (Global Diversity Lines Beijing 04; Ithaca 16; Netherlands 01; Tasmania 01 and Zimbabwe 184 – the latter line was collected in Africa, but turned out to be a recent migrant) (Grenier et al., 2015). These five lines were chosen because of their low levels of heterozygosity, which should limit within-line phenotypic and transcriptional variation. Flies were maintained on standard yeast/glucose media on a 12h light/ dark cycle at 25° C. Virgin females and males were aged 3-5 days in single-sex groups before the start of each experiment. For fecundity and hatchability assays, females were supplemented with live yeast during aging and for the duration of the assays.

Mating scheme and sample collection

Virgin females from each line were singly-mated to virgin males from each of the five inbred lines, similar to a 5x5 full factorial design (fig.2.1). We refer to each cross by the female used, followed by the male used, and replicate number. All matings were observed and males were removed at the end of copulation. For RNAseq and qRT-PCR, mated females were flash frozen 5 to 6h after the start of mating. Age-matched virgin females were flash-frozen in parallel. This time point was chosen to ensure a robust response with transcriptional changes of larger magnitude, compared to earlier time points (Mack et al., 2006; McGraw et al., 2008). For RNAseq, three independent biological replicates were generated for each of the 25 mating combinations and for

virgin females of each genotype (90 samples total). Flies from each replicate were collected from separate bottles, and matings for all three replicates were set up simultaneously. RNA was extracted from five to ten pooled females per replicate. Note that this experimental design resembles a diallel cross. However, gene expression was measured in the females after mating, rather than in the F_1 progeny.

For qRT-PCR, three to four independent biological replicates were collected, with ten females pooled per replicate. Three of the genes tested using qRT-PCR are involved in the immune response (*Dro*, *Def*, *AttB*). We were interested in determining if the expression of these immune genes was affected by female and male genotype. Because immune gene expression is also highly dependent on unmeasured environmental factors such as wild microbial contamination (Gibson, 2008), independent biological replicates were collected from two independent cultures of flies of the same genotype, raised in parallel (fig. S2.12).

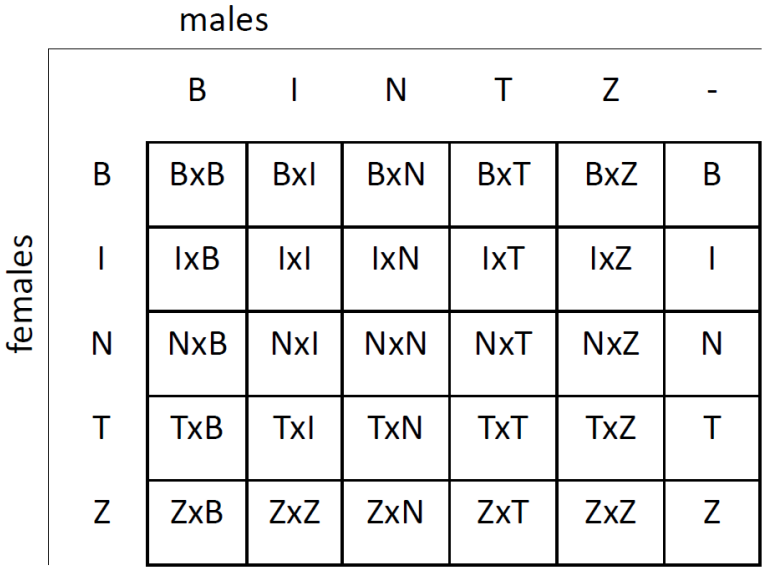

Figure 2.1: Crossing scheme for the five *Drosophila melanogaster* inbred lines (B=Beijing, I=Ithaca, N=Netherlands, T=Tasmania, Z=Zimbabwe). The last column represents virgin females. Cross names list the female's genotype first. After the mating within each cell of the table, RNA was isolated from females only.

Transcript detection

RNA was extracted from whole flies using Trizol (Rio et al., 2010). Whole flies were used because we did not have prior expectations of which tissue(s) might be most important, and because previous studies had shown that even spermathecae-specific genes were readily detected in whole-fly transcriptome analyses (e.g. McGraw et al. 2008). RNAseq libraries were prepared using Illumina's Truseq RNA Library Preparation Kit v2 (cat# RS-122-2001, RS-122-2002). Samples were sequenced in a single-end 100 bp run on a HiSeq2000, at the Genomics Facility in the Cornell Biotechnology Resource Center. For qRT-PCR, RNA was DNase-treated using RQ1

RNase-Free DNase from Promega and cDNA was synthesized using Clontech SMARTScribeTM Reverse Transcriptase. Quantitative PCR was done using the LightCycler 480 SYBR Green I Master from Roche and a Roche LightCycler 480 instrument. *Rp49* was used as a control gene for normalization in qRT-PCR assays (Ponton et al., 2011). *Rp49* transcript levels were found not to change after mating in our dataset (table S2.1). Primer sequences were designed using NCBI primer-BLAST (table S2.2). Results were analyzed using the ΔΔCt method (Livak and Schmittgen, 2001), based on three technical replicates for each biological replicate.

RNAseq data processing and analysis

Read processing and alignment:

FastQC was used to assess the quality of the libraries (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc). One library (I female x Z male, replicate2) was discarded because it contained a very low number of reads (106,087 reads compared to an average of 21 million reads per library). Bases at the 5' end of reads with a phred score lower than 20 were removed using Trimmomatic (Bolger et al., 2014). Reads shorter than 32 bp were discarded. Reads were aligned to the *D. melanogaster* reference genome (dm3) using TopHat2 (Kim et al., 2013). We used the default settings and did not include novel splice discovery, similar to Trapnell et al. (2012). HTseq-count (Anders et al., 2015) was used to determine the raw number of read counts per gene.

Sample quality control:

Biological replicates were compared using MA plots and MDS plots, which indicated ten outlier replicates (B x I-2; B x N-2; B x Z-1; I x I-3; I x N-1; I x T-3; I x Z-1; I x Z-3; N x B-2; N x I-2; fig. S2.1-S2.5). Eight of the ten outlier replicates did not cluster as expected by female genotype or mating status. Two of the ten outlier replicates showed 2-fold or higher differences in expression for over 1000 genes, relative to their biological replicates. These ten replicates were removed from the dataset before filtering out lowly expressed genes. Because all three I x Z samples were removed due to low quality or as outliers, the I x Z combination was completely removed from our dataset. This left 24 mating combinations whose gene expression was to be analyzed.

Differential expression analyses:

EdgeR was used to analyze differential mRNA abundance (McCarthy et al., 2012; Robinson et al., 2010). Read counts were normalized using the CPM (counts per million) function with TMM normalization, to control for size differences among libraries. Based on the normalized counts, lowly expressed genes were removed: a gene was kept in the dataset if it had a CMP > 3 in at least 3 samples. This filtered the dataset down from 14,522 genes to 9,484 genes (fig. S2.6).

Four distinct differential expression analyses were conducted, using linear models, each with its own design matrix. All models were controlled for batch effects, because MDS plots demonstrated a clustering of samples that were processed simultaneously (fig. S2.4). For each linear model, contrasts were set up to find

differentially expressed genes for the comparisons of interest. Differential expression analyses were performed to answer four distinct questions (fig. S2.7):

1. Which genes respond to mating regardless of female or male genotype?

All mated females were compared with all virgin females. (1 contrast total)

2. Which genes respond to mating in a female x male genotype interaction-dependent manner?

The response to mating in a female mated to a particular male was compared with the average response to mating across all combinations of females and males. (24 contrasts total; we did not include the I x Z combination)

3. Which genes respond to mating in a female genotype-dependent manner?

The response to mating in a particular female genotype was compared with the average response to mating across all females. (5 contrasts total)

4. Which genes respond to mating in a male genotype-dependent manner?

The response to mating in females mated to a particular male genotype was compared to the average response to mating across all females mated to all males. (5 contrasts total)

For questions 2, 3 and 4, it is important to note that we were not interested in directly comparing gene expression between females from different lines. Instead, we were interested in detecting differences in the response to mating. Because of this, we always compared females with their respective virgins, before comparing between lines.

For each of the 35 contrasts, we retrieved genes with q-values < 0.05 (p-values corrected for multiple testing using the Benjamini-Hochberg method; Benjamini and Hochberg, 1995; raw p-value quantile-quantile plots: fig. S2.8-S2.11). Flybase and FlyAtlas were used to retrieve information on gene function and tissue-specific expression (Attrill et al., 2016; Chintapalli et al., 2007). DAVID was used to test for enrichment of functional classes among the differentially expressed genes (Huang et al., 2008, 2009). A 5 by 5 factorial ANOVA was used as a different method to address the roles of female and male genotype on post-mating gene expression changes (Supplementary Information p. 15).

Permutation tests:

Permutation tests were performed to ensure that the number of differentially regulated genes detected for questions 2, 3 and 4 differed significantly from the number of differentially regulated genes found by chance. Random sampling was done in R to permute the RNAseq dataset 500 times. Permutations were done within replicate 1, 2 or 3 to still permit for batch effect control in the linear models. The edgeR analyses for questions 2, 3 and 4 were repeated 500 times. For each of the 34 contrasts, we calculated the likelihood of finding a number of differentially regulated genes equal to or larger than the number of differentially regulated genes observed for that contrast based on the original dataset.

Wolbachia:

Four of the five lines we used carry the bacterial endosymbiont *Wolbachia pipientis*. Only the line from the Netherlands is uninfected. Additional analyses were

performed to assess whether the female x male genotypic interactions we observed were due to the presence or absence of *Wolbachia*. The results and discussion of these analyses can be found in Supplementary Information p. 16-18.

Phenotypic assays

Fecundity and hatchability assays:

Singly-mated females were allowed to lay eggs for 24h and were then transferred to a new vial. This was repeated for a total of five days (five vials per female), and eggs were counted daily as a measure of fecundity. Per-vial hatchability was determined as the proportion of eggs that developed into pupae. A total of three independent assays were set up. Egg count and hatchability data were collected from 543 females, yielding an average of 21.7 females for each of the 25 mating combinations. Data from females that died during the experiment, and data from six females that produced fewer than 10 eggs over the course of 5 days were excluded. Egg count data were analyzed in R version 3.3.2 using the lme4 and lsmeans packages (Magezi, 2015, Lenth, 2016). We tested whether the number of eggs produced by a female differed depending on (1) female genotype, (2) male genotype, (3) time, or (4) all possible interactions between these three main factors. Data were fitted using a linear mixed effects model, which assumes a normal error distribution (fig. S2.13). To control for repeated measures on the same female (daily egg counts) “female_ID” was included as a random effect. When analyzing the three assays separately, comparable results were found. Because of this, all three assays were analyzed simultaneously, and “block” was added as an additional random effect to the model. The proportion of hatched eggs was analyzed using a

similar model (fig. S2.14). In these models, *i* represents the effect of the i^{th} female genotype, *j* represents the effect of the j^{th} male genotype, *k* represents the effect of the k^{th} day, *l* represents the effect of the l^{th} block and *m* represents the effect of the m^{th} individual female.

$Phenotype_{ijklm} \sim female_i + male_j + day_k + (female_i * day_k) + (male_j * day_k) + (female_i * male_j) + (female_i * male_j * day_k) + (1|block)_l + (1|female\_ID)_m + \varepsilon_{ijklm}$

Female refractoriness to re-mating:

At twenty-four hours and at four days after the first mating with a male from a Global Diversity Line, a single 3 to 5 day-old virgin *Canton-S* male was aspirated into a vial with one mated female. Pairs of females and males were observed for 1h, and the number of females that started mating with the *Canton-S* male within that hour was recorded. Five assays were conducted to test refractoriness to re-mating on day 1 after the initial mating, and four assays were performed to test refractoriness to re-mating on day 4 after the initial mating. In total, an average of 32 females was tested per female x male combination. We tested whether the number of females that re-mated within 1h differed depending on (1) female genotype, (2) male genotype, or (3) the interaction between female and male genotype. Refractoriness on day 1 and day 4 after mating was analyzed separately. The assays were analyzed using a linear mixed effects model assuming a normal error distribution (fig. S2.15). In these models, *i* represents the effect of the i^{th} female genotype, *j* represents the effect of the j^{th} male genotype, and *k* represents the effect of the k^{th} block.

$$\text{Proportion re-mated}_{ijk} \sim \text{female}_i + \text{male}_j + (\text{female}_i * \text{male}_j) + (1|\text{block})_k + (1|\text{block}_k * \text{female}_i) + (1|\text{block}_k * \text{male}_j) + \varepsilon_{ijk}$$

Correlations between reproductive phenotypes and the transcriptional response to mating.

Correlations between the transcriptional response to mating and reproductive phenotypes were investigated using a Spearman rank correlation test. As a measure of the transcriptional response to mating, edgeR's estimates of the $\log_2$ fold changes of mated vs. virgin females were used for each of the 24 female x male combinations (I x Z was excluded). Correlations were investigated between fold changes and (1) the total number of eggs produced over a period of five days, (2) the average number of eggs produced per day, (3) the total number of eggs produced on day 1 after mating, and (4) the proportion of females that re-mated four days after the first mating. Correlation tests were performed first with the genes that were found to be differentially regulated depending on an interaction between female and male genotype. Second, correlation tests were done with all 9484 genes in our filtered dataset. Because correlations were examined for each gene independently, p-values were corrected using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995).

Data availability

RNAseq data is available on the Gene Expression Omnibus (GEO) website. Supplementary figures and tables can be found in a separate electronic supplement, or online at *Journal of Heredity*.

Results

Transcript levels of 272 genes change post-mating across all mating combinations.

To identify female x male genotypic interactions that influence post-mating transcriptional changes, gene expression was analyzed in mated females from 24 different mating combinations. Specifically, gene expression was measured in females from five diverged lines that were singly-mated to males from these five lines (one combination, I x Z, was excluded from our analysis; see Materials and Methods).

First, we investigated the overall transcriptional response to mating, averaged across all 24 combinations. We detected 272 differentially expressed genes in mated females universally, regardless of female and male genotypes (Supplementary file 2). Of these 272 genes, 50 were down-regulated and 222 were up-regulated in mated females. Only a minority of these genes underwent a 2-fold or greater change in RNA abundance (7 out of 50 for the down-regulated genes, 25 out of 222 for the up-regulated genes). Gene Ontology (GO) functions of the 50 down-regulated genes include cytoskeleton dynamics, immune response, chitin metabolism, sugar and fatty acid metabolism, and genes with functions in the ovary. Among the 222 up-regulated genes, a large proportion is exclusively or highly expressed in the ovary (64/222 genes). Twenty-four genes are exclusively or predominantly expressed in the digestive system, and nine are predominantly expressed in the spermathecae (Attrill et al., 2016; Chintapalli et al., 2007). Up-regulated transcripts encode proteins involved in lipid metabolism, odorant binding, protein folding, the endomembrane system, neurogenesis and muscle system processes, the immune response and chitin cuticle structure,

consistent with previous studies (Dalton et al., 2010; Kapelnikov et al., 2008; Mack et al., 2006; McGraw et al., 2008).

Transcript levels of 77 genes are differentially regulated post-mating depending on interactions between female and male genotype.

Seventy-seven genes responded differently to mating in specific female x male combinations, relative to the average response to mating across all 24 combinations (fig. 2.2-2.3, fig. S2.16, Supplementary file 3). This was greater than the number of differentially expressed genes found by chance based on permutation tests (table S2.3). Therefore, the differential expression of these 77 genes likely represents real biological effects caused by female x male genotypic interactions. On average, transcript levels of these 77 genes were 2.3 times more or less abundant in one specific mating combination, relative to the post-mating abundance of those transcripts across all 24 combinations. For the majority of the 77 genes, the female x male genotype interaction was driven by only one mating combination (fig. 2.2). Only *CG8343* (differentially expressed in B x I and B x N) and ten genes that were differentially expressed in mated Tasmania females (fig. 2.2) showed differential expression in more than one mating combination.

Validation of six transcripts with post-mating expression changes was tested using qRT-PCR (*AttB*, *Def*, *Dro*, *Cyp4p2*, *CG3088* and *Obp49a*). These genes were selected for qRT-PCR testing based on their q-value (< 0.05) and their fold change after mating (at least 2-fold up or down). QRT-PCR validated the RNAseq results for *Def, AttB*, *Dro, CG3088* and *Cyp4p2* (table S2.6; fig. S2.19 A-E; fig. S2.20), even though

Cyp4p2 CPM values were very low (< 3) in Beijing females. *AttB* mRNA levels were higher in B x I relative to the average *AttB* mRNA levels in the RNAseq dataset, but there was a large disparity in the CPM values of the two B x I replicates for *AttB* (CPM for B x I-1= 487, CPM for B x I-2= 40). Still, qRT-PCR results confirmed a strong and consistent up-regulation of *AttB* transcripts in B x I, based on three biological replicates. *Obp49a* mRNA levels increased strongly after mating in I x N based on the RNAseq data, and the two biological replicates in the RNAseq dataset were very similar (CPM for I x N-1= 18, CPM for I x N-3= 27; table S2.6, fig. S2.19 D). However, only one out of three biological replicates for I x N showed a post-mating increase in *Obp49a* transcript levels in the qRT-PCR experiments.

Female- or male-genotype dependent changes in transcript abundance are uncommon.

In addition to identifying interaction effects, we also assessed whether transcriptional responses to mating differed depending solely on female or male genotype. Only two genes were differentially regulated depending on the genotype of the male a female mated with, but these results were not well supported by permutation tests (table S2.5, Supplementary file 3). Twenty-four genes were differentially regulated in a female genotype-dependent manner, regardless of the male with whom these females mated (Supplementary file 3). One of these genes, *Acer*, a gene involved in the regulation of sleep (Carhan et al., 2011), was differentially regulated in females from the Ithaca line. However, permutation tests showed that this result for females from the Ithaca line was not different from what could be found by chance (table S2.4). The remaining 23 genes were differentially regulated in females from the Tasmania line. This number was larger than the number of differentially expressed genes that were

found by chance based on permutation tests (table S2.4). These 23 genes included two genes with expression in the ovary (*CG12200*, *CR43837*, Chintapalli et al., 2007), two genes involved in sensory perception of taste (*Ir7a* and *Gr9a*), one gene encoding a spermathecae-specific cytochrome (*Cyp12d1-d*; Prokupek et al., 2009), and five genes with high expression in the digestive system or Malpighian tubules (*CG10477*, *Cyp12a5*, *CG1139*, *CG11034*, *CG17752,* Chintapalli et al., 2007). One gene, *CG13749*, is up-regulated in infected virgin females (Short and Lazzaro, 2013), and was significantly down-regulated in our mated females from the Tasmania line. This suggests that some of the virgin samples from the Tasmania line carried a pathogen. Indeed, virgin samples from the Tasmania line (and the Zimbabwe line as well) showed high CPM values for a set of antimicrobial peptides (AMPs) (fig. S2.18). That some of our virgin samples might have carried pathogens suggests that caution might be needed with the interpretation of the post-mating changes observed in females from the Tasmania line.

Specific tissues and gene functional classes are affected by female x male interactions at the transcript level.

The 77 genes that were differentially regulated post-mating depending on female and male genotype tend to fall into specific functional classes, or are highly expressed in particular tissues. These tissues and biological functions likely represent molecular mechanisms that underlie variation in female phenotypic post-mating responses and reproductive success. The 77 genes were significantly enriched in genes encoding proteases (DAVID EASE score 1.1×10^{-3}) and immune response genes (DAVID EASE score 1.8×10^{-4}). Specifically, twelve of the 77 genes play a role in the immune response (fig. S2.17). These include antimicrobial peptides (*AttB, Def, Dro, Drs*), three

endopeptidase inhibitors (*Tep1, Tep2, Spn28Dc*), two proteases (*Jon65Aii, CG5909*), a peptidoglycan recognition receptor (*PGRP-SC2*), a protease involved in hemolymph coagulation (*CG11313*), and a gene with unknown molecular function (*edin*). Twenty-two of the 77 genes are expressed exclusively or predominantly in the head (Attrill et al. 2016, Chintapalli et al. 2007). These include serine-type endopeptidases (*CG7829*, *CG9676*, *CG3088*), odorant-binding proteins (*Opb56g*, *Obp49a*, *Obp56h*), one gene involved in neurogenesis (*CG12158*), a G-protein coupled receptor involved in phototransduction (*Rh6*), an olfactory receptor (*Snmp1*), and carbohydrate-binding proteins (*CG8343*, *CG11211*). The latter two are also predicted to function as non-self-recognition proteins in the immune response (Theopold et al., 1999). In addition, four of the 77 genes are highly expressed in the ovary, fifteen genes have expression bias to the digestive system, two genes encoding G-protein coupled receptors have high expression in the thoracic-abdominal ganglion, and seven genes are highly expressed in the spermathecae (Chintapalli et al., 2007). The latter included the serine-type endopeptidase *CG32277*, *Esp* and *CG8329*. A total of 43 out of the 77 genes were previously reported to respond to mating in *Drosophila* (Bono et al., 2011; Dalton et al., 2010; Hollis et al., 2016; Kapelnikov et al., 2008; Mack et al., 2006; McGraw et al., 2004, 2008; Prokupek et al., 2009; Short and Lazzaro, 2013; Zhou et al., 2014).

Phenotypic post-mating responses are influenced by female x male genotypic interactions.

Fecundity, as defined by egg production over the course of five days- and the rate at which egg production decreased over time- differed depending on the mating combination ($p < 7 \times 10^{-6}$, fig. S2.21, table S2.7-S2.8). The strongest interactions

occurred with Beijing, Netherlands or Zimbabwe females that mated to males from the Netherlands or Zimbabwe lines. These combinations produced on average 111 (± 4) eggs over the course of five days, while other combinations produced on average 178 eggs (± 4; fig. 2.4B; fig. S2.21). On day 1 post-mating, strong differences were observed between female genotypes, regardless of male genotype (table S2.8). On day 1, females from the Beijing line produced on average 56 (± 5) eggs, while other female genotypes produced on average 38 (± 5) eggs. This was followed by a rapid decline in egg numbers on day 2 in Beijing females (23 ± 4 eggs), but not in other females (32 ± 4 eggs) (fig. 2.4A).

Similar to the fecundity data, the proportion of hatched eggs on a given day, and the decrease in hatchability over time, differed depending on interactions between female and male genotype (p= 0.01; fig. S2.22; table S2.9-S2.10). Hatchability was consistently high in females from the Zimbabwe line, with an average hatch rate of 77% (± 5) (fig. 2.5A). In other females, hatchability varied depending on the genotype of the male, with hatch rates ranging from 38% in B x T, to 79% in T x Z (± 5; fig. 2.5A). These results exclude females from the Netherlands line, the only line not infected with *Wolbachia*. For females from the Netherlands line who mated to a male that carries *Wolbachia*, most eggs did not hatch due to unidirectional cytoplasmic incompatibility (Hoffmann et al., 1998). Incomplete incompatibility was observed, whereby the incompatibility was stronger in N x I and N x T crosses. This is consistent with the findings of Poinsot et al. (1998), who showed that the effect of *Wolbachia* can differ in distinct genetic backgrounds.

Re-mating rates at 24h after the first mating did not differ depending on female genotype, or on the genotype of the first male with whom she mated (table S2.11). At four days after the first mating, female refractoriness to re-mating differed significantly depending on interactions between female and male genotype (p = 0.002, table S2.12-S2.13). Males from the Beijing line successfully induced refractory behavior in all female genotypes (only 30% of females re-mated, ± 1), except when paired with females from the Netherlands line (71% of females re-mated, ±1; fig. 2.5B). Males from the Netherlands or Zimbabwe lines were worst at inducing refractory behavior in any female, with a re-mating rate of 93% (± 1), relative to 47% (± 1) for females mated to other males. Females more receptive to re-mating consistently produced fewer eggs. Females that produced many eggs had a lower receptivity, although in some cases there seemed to be an uncoupling of these two traits (Spearman rank correlation p-value= 0.001; fig. S2.23).

Variation in transcript abundance after mating is correlated with short-term egg production.

Variation in post-mating fold changes in transcript levels significantly correlated with the number of eggs produced on day 1 after mating. No significant correlations were found with other phenotypes that we measured. Among the 77 genes that were differentially regulated depending on female x male genotype, levels of only one transcript, *CG32277*, correlated significantly with egg production. A more than 2-fold down-regulation of *CG32277* in Beijing females mated to Beijing males was correlated with a higher day-one egg production (q=0.03; fig. S2.24). Virgin CPM values for *CG32277* were similar across lines, but only Beijing females down-regulated *CG32277*

transcript levels after mating. *CG32277* encodes a serine-type endopeptidase that is highly expressed in the spermathecae (Chintapalli et al., 2007).

Among all 9484 genes in our dataset, the post-mating fold changes of 235 genes were significantly correlated with egg production on day 1 after mating (Supplementary file 3). Among the 235 genes, many have high expression in the ovary and known functions in oogenesis or ovulation, such as egg shell formation or octopamine signaling. In addition, the 235 genes included genes with GO terms related to metabolism, transcription and translation, cell division, nervous and muscle system processes, sensory perception and the immune response. Based on the phenotype data, females from the Beijing line produced the highest number of eggs on day 1, whereas females from the Netherlands line produced the lowest number of eggs. For the 235 genes, females from these two lines also differed most in their post-mating fold changes. For example, Beijing females underwent the strongest down-regulation of *Tbh*, a gene involved in ovulation (Monastirioti et al., 1996), while Netherlands females underwent the strongest up-regulation of *Tbh*. However, the differences in transcript-level fold changes between Beijing and Netherlands females were not significant.

Variation in female post-mating responses is not correlated with divergence time between the female genotype and the genotype of her mate.

No clear correlation was found between the number or type of differentially regulated genes detected in our RNAseq data and whether the cross was intra- or inter-population. In intra-population crosses, an average of two differentially regulated genes were detected. In inter-population crosses, an average of 4.6 differentially regulated

genes were found. However, the variance within each of these groups was large (table S2.3). Nevertheless, gene expression changes in Zimbabwe females never differed from the average. On the other hand, post-mating transcriptional changes in females from the Tasmania line were more prone to differ from the average due to female x male interactions (table S2.3).

Likewise, no obvious correlations were found between variation in post-mating phenotypes and divergence time. Measures of fecundity were comparable between intra- and inter-population crosses, with an average of 32 (± 7) eggs produced per day in an inter-population cross, and an average of 34 (± 9) eggs produced per day in an intra-population cross (fig. 2.4B, fig. S2.21). Females mated to males from their own population did not consistently demonstrate a higher refractoriness to re-mating (fig. 2.5B). We expected that hatchability would be higher in intra-population crosses, compared to inter-population crosses. However, this was not the case for the Beijing, Ithaca and Tasmania lines (fig. 2.5A).

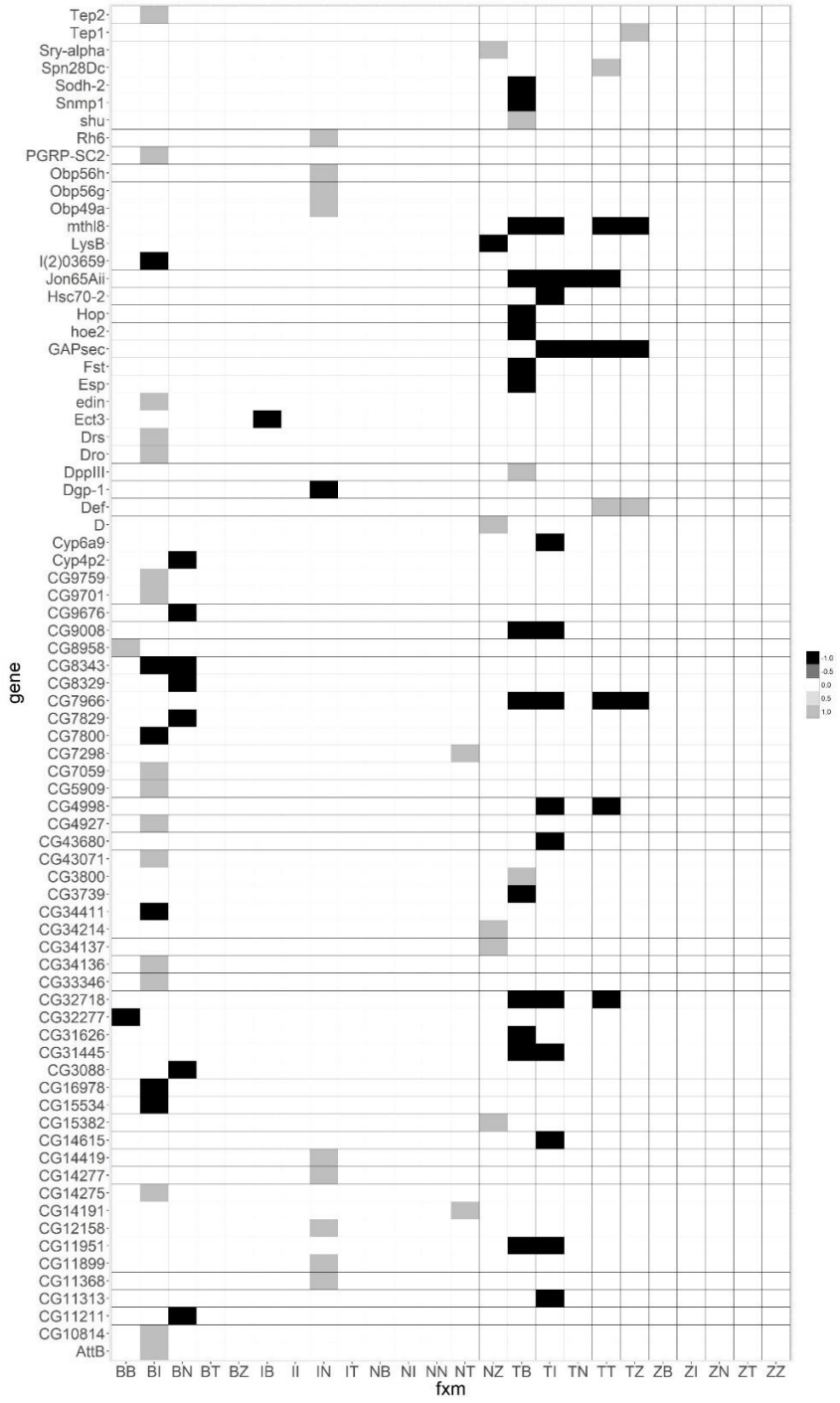

Figure 2.2: For 77 genes, the mating-induced transcriptional changes differed depending on interactions between female and male genotype ($q < 0.05$). Colored cells indicate the 77 genes with respective female x male combinations with significant up- (grey) or down- (black) regulation relative to the average post-mating response across all mating combinations.

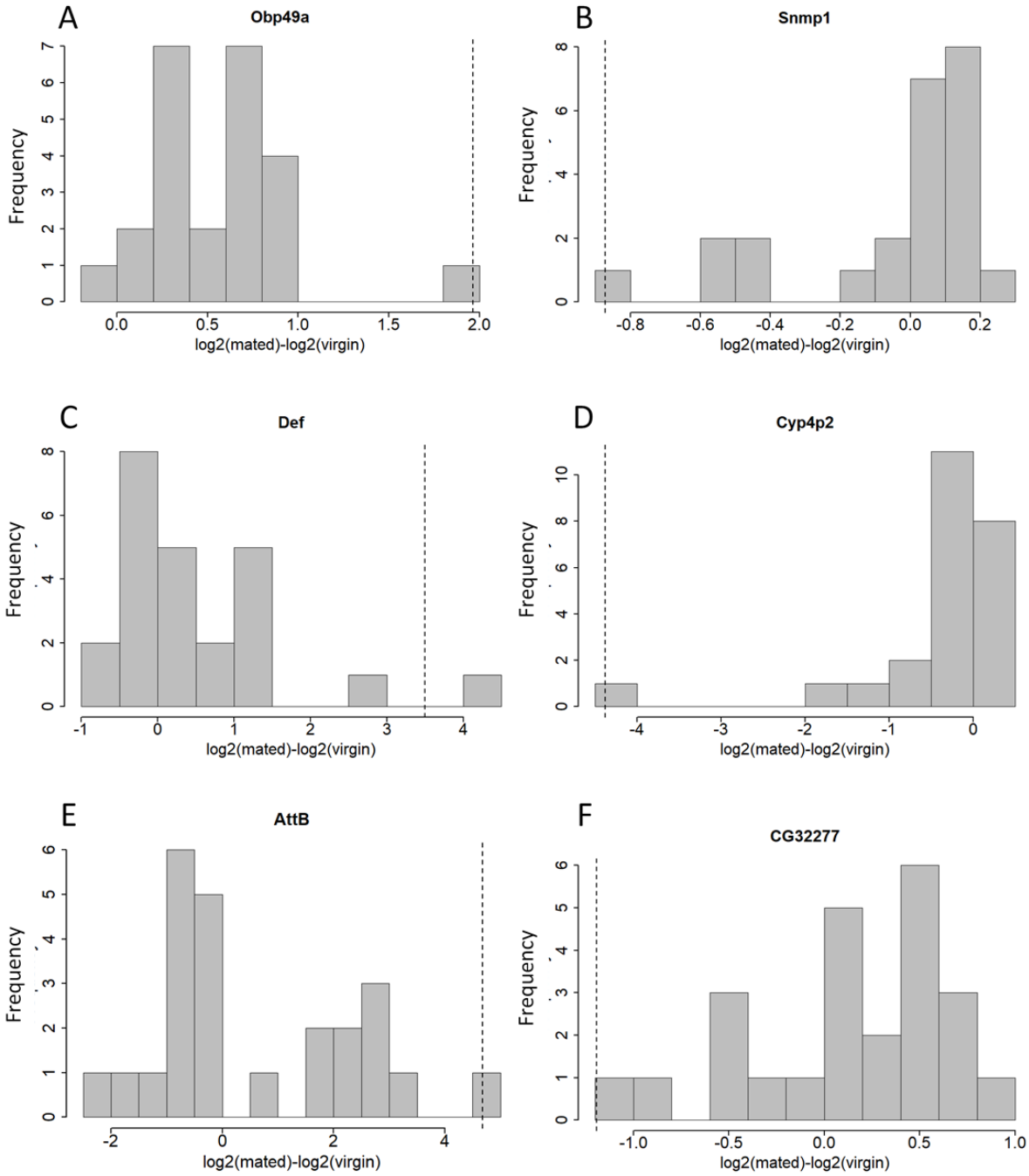
A
Obp49a
Frequency
log2(mated)-log2(virgin)
B
Snmp1
Frequency
log2(mated)-log2(virgin)
C
Def
Frequency
log2(mated)-log2(virgin)
D
Cyp4p2
Frequency
log2(mated)-log2(virgin)
E
AttB
Frequency
log2(mated)-log2(virgin)
F
CG32277
Frequency
log2(mated)-log2(virgin)

Figure 2.3: Distribution of post-mating fold changes across all female x male combinations, for six genes. The dotted line represents the fold change in the genotype(s) that differed significantly from the average post-mating fold change. A: *Obp49a* transcript levels were up-regulated higher than average in I x N (q= 0.015). B: *Snmp1* transcript levels were down-regulated more than average in T x B (q= 0.048). C: *Def* transcript levels were up-regulated more than average in T x T (q= 0.19) and T x Z (q= 6 x 10^{-6}). D: *Cyp4p2* mRNA levels were down-regulated more in B x N (q= 1.5 x 10^{-5}). E: *AttB* transcripts were up-regulated more than average in B x I (q= 0.041). F: *CG32277* mRNA was down-regulated more than average in B x B (q= 0.006).

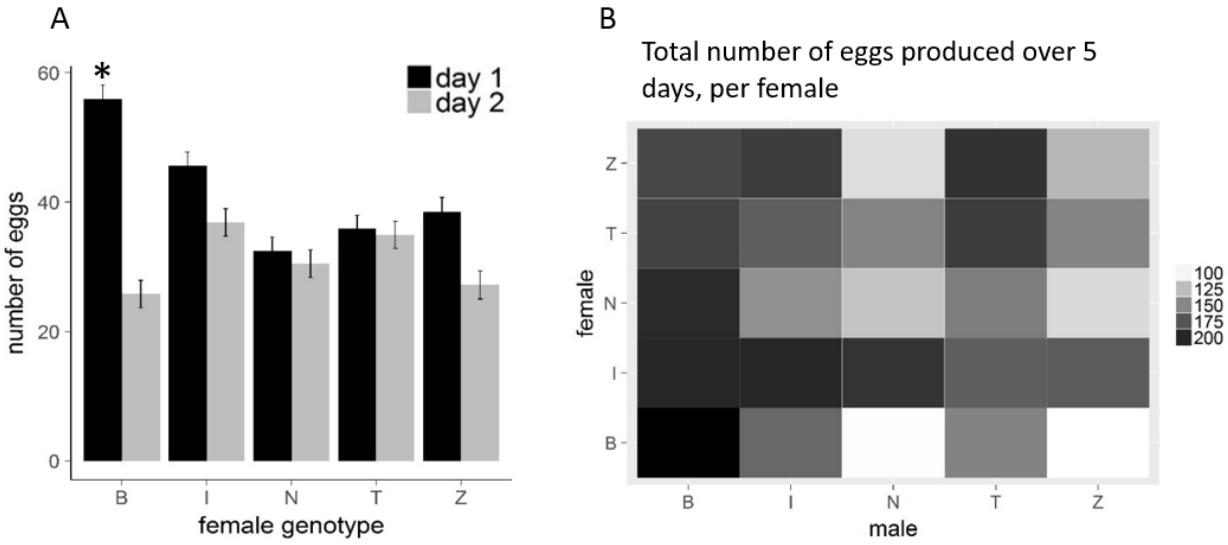

Figure. 2.4: A: Egg production on day 1 and day 2 after mating, for the five female genotypes. On day 1 after mating, females from the Beijing line produced significantly more eggs compared to all other females. Due to a rapid decline in egg numbers on day 2, this significant difference disappeared on day 2 after mating (* $p < 0.05$; error bars indicate standard errors). B: Total number of eggs per female, produced over the course of five days, for all 25 mating combinations. Female x male genotype interactions affected the total number of eggs produced over a total of 5 days (p= 1.6 x 10^{-5}; average n for each of the 25 combinations= 21.7).

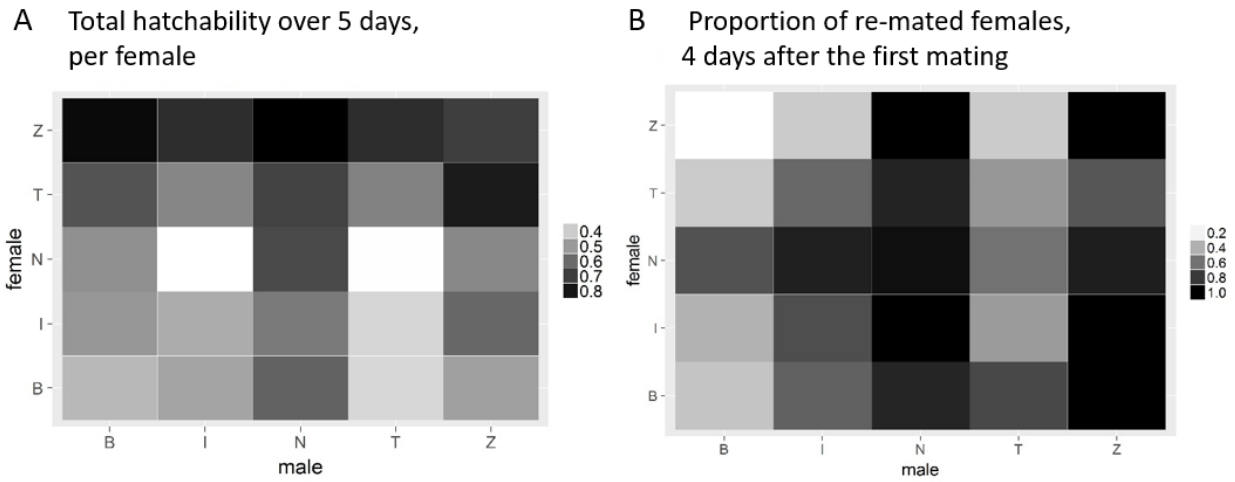

Figure 2.5: A: Proportion of hatched eggs relative to the total number of eggs produced over the course of five days. Female x male genotypic interactions affected hatchability (p= 0.01; average n for each of the 25 combinations= 21.7). B: Proportion of females that re-mated with a standard male, four days after the first mating with a male from the Global Diversity Lines. The tendency to re-mate with a standard male on day four after the first mating, differed depending on female genotype, and depending on the genotype of the male she mated with for the first mating (p= 2.8×10^{-5}; average n for each of the 25 combinations= 32).

Discussion

In this study, we used natural variation in the Global Diversity Lines to assess female x male genotype interaction effects on egg production, hatchability, receptivity and female transcriptional responses to mating, in *Drosophila melanogaster*. Significant female x male interactions were observed for all phenotypes measured here. Our RNAseq analysis identified molecules in females that might underlie female x male genotype-dependent variation affecting reproductive success.

Female x male genotypic interactions affect post-mating responses in the Global Diversity Lines.

Strong interactions between female and male genotype affect phenotypic post-mating responses in the Global Diversity Lines, consistent with previous observations in other lines (Chow et al., 2010; Giardina et al., 2011; Lüpold et al., 2013; Reinhart et al., 2015). The strongest effects in our study were observed for males from the Netherlands and Zimbabwe lines. These males were unable to induce long-term refractoriness to re-mating in all females. In addition, they failed to stimulate long-term egg production in multiple, but not all female backgrounds. Variation across the Global Diversity Lines in the male seminal fluid protein Sex Peptide (SP) might underlie the observed phenotypic responses. SP is crucial for the initiation and maintenance of long-term post-mating responses (Chapman et al., 2003; Liu and Kubli, 2003). Genetic variation might affect SP transfer, storage, or signaling (Chapman et al., 2003; Chow et al., 2010; Cirera and Aguadé, 1997; Liu and Kubli, 2003; Smith et al., 2012; Yapici et al., 2008). Refractoriness to re-mating was variable at 24h after the first mating. Shortly after mating, female receptivity is affected by seminal fluid proteins (Chapman et al., 2003; Saudan et al., 2002) and pheromones (Laturney and Billeter, 2016; Lebreton et al., 2014). Because multiple factors contribute to short-term re-mating rate, stochastic variation in these factors could mask any female or male genotypic effects.

In addition to measuring post-mating phenotypes, we also measured post-mating transcript-level changes in the 24 mating combinations. Several studies have characterized post-mating changes in female transcript-abundance within one line (Kapelnikov et al., 2008; M. K. Lawniczak and Begun, 2004; Mack et al., 2006; McGraw

et al., 2004). We averaged post-mating gene expression changes across diverged lines, and found 272 differentially regulated genes. Sixty-one percent of these differentially expressed genes had previously been reported to respond to mating (Supplementary files 1-2). These consistent gene expression changes might represent the essential transcriptional response to mating, instead of gene expression changes specific to one genetic background.

Female x male interactions at the transcript level would provide a mechanism that underlies the interactions that are observed at the phenotypic level. Unlike previous studies, we identified 77 genes whose change in post-mating transcript level deviated significantly from the average response to mating, depending on the combination of female and male genotype. McGraw et al. (2009) found negligible female x male interaction effects when using microarrays to measure post-mating transcriptional responses in whole females, 1 to 3h after they mated with a male from their own strain or a male from a different inbred lab strain (*Oregon R* and *Canton-S*). Several explanations might account for the discrepancy between the prior and current study. First, shortly after mating, transcriptional changes may not occur because females are "poised" for reproduction, and males simply switch on proteins, RNAs, and molecules that are already present (McGraw et al., 2009). Given that our experiment identified female x male genotype effects at 5 to 6h post mating, interactions on the transcript level potentially occur after the 1 to 3h window examined by McGraw et al. Additionally, stronger interactions might be induced by strains that are genetically more diverged (such as the Global Diversity Lines used here), compared to the two inbred lab strains used by McGraw et al. Antagonistic co-evolution of genes involved in these interactions

could affect reproductive compatibility between diverged populations (e.g. Gavrilets, 2014; Jennings et al., 2014; Sirot et al., 2015; Ting et al., 2001).

Transcripts that are sensitive to female x male genotypic interactions likely underlie variation in phenotypic post-mating responses, and play a role in sexual conflict.

The 77 genes that are sensitive to female x male interactions likely point to mechanisms and biological processes that underlie variation in post-mating phenotypes, possibly through direct interaction with, or downstream responses to, male seminal fluid proteins, several of which have allelic variants known to cause alterations in phenotypic post-mating responses (Chow et al., 2010; Clark et al., 1999; Fiumera et al., 2006, 2005; Greenspan and Clark, 2011; Hughes, 1997; Lüpold et al., 2012a; Zhang et al., 2013) .

These 77 genes might act in a variety of tissues in order to mediate these responses. For example, variation in post-mating transcript levels of genes expressed in the spermathecae and their associated secretory cells could impact sperm storage (Lüpold et al., 2012a, 2013) and maintenance (Schnakenberg et al., 2011; Sun and Spradling, 2013). Differential regulation of genes expressed in the ovary could reflect a male's capacity to induce egg production in a particular female background (Heifetz et al., 2001). Alterations in post-mating transcript levels of genes expressed in the digestive system potentially influence a female's nutrient uptake and metabolism (Shingleton, 2015), which might in turn influence female investment in egg production (Terashima et al., 2005). Differential expression of genes expressed in the head and/ or genes that have sensory functions (vision and olfaction), could alter a female's

response to food or guide her to suitable oviposition sites (Gioti et al., 2012; Harada et al., 2008; Matsuo et al., 2007). Alternatively, differential expression of vision and olfaction-related genes could impact a female's response to other females or males. Mating changes the abundance of both transcripts that encode odorant binding proteins, and odorant binding proteins themselves in females (Findlay et al., 2008; McGraw et al., 2004). Proper functioning of odorant binding proteins and odorant receptors is associated with female sensitivity to male pheromones and re-mating rate (Giardina et al., 2011; Lebreton et al., 2014). *Snmp1*, which is involved in the female response to the male pheromone 11-cis-vaccenyl acetate (Jin et al., 2008), was differentially regulated in our dataset depending on female and male genotype. This suggests that depending on female genotype, some male genotypes have stronger effects on female sensitivity to other males.

Female x male genotypic interactions also affected the expression of immune gene transcripts. Many studies report the up-regulation of immune gene expression after mating, in *Drosophila* (Kapelnikov et al., 2008; Mack et al., 2006; McGraw et al., 2004, 2008; Short and Lazzaro, 2013), other insects (e.g. Baer et al., 2006; Shoemaker et al., 2006), and in vertebrates, including humans (Johansson et al., 2004; Richard et al., 2012; Robertson, 2005; Schjenken and Robertson, 2014). Our study is the first to observe that the intensity of this post-mating up-regulation of immune transcripts depends on an interaction between female and male genotype in *D. melanogaster*. Even though an immune response seems to be an inherent part of the post-mating response, whether it is adaptive in *D. melanogaster* remains speculative. The post-mating up-regulation of immune transcripts could prepare females to fight off sexually

transmitted diseases. In this case, the response is beneficial for both sexes (Lawniczak et al., 2007; Lung et al., 2001; Samakovlis et al., 1991; Zhong et al., 2013). Alternatively, females might induce an up-regulation of AMPs after mating to compensate for the toxic effects of some seminal fluid proteins (Chapman et al., 1995; Innocenti and Morrow, 2009; Morrow and Innocenti, 2012; Mueller et al., 2007; Wigby and Chapman, 2005). It is also possible that females employ the immune response to assess male quality or compatibility (Lawniczak et al., 2007).

The variation observed in the phenotypes and transcript levels described above could be the consequence of sexual conflict. In terms of the immune response, recently mated females have higher AMP mRNA levels, but they are less resistant to systemic bacterial infection than are virgin females, and this difference depends on the transfer of the ejaculate (Fedorka et al., 2007; Short et al., 2012). Genes that impact female olfactory behavior can alter female receptivity to future matings, while polyandry is thought to be beneficial for females and not males. Male influence on female egg production potentially causes sexual conflict as well. Males benefit if females produce many eggs shortly after mating, to ensure the female uses as much of the male's sperm before mating with another male. On the other hand, females might suffer reduced lifetime reproductive output when investing many resources in egg production in a brief period of time (Sirot et al., 2015). We found that down-regulation of the spermathecal endopeptidase *CG32277* in Beijing females correlated with a high day 1 egg production, a trait thought to benefit mainly males. Sexual conflict over *CG32277* expression levels could have resulted in a transcriptional post-mating response that benefits males in the Beijing line. As *CG32277* is a secreted peptidase, it has the opportunity to interact with

male molecules transferred during mating, exposing *CG32277* directly to pressures arising from sexual conflict. Similarly, *Esp* and *CG8329* form potential targets of sexual conflict. *Esp* is a member of the “Sex Peptide network”, a network of male and female proteins required to bind the male seminal fluid protein Sex Peptide to sperm. This process is crucial to mediate long-term post-mating responses, including a long-term reduction in receptivity, in females (Findlay et al., 2014). Expression of *CG8329* occurs both in the head (Chintapalli et al., 2007) and in spermathecae (Prokupek et al., 2009), and is regulated by the seminal proteins *Acp62F* and *Acp29Ab* at 1 to 3h after mating (McGraw et al., 2008).

Validation using qRT-PCR.

For five out of six genes, our RNAseq results were well validated using qRT-PCR. The exception was *Obp49a,* for which only one of three qRT-PCR replicates confirmed the findings from the RNAseq analysis. Although we did not dissect the causes for this, it is most likely due to unmeasured microenvironmental variation, such as might be caused by differences in the medium or microbial contamination. Immune genes were up-regulated more than average in T x T and T x Z in the RNAseq dataset, but Tasmania virgins also had higher CPM values for a range of antimicrobial peptides (fig. S2.17). This raised the concern that variation in immune gene transcripts post-mating was caused by infection rather than mating. Validation using a separate QRT-PCR assay was necessary to ensure that the observed results in T x T and T x Z were reproducible, and were not due to the presence of pathogens in the Tasmania stocks used for RNAseq sample collection. The up-regulation of *Def* in T x T was validated using qRT-PCR, and mRNA levels for antimicrobial peptides were not found to be

higher in Tasmania virgins relative to Beijing and Ithaca virgins in the qRT-PCR assays. This suggests that the observed up-regulation of *Def* is in fact due to genotypic interactions.

Female x male genotypic interactions are more prevalent than male- or female-genotype dependent effects.

No significant differences were found in the transcriptional response to mating depending on male genotype alone. A probable reason is that the role of the male is thought to be limited to triggering the post-mating response. Once the switch from "unmated" to "mated" has been made in the female (and this switch occurs before 5 to 6h after mating), robust female responses take over (Carmel et al., 2016; Heifetz et al., 2014; Heifetz and Wolfner, 2004; Mattei et al., 2015). Additionally, male-only genotype effects might be rare, as the effect of variable seminal fluid protein composition across diverged males would also depend on female genotype-specific sensitivity to this variation in protein composition.

Genes differently regulated depending on only the female genotype (and averaged across male genotypes) were only found in females from the Tasmania line. These differentially regulated genes encoded among others proteases and digestive system-specific proteins, suggesting that post-mating metabolism differs by female genotype. Additionally, the differential regulation of two genes encoding proteins involved in the perception of taste, suggests that female genotypes vary in post-mating gustatory processes. Mating affects these processes, presumably to enhance nutrient intake and reproductive output (Walker et al., 2015). In general, females from the

diverged, geographically isolated populations used here did not show drastic differences in their response to mating. Mating causes systemic changes that affect complex, polygenic traits (Apger-McGlaughon and Wolfner, 2013; Carvalho et al., 2006; Cognigni et al., 2011; McGraw et al., 2004; Reiff et al., 2015; Short and Lazzaro, 2013). Females from different populations might differ slightly in the timing of regulation of the genes involved, or might favor one gene over the other if there is redundancy. Overall, transcriptional changes caused by mating appear to be robust with respect to female genotype.

Variation in female post-mating responses is not correlated with divergence time between female and male genotypes of the Global Diversity Lines.

We expected to see evidence of co-evolution in gene expression patterns, where inter-population crosses, or crosses between more diverged lines would lead to stronger effects on post-mating phenotypes. For example, in ants, queens mated to an allopatric male had higher levels of immune response mRNAs, but this did not happen if the queen had mated with a sympatric male (Schrempf et al., 2015). Among the five lines used in this study, the lines from Ithaca and Tasmania diverged more recently than the lines from Beijing and the Netherlands (Grenier et al., 2015). *Drosophila melanogaster* originated in Africa (Lachaise et al., 1988), but the line from Zimbabwe used here was a recent migrant (Grenier et al., 2015). In our study, we did not see correlations between the strength of post-mating responses and divergence time. We observed, however, that females from distinct lines differed in their sensitivity to female x male genotypic interactions. This suggests that at the transcriptome level, females from isolated populations differ in how sensitive they are to male input. In addition, the timing of

female x male genotype-dependent transcriptional responses might vary across females from isolated populations. Finally, we detected low intra-population hatchability in the Beijing, Tasmania and Ithaca lines, likely due to some degree of inbreeding depression. Kao et al. (2015) have suggested that low hatchability due to hybrid incompatibility could contribute to incipient speciation between *D. melanogaster* lines, but we saw no evidence for hybrid incompatibility among the lines we tested.

Conclusions

Our results demonstrate the widespread effects of female x male genotypic interactions in processes related to reproduction, going from post-mating transcriptional responses to physiological and behavioral changes. Genes affected by female x male genotypic interactions included ones involved in the immune response, ones that might impact egg laying, and ones that are likely involved in post-mating behavioral changes. Future work is needed to uncover the precise roles these genes play in the female post-mating response, to determine to what extent these genes are indicators of female x male compatibility on a molecular level, and to determine if they are affected by (antagonistic) sexual selection.

Funding

This work was supported in part by the National Institutes of Health (R01 HD059060 to AGC and MFW). For part of this work, SYND was supported by a fellowship from the Belgian American Educational Foundation and CYC was supported by a traineeship from the National Institutes of Health (training grant T32-HD052471).

Acknowledgements

We thank E.J. Cosgrove, L.M. Johnson (CSCU), J. Grenier, J. Pleiss, Y. Ahmed-Braimah, Y. Hafezi and E. Davenport for helpful discussions and advice on statistical analyses, A. Manfredo for the RNA-seq library preparation, Z. Waseem for help with fecundity and hatchability assays, and S. Wigby and anonymous reviewers for the helpful comments and suggestions.

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CHAPTER 3:

FEMALE GENETIC CONTRIBUTIONS TO SPERM COMPETITION IN *DROSOPHILA MELANOGASTER*[2]

Abstract

In many species, sperm can remain viable in the reproductive tract of a female well beyond the typical interval to remating. This creates an opportunity for sperm from different males to compete for oocyte fertilization inside the female's reproductive tract. In *Drosophila melanogaster*, sperm characteristics and seminal fluid content affect male success in sperm competition. On the other hand, although genome-wide association studies (GWAS) have demonstrated that female genotype plays a role in sperm competition outcome as well, the biochemical, sensory and physiological processes by which females detect and selectively use sperm from different males remain elusive. Here, we functionally tested 26 candidate genes implicated via a GWAS for their contribution to the female's role in sperm competition, measured as changes in the relative success of the first male to mate (P1). Of these 26 candidates, we identified eight genes that affect P1 when knocked down in females, and showed that five of them do so when knocked down in the female nervous system. In particular, *Rim* knockdown

[2] This chapter was published in Genetics as Chen, D.S.*, Delbare, S.Y.N.*, White, S.L.*, Sitnik, J., Chatterjee, M., Dobell, E., Weiss, O., Clark, A.G., Wolfner, M.F. (2019). Female genetic contributions to sperm competition in *Drosophila melanogaster*. *Genetics* 212(3): 789-800 and is reprinted here with permission. Chen DS, Delbare SYN and White SL are co-first authors. White SL performed the majority of sperm competition experiments, with help from Sitnik J and Chatterjee M. Chen DS and Delbare SYN performed sperm competition experiments with help from Dobell E and Weiss O, and wrote the manuscript. Chen DS. performed the final analysis of the sperm competition data. Wolfner M.F. and Clark A.G. provided the initial ideas and design, provided advice throughout the project and provided part of the funding.

in sensory *pickpocket* (*ppk)*$^{+}$ neurons lowered P1, confirming previously published results, and a novel candidate, *caup*, lowered P1 when knocked down in octopaminergic *Tdc2*$^{+}$ neurons. These results demonstrate that specific neurons in the female's nervous system play a functional role in sperm competition and expand our understanding of the genetic, neuronal and mechanistic basis of female responses to multiple matings. We propose that these neurons in females are used to sense and integrate signals from courtship or ejaculates, to modulate sperm competition outcome accordingly.

Introduction

Natural and sexual selection increase the frequencies of alleles that boost an organism's reproductive success. Sexual selection acts on pre-copulatory traits, such as male courtship behavior and female mate choice, as well as on post-copulatory processes. Sperm competition is one of these post-copulatory processes. Across vertebrates and invertebrates, it can be beneficial for females to obtain multiple mates (Jennions and Petrie 2000). If multiple mating occurs at a high enough frequency, and/or if sperm is stored long term, ejaculates from rival males will compete for oocyte fertilization (Parker 1970). This type of male-male post-copulatory sexual selection mediates the evolution of adaptations in males to mitigate the risk of sperm competition. One form of adaptation is to lower the chances of female remating with other males through transferring mating plugs (e.g. Parker 1970; Orr and Rutowski 1991) or seminal fluid proteins (e.g. Chapman *et al.* 2003; Liu and Kubli 2003), since the last male to mate often sires most of a female's progeny. If a female does remate, characteristics of sperm and seminal fluid proteins influence a male's ability to compete with ejaculates

from other males. *Drosophila melanogaster* has proven to be an especially informative model to study these male × male genotypic interactions. Generally, longer and slower sperm are better at withstanding displacement in *D. melanogaster* (Lüpold *et al.* 2012). Genome-wide association studies (GWAS) further uncovered the genetic basis of male competitive ability. Besides genes encoding sperm components (Yeh *et al.* 2012), genes encoding seminal fluid proteins were discovered to play a role in sperm competition (Clark *et al.* 1995; Fiumera *et al.* 2005, 2007; Greenspan and Clark 2011). These proteins have a variety of functions, such as inducing female refractoriness to remating, stimulating egg laying (e.g. Sex peptide; Chapman et al. 2003; Liu and Kubli 2003) and promoting sperm storage (e.g. Acp36DE; Neubaum and Wolfner 1999; Acp29AB; Wong *et al.* 2008; and Acp62F; Mueller *et al.* 2008). Interestingly, many seminal fluid proteins evolve rapidly (reviewed in Swanson and Vacquier 2002), and some were found to be harmful to females (Civetta and Clark 2000; Wigby and Chapman 2005; Mueller *et al.* 2007), suggesting that their evolution is mediated by sexual conflict: what makes a male a better competitor might actually be disadvantageous to females (Wigby and Chapman 2005; Hollis *et al.* 2019).

Although most studies of sperm competition focused on the role of the male, a number of studies have argued that females are not “passive vessels” in this process. Cryptic female choice, whereby a female selectively uses sperm from ejaculates she received from multiple males, has been proposed as a powerful mechanism for female contributions to sperm competition (Eberhard 1996). A classic example of such female contribution has been observed in junglefowl, in which females were seen to eject sperm from subdominant males after forced copulation (Pizzari and Birkhead 2000).

Studies in *D. melanogaster* with standard male genotypes and varying female genotypes also illustrate that male success depends not only on his genotype and the genotype of his competitor, but also on the genotype of the female (Clark *et al.* 1999, 2000; Lawniczak and Begun 2005; Chow *et al.* 2010; Giardina *et al.* 2011; Zhang *et al.* 2013; Lüpold *et al.* 2013; Reinhart *et al.* 2015). These three-way interactions have been suggested to be important for maintaining polymorphisms in populations (Clark *et al.* 2000; Clark 2002). However, despite the observation that female genotype plays a role, it has been difficult to disentangle female control from female × male interactions, and to identify the genetic loci involved. Recent studies in *Drosophila* have begun to provide a way to dissect the female's role in sperm competition, and to determine the genes and mechanisms that contribute to differences in sperm competition outcome. First, *D. melanogaster* males carrying sperm protamines labeled with GFP or RFP enabled direct observation of competing sperm inside the female reproductive tract (Manier *et al.* 2010) and measurements of heritable variation across female genotypes in sperm ejection, storage and displacement (Lüpold *et al.* 2013). Second, initial studies have been done of the female's genetic makeup underlying variation in her contribution to sperm competition. Chow *et al.* (2013) identified SNPs whose presence in the female was associated with sperm competition outcome by performing sperm competition assays using two standard tester males and females from 39 DGRP lines, a panel of wild-derived inbred lines whose genome sequences are available (Mackay *et al.* 2012). They found variation in the proportion of first male offspring (P1) across DGRP females, and a GWAS revealed correlations between P1 and SNPs in or close to 33 genes (Chow *et al.* 2013). However, roles for the majority of these genes in sperm competition

were not known. Intriguingly, 15 of the 33 candidate genes identified by Chow et al. (2013) have expression biased to the nervous system or have known neural functions, encoding proteins such as ion channels, transcription factors involved in proneural development, or proteins with roles in vesicle trafficking. Moreover, when Chow et al. (2013) knocked down 4 of the 33 candidate genes in female sensory *pickpocket* (*ppk*$^{+}$) neurons, which are required for female postmating responses (Yapici *et al.* 2008; Häsemeyer *et al.* 2009; Yang *et al.* 2009; Rezával *et al.* 2012), they found that knockdown of 3 of these 4 candidates mediated changes in P1, demonstrating a direct role for the female nervous system in impacting the paternity share of each male (Chow et al. 2013). This result supported the hypothesis from Arthur *et al.* (1998) that the female nervous system might influence sperm competition, based on the observation that the female nervous system is required for proper sperm storage. The importance of the female nervous system in sperm competition is further supported by findings regarding *sex peptide receptor* (*SPR;* Chow et al. 2010) and *Neprilysin 2* (Sitnik et al. 2014), which are two additional genes known to affect female contributions to sperm competition. This was determined in experiments that knocked down *SPR* or *Neprilysin 2* in females ubiquitously, but both genes are known to be expressed in the female nervous system.

Nevertheless, many questions remain to be answered regarding the female's involvement in sperm competition. For example, the relative contributions of neuronal versus non-neuronal tissues to a female's influence on sperm competition remain to be elucidated. In addition, we do not know if other neurons besides *ppk*$^{+}$ neurons are involved. For example, the neuromodulator octopamine is required for sperm release

from storage (Avila *et al.* 2012; Sitnik *et al.* 2014), and both octopamine and octopaminergic *Tdc2*$^{+}$ neurons are required for ovulation (Monastirioti *et al.* 1996; Monastirioti 2003; Cole *et al.* 2005; Rubinstein and Wolfner 2013; Rezával *et al.* 2014) and refractoriness to remating (Rezával *et al.* 2014), suggesting a potential role for octopamine and *Tdc2*$^{+}$ neurons in sperm competition. Here, we aim at determining whether other candidate genes put forward by Chow et al. (2013) influence sperm competition, and whether or not most of them do this by acting through the female nervous system. We individually knocked down candidate genes using RNA interference (RNAi) in females, either ubiquitously or in the nervous system. Knockdown and control females were mated consecutively to two distinct tester males and we assessed the effect of knockdown on paternity ratios. Of 26 genes tested, 8 genes were found to affect the ratio of offspring sired by each male, significantly expanding the number of genetic loci known in females to influence sperm competition. The majority of these genes (5 out of 8 genes) affected sperm competition outcome when knocked down in the female nervous system, and we identified a role for not only *ppk*$^{+}$, but also *Tdc2*$^{+}$ neurons in sperm competition. Our results provide functional evidence that further emphasizes the crucial role of the female nervous system in sperm competition. These results will allow detailed dissection of the mechanisms of cryptic female choice and sperm competition inside the female reproductive tract, and by extension effects of post-mating pre-zygotic sexual selection and sexual conflict.

Materials and Methods

Fly stocks and husbandry

The UAS/GAL4 system (Brand and Perrimon 1993) was used to individually knock down candidate genes ubiquitously, pan-neuronally or in subsets of neurons in the female nervous system. Driver lines used were: ubiquitous drivers *Tubulin-GAL4/TM3, Sb* and *Tubulin-GAL80^{ts}; Tubulin-GAL4/TM3, Sb*, nervous system-specific drivers *nSyb-GAL4* (Hindle *et al.* 2013), *ppk-GAL4* and *Tdc2-GAL4* (Cole *et al.* 2005). UAS-RNAi lines were ordered from the Vienna *Drosophila* Research Center (VDRC) for each candidate gene identified in a GWAS (Chow et al. 2013) with the following exceptions: *CG34027*, *CG10858*, *RFeSP* and *sti* (no VDRC lines were available for these genes), and *CG13594* (the only available VDRC line has 94 predicted off-targets). VDRC IDs for all VDRC lines are available in table S3.1. Lines were used from both the KK (*attP* background) and GD (*w^{1118}* background) RNAi libraries. Males used for the sperm competition assay had the *cn bw* or *bw^{D}* genotypes. Males and virgin knockdown and control females were aged 3-7 days in single-sex vials before the start of each experiment.

Fly stocks were maintained at room temperature on standard yeast/glucose media on a 12 hr-light/dark cycle. When using *Tubulin-GAL80^{ts}; Tubulin-GAL4/TM3, Sb*, crosses were set up at room temperature, and knockdown and control virgin females were aged at 29°C and maintained at 29°C throughout the sperm competition assay.

Verification of knockdown level

To verify knockdown level, UAS-RNAi lines were crossed to *Tubulin-GAL4/TM3, Sb* to generate *Tubulin-GAL4>*UAS-RNAi knockdown flies. Control flies were generated in one of two ways: 1) *TM3, Sb*; UAS-RNAi siblings obtained from the same crosses, or 2) *Tubulin-GAL4>w^{1118}* or *Tubulin-GAL4>attP* flies generated by crossing *Tubulin-GAL4/TM3, Sb* to *w^{1118}* or *attP* (for GD and KK lines, respectively). Age-matched *TM3, Sb*; UAS-RNAi siblings or *Tubulin-GAL4>w^{1118}* or *Tubulin-GAL4>attP* flies were collected at the same time as knockdown flies and tested as controls. Five candidate genes did not yield viable *Tubulin*-GAL4>UAS-RNAi F1 progeny, suggesting that ubiquitous knockdown of the target gene was lethal and that the RNAi-mediated knockdown was successful at perturbing gene expression. For crosses that yielded viable *Tubulin-GAL4>*UAS-RNAi F1 progeny, RT-PCR was used to assess knockdown level of each UAS-RNAi line (methods described in Ravi Ram *et al.* 2006; Table S3.1). Briefly, total RNA was isolated from 10-20 knockdown and control females using TRIzol according to manufacturer's instructions. RNA was Dnase treated (Promega) and cDNA was synthesized (Clontech). PCR was used to amplify genes of interest and a housekeeping gene (*Actin5C* or *Rp49*), and the results were analyzed on a 1-2% agarose gel using gel electrophoresis. Dilutions were made of cDNA from knockdown and control females to compare their relative levels of expression.

Sperm competition experiments

In each experiment, knockdown females were generated by crossing UAS-RNAi lines for each candidate gene to a GAL4 driver. To obtain control females with wild-type

gene expression, flies from the appropriate background stock (*attP* or w^{1118}) were crossed with flies from the same GAL4 driver. Control and knockdown females were mated to *cn bw* males in single pair matings on day 0 in vial 1. Copulations were observed. Males were removed after copulation ended and mated females were retained in the individual vials. In the evening of day 1, two bw^{D} males were added to each vial and left with the female overnight. Both bw^{D} males were removed in the morning of day 2, and each female was transferred to vial 2. Each female was transferred again every 48 hrs to vials 3, 4 and 5 (on days 4, 6 and 8, respectively). All females were discarded on day 10. Progeny from eggs laid in vials 1-5 were reared to adulthood and the paternity of F1 female progeny was scored based on eye color: female offspring of *cn bw* males had red eyes, and female offspring of bw^{D} males had brown eyes. Male progeny were not scored because they were *w*, making it impossible to use eye color to assess their paternity. On average, each experiment consisted of 71.8 ± 25.1 control females and 65.9 ± 24.3 knockdown females who had mated at least once (mean ± standard deviation). Of these females, 51.9 ± 21.7 control females and 46.9 ± 21.0 knockdown females in each experiment had mated with both males. Sample sizes for each experiment can be found in table S3.2.

Since each female is paired with two bw^{D} males and left overnight for the second mating, there is a chance for multiple remating events to occur, which would affect sperm competition. Nonetheless, in a separate experiment, we found that 0 out of 275 mated females remated twice within a 16-hour period.

Statistical analysis of remating rate, fertility and P1

Remating rate, fertility, and P1 of knockdown and control females were calculated based on the number of first- and second-male progeny. All statistical analyses were performed using base R (version 3.3.1; R Core Team 2016) and the packages lme4 (Cole *et al.* 2005; Bates *et al.* 2015), lmerTest (Kuznetsova *et al.* 2017) and emmeans (https://cran.r-project.org/web/packages/emmeans/index.html). Remating rate was calculated as the proportion of doubly-mated females among all females who mated with the first male. Differences between remating rates of knockdown and control females were compared using Fisher's exact test.

Because we only scored eye color in female offspring, we used the total number of female progeny produced by each doubly-mated female (rather than all progeny) as a proxy for fertility. Females who had mated only once (with the first or the second male, but not both males) were excluded from the analysis because we found a significant study-wide difference between the fertility of singly-mated and doubly-mated females (Fig. S3.1). We compared fertility of control and knockdown females by fitting linear models, or linear mixed models for experiments with multiple replicates, and by performing an ANOVA.

Finally, P1 was calculated for each doubly-mated female as the ratio of the number of female offspring sired by the first male vs. the total number of female offspring sired by either the first or second males in vials 2-5. Vial 1 was excluded from the calculation of P1 because both matings occurred in vial 1, and with this experimental setup, we were unable to determine how many offspring were sired before

the second mating. However, the presence of first- and/or second-male progeny in all vials was used to determine whether a female had mated with both males. For the statistical analysis of P1, we arcsine square-root transformed P1 values before applying linear models, or linear mixed models for experiments with multiple replicates, to the transformed values. Temporal dynamics of P1 between control and knockdown females were compared using linear mixed models, with genotype and vial as fixed effects and individual females as a random effect.

Since we analyzed 26 candidate genes using multiple *GAL4* drivers, we performed a study-wide Benjamini Hochberg (Benjamini and Hochberg 1997) correction for multiple testing. This correction was performed on all nominal p-values for the test of overall P1. For the temporal analysis, we obtained four p-values for each gene/driver combination tested (one for each vial). We performed an FDR analysis on p-values for each of these vials separately, across gene/driver combinations.

Data availability statement

Supplementary figures and table legends can be found in this document. All supplementary tables, R scripts and progeny count data are available in a separate electronic supplement or on figshare (accessible through the Genetics website).

Results

Chow et al. (2013) identified 33 top SNPs associated with sperm competition outcome in females. Not all of these SNPs were located within genes. Thus, to identify genes that directly affect sperm competition, we used RNAi to individually knock down

genes that were put forward as candidates by Chow et al. We tested 26 of the 33 candidate genes for their roles in influencing the female's contribution to sperm competition, which we scored as P1, the proportion of first male progeny among total progeny after the second mating. Five of the genes that were identified by Chow et al. could not be tested because no suitable UAS-RNAi lines were available from the VDRC. RNAi lines for two additional genes, *SK* and *CG33298*, gave no detectable RNAi knockdown (Table S3.1), and thus, we were unable to assess these two genes' role in sperm competition (Fig. 3.1A).

Candidate gene knockdown caused changes in remating rate and fertility.

How readily females remate after the first mating and how fertile they are can influence the risk and intensity of sperm competition. Therefore, we assessed the effect of knocking down each of the 26 genes on remating rate and fertility. Of the 26 genes, ubiquitous knockdown of three genes (*CG10962*, *CG33095* and *Ddr*) reduced female remating rate (Table 3.1, Fig. S3.2). Reduced remating rate observed upon ubiquitous knockdown needs to be interpreted with caution, since ubiquitous knockdowns could directly affect female receptivity to remating, or could have detrimental effects on overall female health or development, making females simply less inclined to mate. Hence, we also analyzed the effects of neuronal knockdown on remating rate. Remating rate was reduced by *Tdc2*$^{+}$ neuron-specific knockdown of *Rab2* and *ppk*$^{+}$ neuron-specific knockdown of *para* (Table 3.1, Fig. S3.2). Finally, *Tdc2*$^{+}$ and *ppk*$^{+}$ neuron-specific knockdown of *hid* led to an increase in remating rate (Table 3.1, Fig. S3.2). Since *hid*

expression stimulates apoptosis (Grether *et al.* 1995), differences in the numbers or innervation patterns of *Tdc2*$^{+}$ and *ppk*$^{+}$ neurons might be responsible for this effect.

Female fertility was affected by many candidate gene knockdowns. Ubiquitous knockdown of 18 of the 26 genes reduced female fertility (Table 3.2, Fig. S3.2). However, as mentioned above, these results could be either direct or indirect consequences of ubiquitous gene knockdown. Consistent with the latter hypothesis, we found that nervous system-specific knockdown of only 5 genes caused a decrease in female fertility (*btsz*, *caup*, *Ddr*, *Rab2*, *Rim*; Table 3.2, Fig. S3.2). Specifically, *Tdc2*$^{+}$ neuron-specific knockdown of *Rab2* mediated a substantial decrease in both fertility and remating rate, to the extent that very few doubly mated females were retrieved for sperm competition experiments (only 8 out of 50 females remated). Because the knockdown was tissue specific, these results strongly suggest that *Rab2* is essential for the proper functioning of *Tdc2*$^{+}$ neurons, which are in turn known to be required for female remating and fecundity (Rezával *et al.* 2014). Interestingly, ubiquitous knockdown of *Zasp66* and *ppk*$^{+}$ neuron-specific knockdown of *hid* significantly increased fertility (Table 3.2, Fig. S3.2).

Although all 26 candidate genes were detected in a GWAS based on sperm competition outcomes, these results suggest that some of the genes also play roles in modulating other female reproductive traits. For the P1 measurements reported below, we found that candidate gene knockdown could affect P1 without affecting fertility (Fig. S3.5G, H), but we also observed cases in which both P1 and fertility differed between control and knockdown females. If fertility was reduced, we observed cases in which the reduction impacted only one of the two males (e.g. Fig. S3.5F), and cases in which it

impacted both males, but one male more than the other (e.g. Fig. S3.5A). Both of these scenarios could lead to a change in P1.

Table 3.1: Remating rate for control and knockdown (KD) females. Remating rate was calculated as the ratio of the number of females that mated with both males vs. the total number of females that mated with the first male. Differences between control and KD females were evaluated using a Fisher's exact test.

Genes	**Drivers**	**Remating rate control**	**Remating rate KD**	**p-value**
CG10962	*Tubulin*	0.88	0.62	0.000
CG33095		0.81	0.63	0.039
Ddr		0.64	0.37	0.004
Rab2	*Tdc2*$^{+}$	0.60	0.21	0.000
para	*ppk*$^{+}$	0.89	0.72	0.006
hid	*Tdc2*$^{+}$	0.53	0.86	0.000
	ppk^{+}	0.66	0.91	0.000

Table 3.2: Mean female fertility for control and knockdown (KD) females. Female fertility was calculated as the total number of female progeny produced by doubly mated females over the course of 10 days. Significant differences between control and KD females were evaluated using linear models. Sd = standard deviation.

Genes	**Drivers**	**Mean fertility control (± sd)**	**Mean fertility KD (± sd)**	**p-value**
Ddr	*ppk*$^{+}$	180.83 (35.11)	161.89 (34.81)	0.004
	Tdc2^{+}	113.07 (20.22)	103.28 (22.98)	0.042
	nSyb	144.53 (29.71)	98.21 (32.63)	0.000
Rim	*Tdc2*$^{+}$	204.57 (47.96)	181.50 (49.24)	0.035
Rab2	*Tdc2*$^{+}$	76.92 (21.09)	14.25 (8.75)	0.000
caup	*Tdc2*$^{+}$	133.95 (28.69)	120.57 (22.99)	0.012
btsz	*nSyb*	142.13 (31.65)	117.89 (52.73)	0.008
	Tubulin	127.08 (21.89)	93.77 (28.61)	0.000
Zasp66	*Tubulin*	82.83 (17.18)	103.48 (25.79)	0.000

hid	*ppk*$^{+}$	177.24 (50.46)	221.67 (41.59)	0.000
CG10962	*Tubulin*	91.28 (35.99)	61.58 (30.12)	0.000
CG15800		113.92 (17.24)	90.60 (21.05)	0.000
CG31872		142.21 (22.79)	117.15 (28.32)	0.000
CG32532		144.13 (22.48)	91.46 (40.95)	0.000
CG32834		113.92 (17.24)	100.37 (20.63)	0.002
CG33095 5-HT2B		128.31 (23.49)	102.55 (28.22)	0.000
CG6163		139.20 (38.85)	72.72 (30.33)	0.000
sona		128.31 (23.49)	113.97 (27.49)	0.007
Cyp313a2		142.16 (30.84)	109.07 (31.19)	0.000
Msp300		135.10 (32.95)	125.90 (26.78)	0.014
Rbp6		91.28 (35.99)	56.27 (15.59)	0.000
Shab		119.46 (19.49)	94.39 (27.48)	0.000
sima		139.20 (38.85)	113.98 (24.63)	0.000
spz5		139.20 (38.85)	108.46 (26.81)	0.000
uif		135.10 (32.95)	111.24 (35.96)	0.000
		142.16 (30.84)	97.57 (22.37)	0.000

Seven genes influence sperm competition outcome upon ubiquitous or pan-neuronal knockdown in females.

Of the 26 candidate genes of interest, three (*para, Rim* and *Rab2*) were reported to affect P1 when knocked down in *ppk*$^{+}$ neurons by Chow et al. (2013). For the 23 remaining candidate genes, in an initial test we knocked down each candidate ubiquitously with *Tubulin-GAL4*. If constitutive ubiquitous knockdown was lethal, and/or if the gene of interest had a known neural function, *Tubulin-GAL4; Tubulin-GAL80*^{ts} or the pan-neuronal driver *nSyb-GAL4* were used instead of *Tubulin-GAL4*. In cases where ubiquitous knockdown produced a significant effect on overall P1, we proceeded to knock down the gene pan-neuronally, with the exception of *CG31872* and *CG32834*. These two genes are not expressed in the nervous system, but are expressed in the female rectal pad and sperm storage organs, respectively (Leader *et al.* 2018). We hypothesize that the effects of knockdown on sperm competition outcome may be due to the importance of these genes' expression in the female reproductive tract or other non-neural tissues.

Ubiquitous knockdown of five genes in females caused reduction of P1 (*btsz, CG31872, CG32834, Ddr, Msp300*; Fig. 3.1B-F, Fig. S3.2), attributable to fewer first male progeny and more second male progeny (*CG31872* and *CG32834,* Fig. S3.5B, C), fewer first and second male progeny (*btsz*, *Msp300,* Fig. S3.5A, E), or fewer first male progeny but similar numbers of second male progeny relative to control females (*Ddr,* Fig. S3.5D). The overall fertility of these knockdown females was also lower than that of control females for all five genes (Fig. S3.2).

Additionally, we found four genes whose pan-neuronal knockdown caused an increase (*hid, Msp300*) or decrease (*btsz, caup*) in P1 (Fig. 3.1G-J, Fig. S3.2). Pan-neuronal knockdown of *btsz* reduced the number of first male progeny without affecting the number of second male progeny, leading to an overall reduction in fertility (Fig. S3.5F). Pan-neuronal knockdown of *caup* and *hid* affected the relative proportions of first- and second-male progeny without influencing overall fertility (Fig. S3.5G, H). Finally, *Msp300* pan-neuronal knockdown females produced more first male progeny but similar numbers of second male progeny compared to control females, but the overall fertility difference between *Msp300* knockdown and control females was not significant (Fig. S3.5I). Intriguingly, ubiquitous knockdown of *Msp300* lowered P1, while pan-neuronal knockdown increased P1 (Fig. 3.1F, J, Fig. S3.2). This suggests that ubiquitous knockdown of *Msp300* could be detrimental to females' health, or that *Msp300* expression in different tissues has distinct effects on sperm competition. Overall, we found 7 genes that had effects on sperm competition when knocked down ubiquitously or pan-neuronally in females.

When analyzing P1 on a temporal, vial-by-vial basis, we found that at least two vials were significantly different between control and knockdown females for each of the genes that had an effect on overall P1 (Fig. S3.4). *Ddr*, which affected overall P1 upon ubiquitous knockdown only, also showed significant effects on P1 in vials 2 and 3 with pan-neuronal knockdown (Fig. S3.3F), suggesting some neuronal function for *Ddr* as well. Finally, five genes (*sima, sona, spz5*, *CG33095* and *Zasp66*) did not change overall P1 when knocked down ubiquitously, but significantly affected P1 in at least one vial when ubiquitous knockdown was analyzed on a vial-by-vial basis (Fig. S3.3A-E).

This result could have several explanations. The products of these five genes might influence processes that are important for sperm competition at specific times after the second mating. Alternatively, these gene products or the processes they mediate might have small roles or are redundant players in sperm competition.

Tdc2^{+} *and ppk*$^{+}$ *neurons play roles in sperm competition*

Informed by the results of the initial test, we further asked in which of the female's neurons the products of *btsz, caup, hid, Msp300* and *Ddr* act to modulate sperm competition. In particular, we assessed the functions of these five genes in octopaminergic *Tdc2*$^{+}$ neurons and proprioceptive *ppk*$^{+}$ neurons, which have been implicated in female responses to mating (Cole *et al.* 2005; Yapici *et al.* 2008; Häsemeyer *et al.* 2009; Yang *et al.* 2009; Avila *et al.* 2012; Rezával *et al.* 2012, 2014; Rubinstein and Wolfner 2013). In addition to the five neural genes we identified from the initial test, three other genes had been reported to modulate sperm competition outcome through *ppk*$^{+}$ neurons (*para, Rab2, Rim*; Chow et al. 2013). Therefore, in the secondary test, we assessed the effect of knocking down each of these eight genes in *Tdc2*$^{+}$ neurons and *ppk*$^{+}$ neurons.

Of these eight genes, *caup* was the only gene that affected P1 when knocked down in *Tdc2*$^{+}$ neurons (Fig. 3.1K). Knockdown females produced much fewer first male progeny and slightly more second male progeny than control females over the course of the assay, resulting in an overall reduction in fertility and a significant decrease in P1 in vials 2-5 (Fig. S3.4, Fig. S3.5J). *Hid*, one of the genes that affected P1 when knocked down pan-neuronally, had no overall effect on P1 when knocked down in *Tdc2*$^{+}$

neurons. However, on a vial by vial basis, P1 in vials 2 and 3 was significantly higher, and in vial 5 significantly lower, in *hid* knockdown females relative to the P1 of controls (Fig. S3.3G). This result suggests a weaker, but significant role for *hid* in *Tdc2*$^{+}$ neurons on sperm competition outcome. Similarly, *Rab2* knockdown in *Tdc2*$^{+}$ neurons mediated a significant increase in P1 only in vial 2 (Fig. S3.3H).

We also corroborated earlier findings and showed that *ppk*$^{+}$ neuron-specific knockdown of *Rim* caused females to have a lower P1 (Fig. 3.1L), specifically by reducing the number of first male progeny produced (Fig. S3.5K). Temporal effects on P1 were observed for *Ddr* and *hid* knockdown in the *ppk*$^{+}$ neurons, for which P1 was lower in knockdown females compared to controls in vial 5 only (Fig. S3.3I-J). None of the other genes affected P1 when knocked down in the *ppk*$^{+}$ neurons. This included *Rab2* and *para*, two genes that had been reported to affect P1 upon knockdown in *ppk*$^{+}$ neurons by Chow et al. (2013). The previous study used a *ppk*-GAL4 driver generated in a different genetic background compared to the one used in our study, possibly explaining the discrepancy; alternatively, variable environmental factors could be the cause.

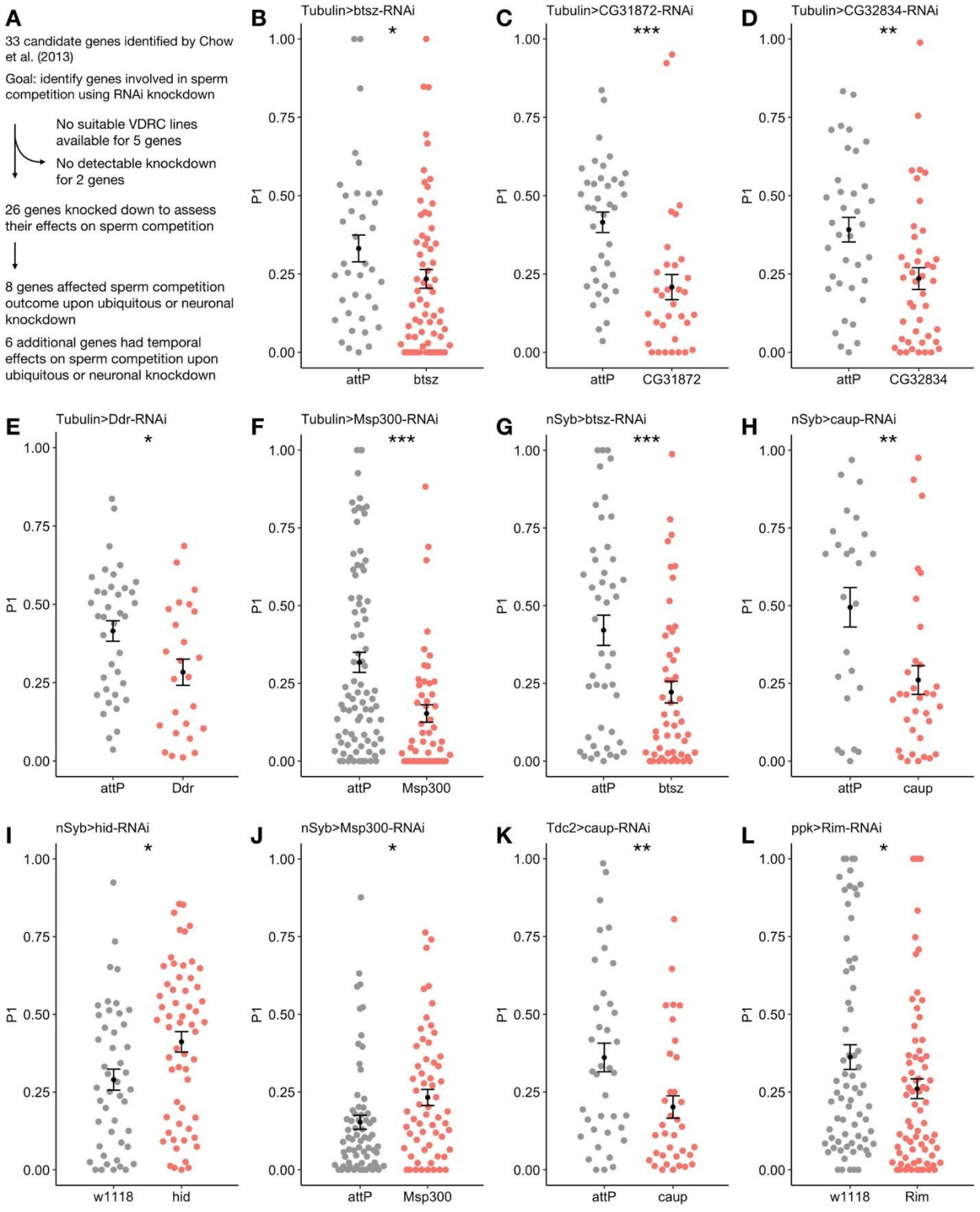

Figure 3.1: Out of 26 candidate genes tested, ubiquitous or tissue-specific knockdown of eight genes affects sperm competition. A: Overview of the candidate genes from

Chow et al. (2013) that were tested using RNAi knockdown. B-L: Sperm competition was measured as the proportion of offspring sired by the first male (P1) over the course of eight days. For all genes, controls were generated by crossing the appropriate background strain (*attP* or w^{1118}) to the respective GAL4 driver line. Each dot represents overall P1 in vials 2-5 for an individual control or knockdown female. Significant differences in P1 between control and knockdown females were determined using linear models. Error bars represent the standard error of the mean. Asterisks (*) indicate $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***). B-F: Changes in P1 mediated by ubiquitous knockdown (*Tubulin-GAL4/TM3, Sb*). G-J: Changes in P1 mediated by pan-neuronal knockdown (*nSyb-GAL4*). K, L: Changes in P1 mediated by $Tdc2^{+}$ neuron- or ppk^{+} neuron-specific knockdown.

Multiple testing correction

We applied Benjamini Hochberg correction for multiple testing on i) the p-values obtained for measurements of overall P1 and ii) the p-values obtained for the analysis of temporal differences in P1. Out of 42 tests that were performed for overall P1, 11 tests had a nominal p-value < 0.05 (eight unique genes). Ten of these tests had an adjusted p-value < 0.1 (Table S3.2; this set still includes the eight unique genes). At an FDR of 0.1, we expect 9 out of these 10 tests to be true positives. For the temporal analysis, we treated each vial as a separate phenotype and performed 42 tests on each of the 4 vials to analyze differences in P1 between control and knockdown females in each vial. Out of 168 tests, 44 had a nominal p-value < 0.05. Of these 44 tests, 31 had an adjusted p-value < 0.1 (Table S3.3). The only candidate gene that fell outside of the 0.1 FDR cutoff for the temporal analysis was *spz5*. Of the 31 tests with an adjusted p-value < 0.1, we expect around 28 to be true positives.

Discussion

A number of approaches have suggested that females play an active role in sperm competition (Arthur *et al.* 1998; Chow *et al.* 2010, 2013; Sitnik *et al.* 2014), but the underlying mechanisms and genetics still remain poorly understood. Our group previously showed that the knockdown of three genes in the female nervous system changed the relative paternity success of two males (Chow *et al.* 2013). Here, we present eight genes that are important for the female's involvement in sperm competition, including one gene reported in the previous study. We also show that the actions of five of these genes are required in the female nervous system, indicating a major role for the female nervous system in sperm competition. Further, we report that the ubiquitous or tissue-specific knockdown of six additional genes had time-specific effects on the outcome of sperm competition. Knockdown of 12 remaining candidate genes tested either had no detectable effect on sperm competition (perhaps another gene near the SNP is involved), or their role in sperm competition could not be identified given limitations of the RNAi method. Specifically, the SNPs were originally identified in DGRP lines, where their variation could have more subtle effects on the spatiotemporal dynamics of candidate gene expression. RNAi, however, reduces gene expression continuously in all tissues where the *GAL4* driver is active.

Understanding the functions of the genes we found to be involved in sperm competition can shed light on the mechanisms by which females contribute to this process. In this section, we discuss the potential biological significance of the genes we identified, in terms of the mechanism through which they could influence sperm competition, and in terms of the evolutionary significance.

From a mechanistic point of view, most of the genes we found to affect P1 upon neuronal knockdown act during development or facilitate basic neuronal processes: *btsz* is a synaptotagmin-like protein involved in membrane trafficking (Serano and Rubin 2003), *caup* is involved in neuronal development (Gómez-Skarmeta and Modolell 1996), *Msp300* has previously been found to play a role at the neuromuscular junction (Morel *et al.* 2014), the general function of *Rim* in the nervous system is to mediate efficient neurotransmitter secretion (Graf *et al.* 2012; Müller *et al.* 2012) and *hid* stimulates apoptosis (Grether *et al.* 1995). *Rab2* has a temporal effect on sperm competition upon *Tdc2*$^{+}$ neuron-specific knockdown, and it encodes a GTPase involved in vesicle trafficking (Gaudet *et al.* 2011). We found that eight other genes affected sperm competition, overall or in specific vials, when knocked down ubiquitously. Of these eight genes, four have known functions in the nervous system or during development, and the other four are likely important in the female reproductive system. *Ddr* belongs to the family of receptor tyrosine kinases, but its exact function is unknown (Sopko and Perrimon 2013); *spz5* is known for its function in the immune response, but is also involved in the development of the nervous system (Zhu *et al.* 2008); *Zasp66* plays a role in muscle development (Katzemich *et al.* 2013); and *sona* encodes a metallopeptidase and is involved in Wg signaling (Kim *et al.* 2016). Based on their functions and the effects of knockdown in the female nervous system, it is likely that the genes themselves are not directly involved in sperm competition. Rather, knockdown of these genes can impair the function or connectivity of the female nervous system, which could then directly influence sperm competition. Potentially, knockdown of other genes that are required for neuronal development or signaling, or experimental manipulation of

neuronal activity, can affect sperm competition in a similar way. For the same reason, neuronal or tissue-specific knockdowns are more likely to have direct effects on sperm competition, while ubiquitous knockdowns could lead to more widespread problems in female physiology.

Through which mechanisms can the female nervous system influence sperm competition? First, after mating, uterine conformational changes modulated by muscle contractions are needed to store sperm (Adams and Wolfner 2007; Mattei *et al.* 2015), and sperm in storage needs to be maintained (Schnakenberg *et al.* 2012). At the same time, females are exposed to pheromones (Smith *et al.* 2017) and seminal fluid proteins like sex peptide (SP; Chapman *et al.* 2003; Liu and Kubli 2003). These molecules can affect her receptivity to remating and directly impact the risk and intensity of sperm competition. The later she remates, the higher the success of the first male. Once a female is doubly mated, the timing of sperm ejection after the second mating affects which sperm are stored and therefore contribute to the fertilization set (Manier *et al.* 2010; Lüpold *et al.* 2013). The *diuretic hormone 44 (Dh44)*$^{+}$ neural circuit controls sperm ejection (Lee *et al.* 2015). However, we previously found that the pan-neuronal knockdown of *Dh44* did not influence sperm competition (White 2017). Finally, once sperm from both males is stored in female sperm storage organs, there is an equal chance for each sperm to be used, regardless of the male of origin, according to the fair raffle hypothesis (Parker *et al.* 1990; Manier *et al.* 2010).

It is conceivable that our nervous system-specific gene knockdowns impact neuronal signaling and consequently female physiology, behavior, or muscle contractions, allowing for any of these female-mediated aspects of sperm competition to

be affected. Since these aspects of sperm competition are important at different time points after both matings take place (e.g. sperm ejection and displacement occur early after the second mating, while sperm maintenance and use continue over the course of multiple days), we expect that the knockdown of some candidate genes would affect sperm competition outcome only in some vials. We performed a P1 by vial analysis to address this possibility, and we indeed identified candidates with such time-specific effects.

In line with the hypothesis that the female nervous system influences sperm competition, we identified a role for both sensory *ppk*$^{+}$ neurons and octopaminergic *Tdc2*$^{+}$ neurons in mediating sperm competition outcome. A population of sexually dimorphic *Tdc2*$^{+}$ neurons located in the abdominal ganglion innervate the female reproductive tract extensively and regulate post-mating responses (PMR) including remating refractoriness and ovulation (Monastirioti *et al.* 1996; Rubinstein and Wolfner 2013; Rezával *et al.* 2014). Innervation of *Tdc2*$^{+}$ neurons in the female sperm storage organs (seminal receptacle and paired spermathecae; Avila et al. 2012, Rezával et al. 2014) suggests that *caup, hid or Rab2* might affect development or function of *Tdc2*$^{+}$ neurons, which in turn could modulate sperm storage and sperm competition. In addition, sensory *ppk*$^{+}$ neurons are also crucial for female PMR (Häsemeyer et al. 2009, Yang et al. 2009). The male seminal fluid protein SP binds to the SPR expressed in female *ppk*$^{+}$ neurons to silence these neurons and elicit PMR (Yapici et al. 2008, Häsemeyer et al. 2009, Yang et al. 2009, Rezával et al. 2012, Lee *et al.* 2016). Both SP and SPR are known to influence sperm competition outcome (Chow et al. 2010, Castillo and Moyle 2014). *Rim* knockdown in the *ppk*$^{+}$ neurons could affect these neurons'

signaling capabilities, thereby mediating a change in P1. SP and SPR silence the *ppk*$^{+}$ neurons to induce increased egg production and lower remating rate. In this regard, it might be surprising that *Rim* knockdown does not mediate these PMR. However, all females in our experiments are mated and thus exposed to SP, so the effect of *Rim* knockdown in a mated female might not have extra effects on PMR in addition to the *ppk*$^{+}$ neuron-silencing effects SP already has. Finally, although the female reproductive tract is extensively innervated, seminal fluid proteins can also enter the female's hemolymph (Monsma *et al.* 1990; Lung and Wolfner 1999; Ram and Wolfner 2005; Pilpel *et al.* 2008) and thus have the opportunity to directly interact with *Tdc2*$^{+}$ or *ppk*$^{+}$ neurons throughout the female body.

Besides a role for the female nervous system in sperm competition, other tissues are likely involved as well. Two of the eight genes that affected overall P1 were only tested with ubiquitous knockdown. *CG32834*, a predicted serine-type endopeptidase, is spermathecae-specific (Leader et al. 2018). The spermathecae are long-term sperm storage organs whose secretions affect sperm motility (Schnakenberg *et al.* 2011), so *CG32834* has the potential to affect sperm storage, maintenance or release from storage. In addition, a previous study found that *CG32834* knockdown results in lower egg production and increased remating (Sirot *et al.* 2014a), in line with the results reported here. *CG31872* is reported to be expressed in the female rectal pad (Leader et al. 2018), but it is not clear what its function is in female reproduction. In addition, two genes with temporal effects on P1 are also involved in female reproduction: *sima* plays a role in border cell migration in the ovary (Doronkin *et al.* 2010), and *CG33095*, a gene with unknown function, is also expressed in the ovary (Leader *et al.* 2018). The roles of

these genes in sperm competition can be further investigated in the future by testing tissue-specific knockdowns.

The question remains whether the genes and neurons we identified are required to assess and respond to male and/or ejaculate quality and effect cryptic female choice, or whether they influence sperm competition independently of male genotype and instead influence the paternity share based on male mating order. Across all tissues tested, six of the eight genes that affected sperm competition outcome when knocked down led to a decreased success for the first male. This suggests that in a wild-type situation, these genes, or the neurons in which they act, play a role in mediating a higher paternity success for the first male (P1) and a decreased success for the second male (P2). It is possible that, when these genes are knocked down, neuronal signaling in response to the first mating is impaired. This could lead to decreased storage of the first male's sperm, increased loss or displacement of the first male's sperm, or an incomplete switch from virgin to mated state. This could also explain the lower overall fertility that we often observed in knockdown females. A second mating, and a second exposure to mating signals, mechanical and/or molecular, might improve the response to mating, leading to a higher success for the second male.

From an evolutionary point of view, since the candidate genes tested here were identified based on natural variation across the DGRP, where females from some isofemale lines naturally have a lower P1 when doubly mated to standard tester males (Chow et al. 2013), it is possible that there is natural variation in how strongly females respond to mating due to variation in neural development or differences in neural gene expression. All SNPs in the 33 candidate genes identified by Chow et al. (2013) are in

noncoding regions or are synonymous substitutions, suggesting that they can indeed affect gene expression. (Durham *et al.* 2014) measured variation in fecundity across young and aged DGRP females and identified associated candidate genes in a GWAS. GO categories overrepresented in those GWAS results included categories associated with neural development (Durham et al. 2014), and five of their candidate genes were also found in the GWAS from Chow et al. (2013) (*Ddr*, *CG32834*, *sima*, *Rbp6* and *CG15765*). Genotype-specific differences in fecundity could exist because the optimal number and timing of egg production can be a source of sexual conflict: it is beneficial for males if a female produces many eggs shortly after mating (and before remating), while more reserved resource allocation can be beneficial for females (Sirot *et al.* 2014b; Wensing and Fricke 2018). In addition, there might be selection in females for a higher P1 and a lower P2. P2, or the paternity share of the second male, is usually high (>50%) because of the mechanics of sperm displacement. It could be beneficial for females to counteract this process and to attempt to balance P1 and P2. Keeping a better balance in sperm from both males gives females the chance to maximize the genetic variation that can be passed on to her offspring.

Finally, an outstanding question in research on sexual conflict is concerned with the interplay of male signals that act on the female's nervous system to influence her physiology and behavior, and the female's processing of and response to male cues (Schnakenberg *et al.* 2012). Our findings regarding sensory *ppk*$^{+}$ neurons and *Tdc2*$^{+}$ neurons, which include neurons innervating the female reproductive tract, form an important step in understanding the mechanistic and molecular basis of that interplay in sperm competition.

Acknowledgements

We thank members of the Clark and Wolfner labs for helpful discussions and comments. We are grateful to NIH R01 HD059060 (to AGC and MFW) for supporting this work. Several authors were supported for all or part of this study by fellowships, for which we are grateful: a SUNY Diversity Fellowship, followed by an HHMI Gilliam Fellowship (SLW), a fellowship from the Belgian American Educational Foundation (SYND), and a summer internship from the NSF-funded EDEN Research Collaboration Network (C. Extavour, PI) for MC. We also thank the editor and the anonymous reviewers for helpful comments and suggestions to improve the manuscript.

Supplementary table legends

Table S3.1: Summary of candidate genes tested, VDRC lines used, the approximate level of gene knockdown relative to genetically matched controls, and primer sequences used for RT-PCR.

Table S3.2: Summary statistics and sample sizes (n) for remating rate, fertility and P1. KD = knockdown; sd = standard deviation; TG4 = *Tubulin-GAL4/TM3, Sb*; TG80 = *Tubulin-GAL80^{ts}; Tubulin-GAL4/TM3, Sb*. Q-values were obtained using Benjamini-Hochberg correction.

Table S3.3: Nominal p-values and Benjamini-Hochberg adjusted p-values by vial for the temporal analysis of P1, for all driver-gene combinations tested. TG4 = *Tubulin-GAL4/TM3, Sb*; TG80 = *Tubulin-GAL80^{ts}; Tubulin-GAL4/TM3, Sb*.

Supplementary figures

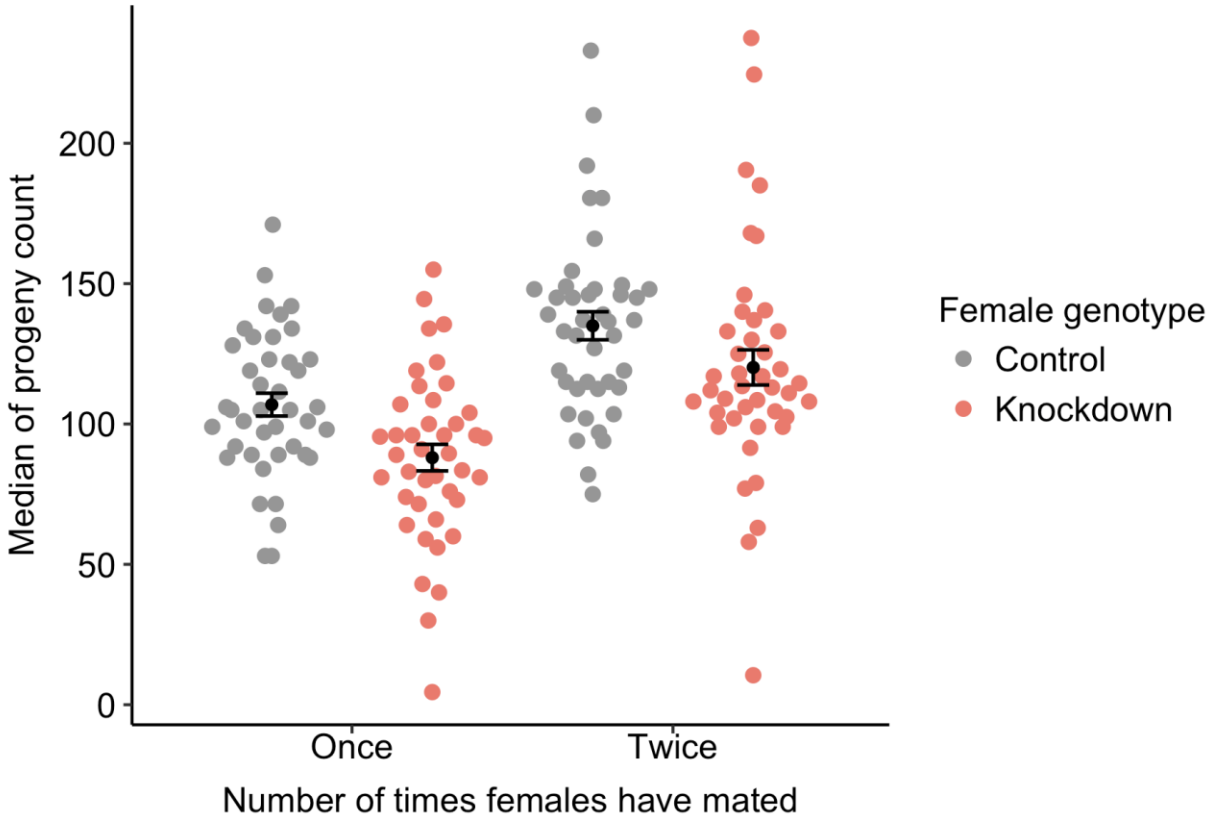

Figure S3.1. Doubly mated females have higher fertility than singly mated females. Fertility of each female was calculated as the total number of progeny she produced over the course of the sperm competition assay. Each dot represents the median fertility of all females used in one experiment, with females grouped by genotype (control and knockdown) and by the number of times they have mated. Error bars represent mean ± standard error. ANOVA revealed significant differences in fertility based on female mating status ($F=34.959$, $p=1.93\times10^{-8}$) and genotype ($F=10.918$, $p=0.00117$) but not their interaction ($F=0.158$, $p=0.692$). In a separate analysis, when we grouped singly mated females by the identity of the males they mated with (first male or second male), we found that there was no significant difference in the fertility of singly mated females depending on the identity of the male they mated with (data not shown).

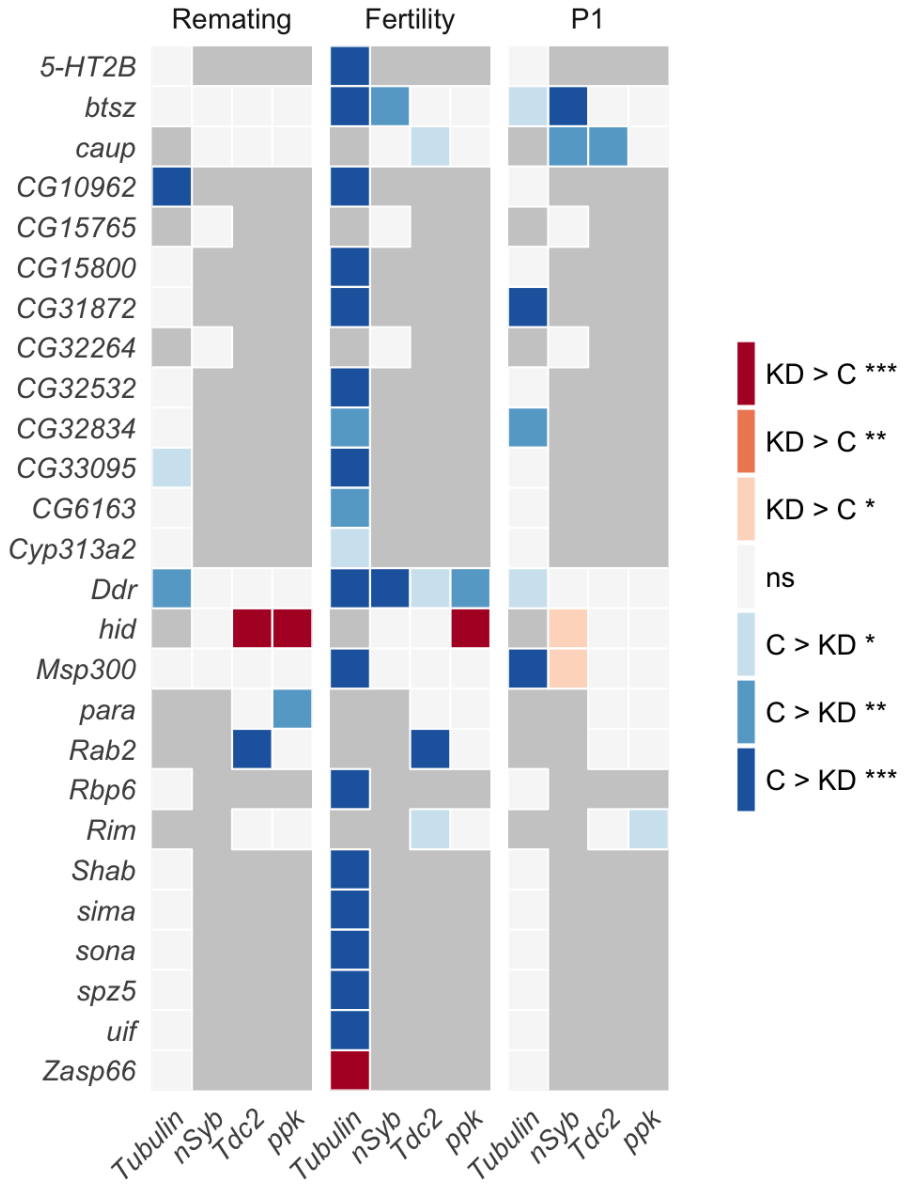

Figure S3.2. Summary of the effects of ubiquitous and tissue-specific gene knockdown on female remating rate, fertility and P1. Each row represents one gene and each column represents one tissue in which the gene is knocked down. Remating rate was measured as the proportion of doubly mated females among females who mated with the first male. Fertility was measured as the total number of offspring produced by a doubly mated female. P1 was measured as the proportion of offspring sired by the first male (P1) upon ubiquitous (*Tubulin-GAL4*) or neuronal knockdown (*nSyb-GAL4, Tdc2-GAL4, ppk-GAL4*). Colors represent the direction of changes and their significance levels (C: control, KD: knockdown, ns: not significant, dark gray: not tested). Asterisks indicate $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***).

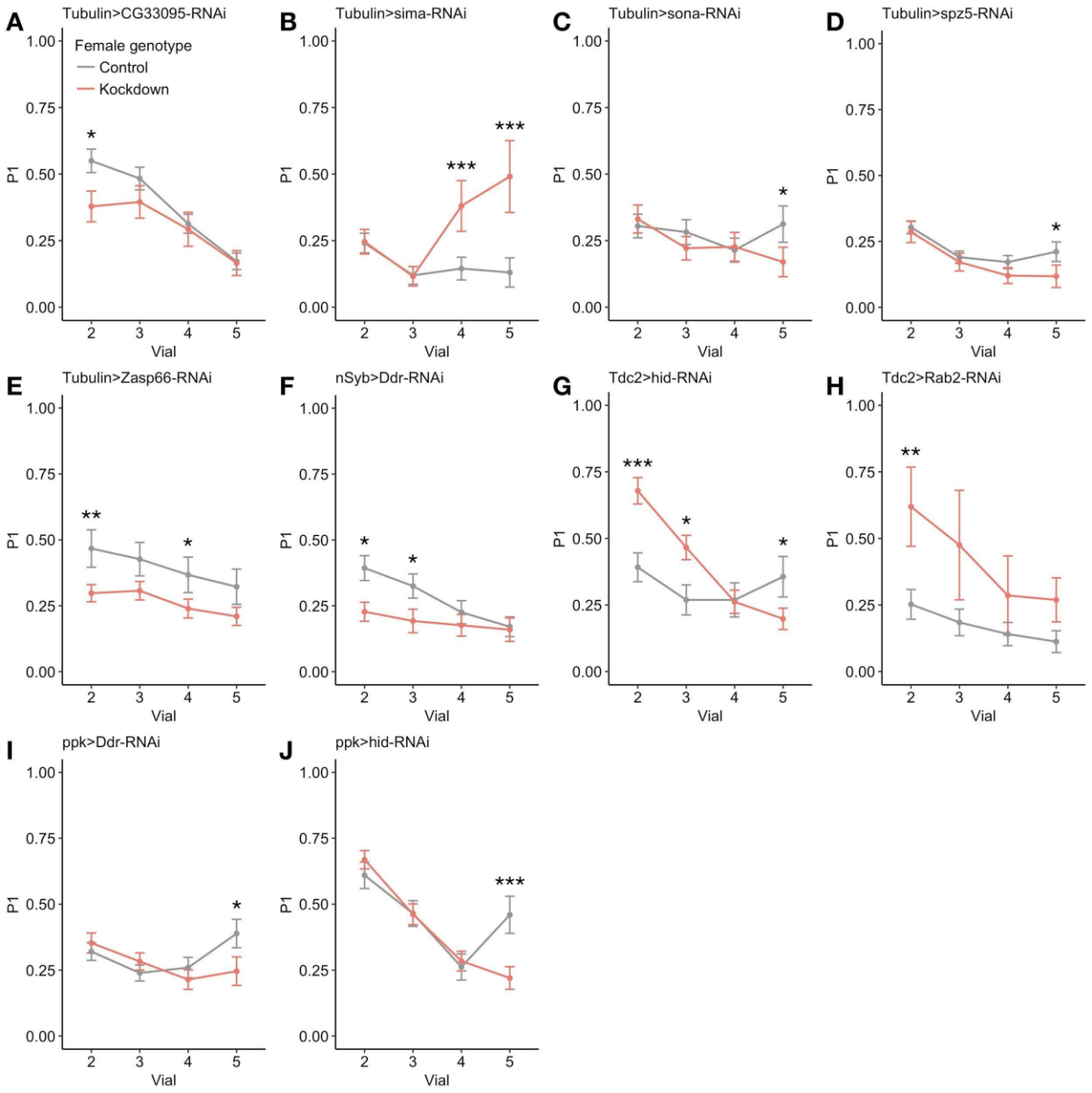

Figure S3.3: Knockdown of eight candidate genes mediated temporal, but not overall, differences in P1. The line plots represent the average relative success of the first male to mate (P1), for knockdown and control females, across vials 2-5. Error bars represent the standard error of the mean. Asterisks (*) indicate $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***). A-E: Significant temporal effects due to ubiquitous knockdown (*Tubulin-GAL4*). F: Effects of pan-neuronal knockdown (*nSyb-GAL4*). G-J: Changes in P1 mediated by *Tdc2*$^{+}$ neuron- (G, H) or *ppk*$^{+}$ neuron-specific knockdown (I, J).

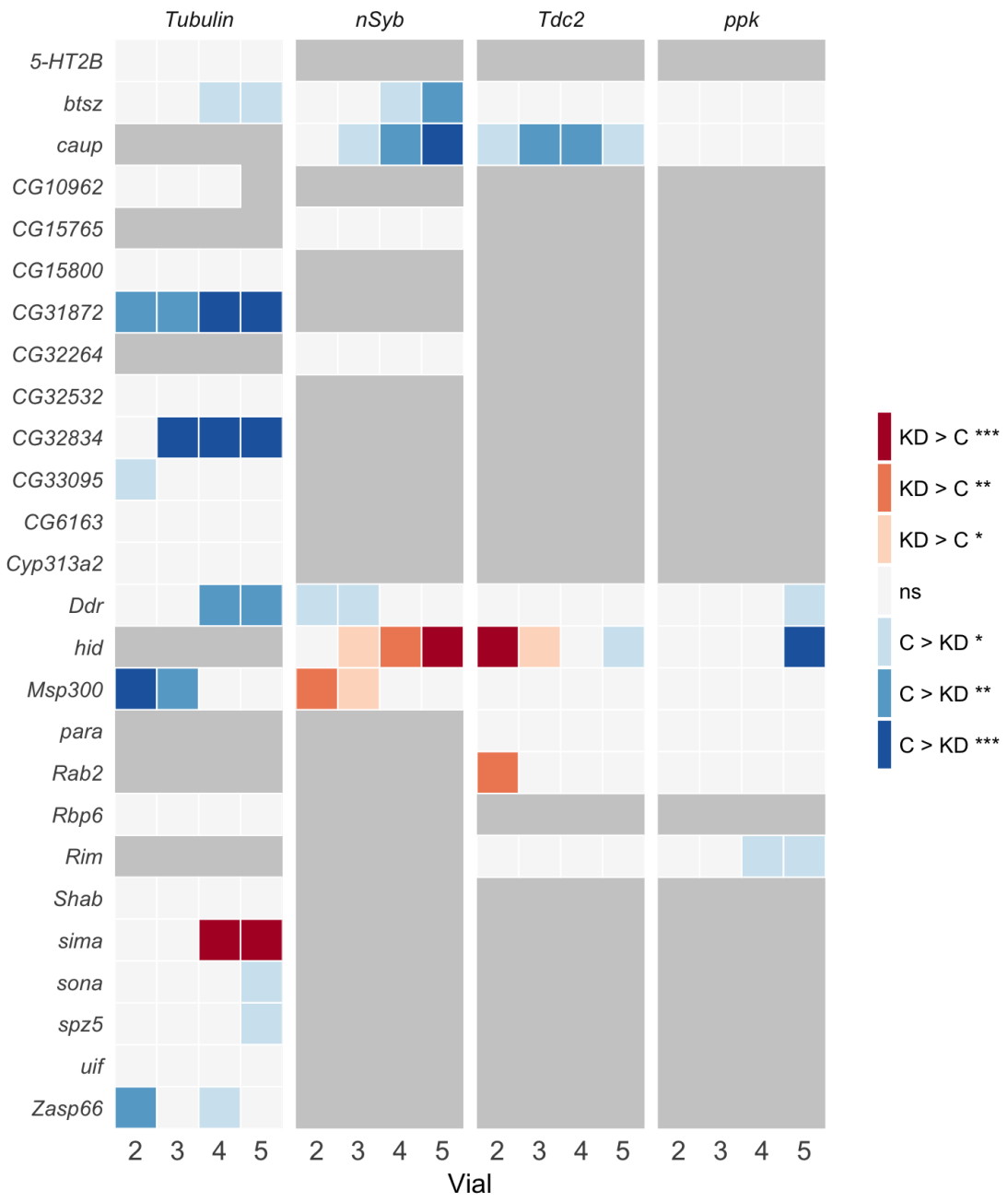

Figure S3.4. Summary of temporal effects of ubiquitous and tissue-specific gene knockdown on P1. Each row represents one gene, each facet represents one tissue in which the gene is knocked down, and each column within each facet represents one vial (range 2-5). Colors represent the direction of changes and their significance levels (C: control, KD: knockdown, ns: not significant, dark gray: not tested). Asterisks (*) indicate $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***).

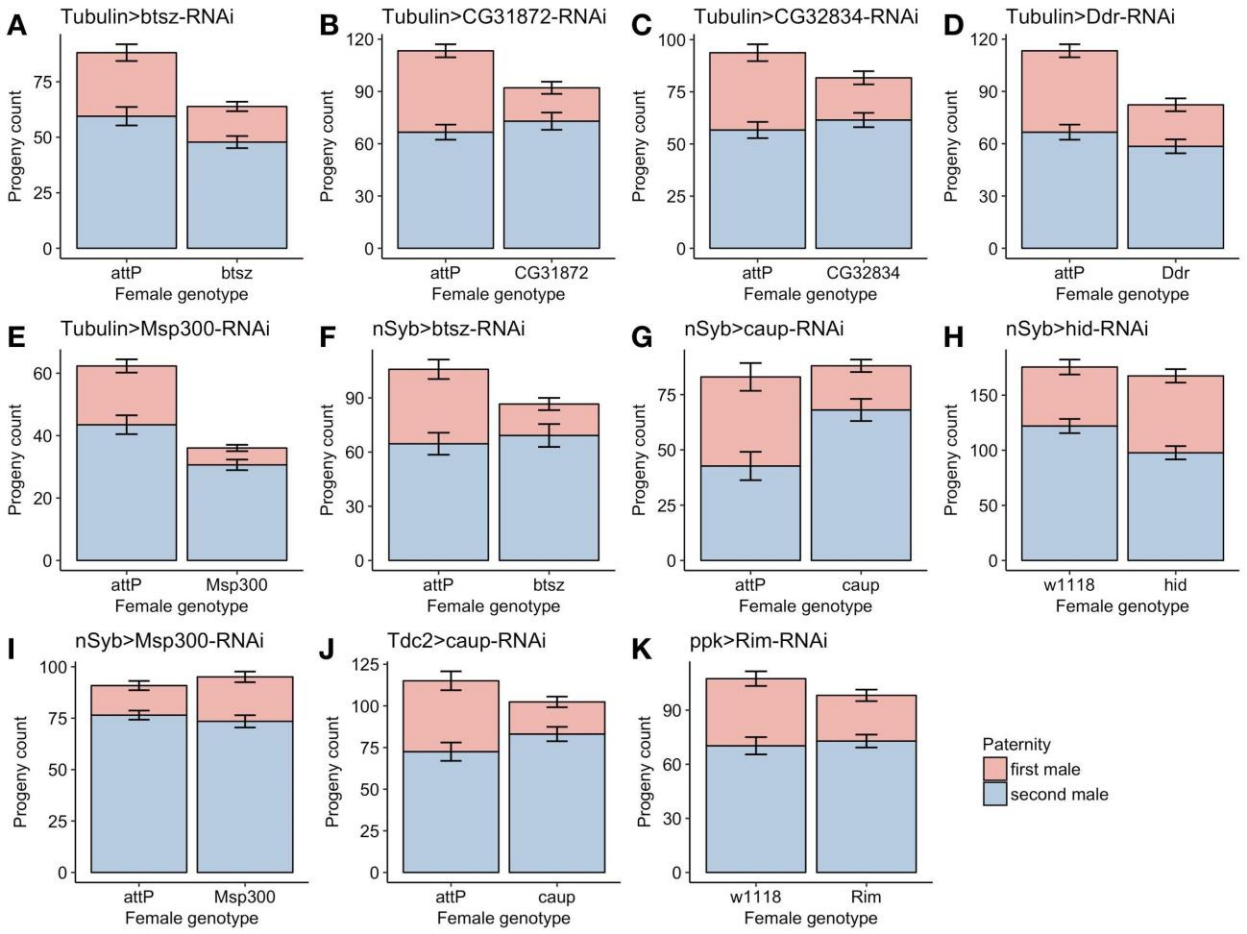

Figure S3.5. Knockdown of 8 genes that affect sperm competition outcome upon ubiquitous or tissue-specific knockdown has different effects on absolute numbers of first- versus second-male progeny. Bar graphs represent the mean progeny count of knockdown and control females in vials 2-5, with colors indicating the paternity of their progeny (red: first male progeny; blue: second male progeny). Vial 1 was excluded in these graphs (and in the calculation of P1) because both matings took place in vial 1, and with our experimental setup, it was not possible to determine which progeny were produced before or after the second mating. Error bars represent mean ± standard error.

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CHAPTER 4:

EARLY TRANSCRIPTOME MEASUREMENTS IN THE LOWER REPRODUCTIVE TRACT OF MATED *DROSOPHILA MELANOGASTER* FEMALES REVEAL CHANGES IN RNAS INVOLVED IN WOUND HEALING AND NEURONAL REMODELING, AND TRANSCRIPTS DERIVED FROM THE MALE.[3]

Abstract

The female's lower reproductive tract is exposed directly to the male's ejaculate, making it a hotspot for mating-induced responses shortly after mating. In *Drosophila melanogaster*, mating induces physiological changes in preparation for fertilization events. Microarray studies have detected modest transcriptome changes at early time points after mating, but no study has examined immediate transcriptome changes of both coding and noncoding RNAs in the female reproductive tract. To more precisely detect the earliest events in the female's lower reproductive tract, we measured transcript abundance in lower reproductive tracts of virgin females and females collected within 10-15 minutes after the end of mating. We observed mating-induced transcriptional changes in several functional categories, including 13 long noncoding RNAs, 15 genes involved in remodeling of the nervous system and synaptic transmission, and 30 genes associated with activation of wound healing. Several additional genes involved in the stress response, including components of the innate immune Toll and Imd signaling pathways, were up-regulated. In addition, using SNPs

[3] A modified form of this chapter will be submitted for publication. Delbare SYN collected samples for RNA-seq, analyzed RNA-seq data and wrote the manuscript. Wolfner MF and Clark AG provided advice throughout the project and provided funding.

we identified transcripts in mated females that had been transferred by their mates. Many of these transcripts likely originate from the male accessory gland, but whether they have a physiological effect in females remains to be determined. Next, we asked what aspect of the male's ejaculate induced early transcriptome changes in females. Recent studies reported that BMP signaling in secondary cells of the male accessory gland influences the composition of the male's ejaculate - but we found no effect of BMP-regulated secondary cell functions on early post-mating transcriptome changes in females. Our results shed light on the molecular changes that accompany very early responses to mating and are useful for further examination of the crosstalk that occurs between female and male molecules inside the reproductive tract.

Introduction

The female lower reproductive tract undergoes significant changes after mating to optimize fertilization. The transcriptome of the reproductive tract is altered post-mating and there is evidence for crosstalk between sperm and seminal fluid proteins and female reproductive tract epithelia in both mammals (Adams et al. 2016; Ferraz et al. 2019; He et al. 2019; Suarez 2016) and insects, including insect vectors of disease (Alfonso-Parra et al. 2016; McGraw et al. 2004; Procházka et al. 2018). Key changes in the reproductive tract have been thoroughly investigated and described in *Drosophila melanogaster* females. In these females, mating induces conformational changes in reproductive tract organs (Avila & Wolfner 2009; Mattei et al. 2015; Rubinstein & Wolfner 2013). Concurrently, modifications occur in the release of vesicles and neuromodulators from neurons that innervate the reproductive tract (Avila et al. 2012;

Heifetz et al. 2014; Heifetz & Wolfner 2004). These processes are essential to mediate sperm storage, which starts already before the end of copulation, and to allow ovulation, a process that starts around 90 min after mating (Bloch Qazi et al. 2003). Soon after insemination starts, male seminal fluid proteins initiate the formation of a mating plug to prevent the ejaculate from leaking out of the female's reproductive tract until sperm storage is complete, which usually happens within six hours after mating (Avila et al. 2015; Lee et al. 2015). Also during copulation, pheromones transferred by the male's cuticle and ejaculate alter the female's pheromonal profile. The transfer of pheromones makes mated females less attractive than virgin females, already 10 minutes after the end of copulation (Laturney & Billeter 2016; Mane et al. 1983; Tompkins & Hall 1981). In addition, studies of dissected tissues and micro-CT scans have shown that copulatory wounding occurs in flies (Kamimura 2007; Mattei et al. 2015). Wounding could be partly responsible for the post-mating up-regulation of immune response transcripts that multiple studies have observed at 1-3 hours and 6-8 hours after mating (Gioti et al. 2012; Kapelnikov et al. 2008; Mack et al. 2006; McGraw et al. 2008; McGraw et al. 2004), but seminal fluid proteins and sperm certainly have a part in this as well (Domanitskaya 2007; Gioti et al. 2012; McGraw et al. 2004; Peng et al. 2005). In addition to immune genes, the transcript abundance of genes involved in among others egg production, odorant binding and metabolism changes 1-3 hours after mating and peaks at 6-8 hours after mating (Kapelnikov et al. 2008; Lawniczak & Begun 2004; McGraw et al. 2008, 2004). One microarray study measured transcript levels in the female reproductive tract within one hour after mating, but observed few changes in gene expression (Mack et al. 2006), contrary to what might be expected based on the

numerous changes the reproductive tract undergoes during and shortly after copulation. These mating-induced responses could also be caused by changes in translation or post-translational modifications of proteins, but this was not measured by Mack et al. (2006) within one hour after mating. Further, transcriptome studies performed on *Aedes* mosquitoes (Alfonso-Parra et al. 2016) and *D. mojavensis* (Bono et al. 2011) at very early time points post-mating, made the intriguing observation that male RNAs were transferred from the male to the female during mating, but none of the studies performed in *D. melanogaster* identified male-derived transcripts. Because of this, we decided to investigate immediate transcriptome changes in the female's lower reproductive tract using more sensitive RNA-sequencing and at a well-defined time point after mating.

In addition to characterizing transcriptome changes, we were also interested in determining what aspect of mating they were caused by. Previous work has shown that transcriptome changes in females, at 1-3 hours after mating, are influenced by copulation itself, by sperm, and by seminal fluid proteins (McGraw et al. 2004). Specifically, McGraw et al. (2004) studied the effects of seminal fluid proteins on the female's transcriptome by ablating the main cells of the male accessory gland -the main producers of seminal fluid proteins (Findlay et al. 2008; Kalb et al. 1993). However, male accessory glands also contain ~40 secondary cells, which are larger than the main cells and are filled with vacuoles (Bairati 1968; Bertram et al. 1992). Recent studies have shown that secondary cells contribute to the composition of seminal fluid in at least two ways. First, they influence the seminal fluid composition of the ejaculate (Gligorov et al. 2013; Sitnik et al. 2016; Hopkins et al. 2019; Monsma et al. 1990).

Second, secondary cells are the source of exosomes, small membrane-bound vesicles involved in intercellular communication (Wilson et al. 2017). These exosomes fuse with sperm and associate with epithelia inside the female's reproductive tract (Corrigan et al. 2014; Leiblich et al. 2012). Males that do not transfer exosomes fail to induce refractory behavior to remating in their mates (Corrigan et al. 2014; Leiblich et al. 2012). The contents of seminal fluid exosomes in *D. melanogaster* have not been characterized and it is unknown whether transferred exosomes influence the female's transcriptome. Several methods are available to study secondary cell function. Secondary cells are unable to fully develop in the absence of the *iab-6* enhancer in the *Abd-B* locus (Maeda et al. 2018). The secretion of exosomes can be prevented by knocking down secretory pathway components (Corrigan et al. 2014), or through the inhibition of BMP signaling by overexpression of the BMP-inhibitor Dad (Corrigan et al. 2014). Aside from blocking exosome secretion, BMP signaling has additional effects on secondary cell function. It is required for the replenishment of dense core granules, whose role in reproduction has not been examined (Redhai et al. 2016) and inhibition of BMP signaling in secondary cells was recently reported to increase the sperm competition success of males if they are first to mate, and was reported to cause small changes in the abundance of certain seminal fluid proteins in the male accessory gland (Hopkins et al. 2019). However, the mechanisms underlying these effects of BMP signaling are not known.

In this study, to obtain a comprehensive view of transcriptome changes after mating, we sequenced coding and noncoding RNAs (small RNA-seq is in progress) of the lower reproductive tracts of virgin females and females collected within 10-15

minutes after the end of mating (or, since *D. melanogaster* mate on average for 20 min, 30-35 min after the start of mating). These females were mated either to wildtype males, or males with inhibited BMP signaling in their secondary cells. Regardless of the male's genotype, we observed that mating altered the transcript abundance of genes involved in neuronal remodeling, muscle development and wound healing, along with long noncoding RNAs and unexpectedly, RNAs encoding seminal fluid proteins. Using SNPs that differed between the female and male lines we used, we were able to establish that RNAs are transferred from the male to the female during mating.

Materials and Methods

Fly husbandry

All females used were from the wildtype stock *Oregon-R-P2* (Waring et al. 1983). Control males were either from the stock w^{1118} or were offspring with wildtype phenotype from w^{1118} males crossed to *w;esg-GAL4 tub-GAL80ts UAS-FLP/CyO;UAS-GFP nls, act>CD2>GAL4/TM6* (*esg*^{ts} *F/O*) females (Leiblich et al. 2012). Males with inhibited BMP signaling in the secondary cells ("Dad males") were generated by crossing *esg*^{ts} *F/O* females to *w;P[w+ UAS-Dad]* (*UAS-Dad*) males (Dad acts as a BMP inhibitor) (Leiblich et al. 2012; Redhai et al.2016; Tsuneizumi et al. 1997). The latter two lines were kindly given to us by Dr. Clive Wilson. Stocks were maintained at room temperature or 29°C on yeast/ glucose food in a 12 hour light/ dark cycle.

RNA-seq sample collection

To investigate short-term transcriptome changes induced by mating, we mated three to five-day old virgin *Oregon-R-P2* females to five-day old virgin w^{1118} males at room temperature. Matings were observed, females were flash frozen within 10-15 minutes after the end of mating and virgin females were collected in parallel.

To investigate whether female gene expression is impacted by BMP-regulated secondary cell contributions to the ejaculate, we crossed either w^{1118} or *UAS-Dad* males to esg^{ts} *F/O* virgin females at 21°C (room temperature). From the offspring of this cross, we selected male pupae once sex combes were visible, just before eclosion (*w;esg-GAL4 tub-GAL80ts UAS-FLP/UAS-Dad;UAS-GFP nls, act>CD2>GAL4/+* for Dad males and *w;esg-GAL4 tub-GAL80ts UAS-FLP/+;UAS-GFP nls, act>CD2>GAL4/+* for control males). Male pupae were placed in groups of 10-15 at 29°C. The initial cross was set up at room temperature to avoid inhibition of BMP signaling during development. Transferring late stage pupae to 29°C will initiate the inhibition of BMP signaling in newly eclosed adult males. This prevents the secretion of secondary cell exosomes into the lumen of the accessory gland, which we verified indirectly using a receptivity assay (see below).

The afternoon before matings were set up, two to four-day old *Oregon-R-P2* virgin females were also placed at 29°C. The next morning, five-day old virgin males were mated to three to five-day old *Oregon-R-P2* virgin females at 29°C. Matings were observed and females were flash frozen within 10-15 minutes after the end of copulation. Virgin females kept at 29°C were collected in parallel. During this sample collection, males were collected for sequencing as well, to characterize differences in

transcript abundance between control males and Dad males and to identify male-specific SNPs. All matings occured from morning to early afternoon. Samples for each treatment were collected simultaneously. This was repeated on three different days, to collect three replicates for each treatment.

Receptivity assay and sperm counts

Males that cannot transfer exosomes are unable to induce the refractory behavior mated *D. melanogaster* females typically exhibit (Corrigan et al. 2014; Leiblich et al. 2012). We conducted a receptivity assay to ensure all *GAL4* and *UAS* constructs were working as expected. We collected and mated Dad and Control males and *Oregon-R-P2* virgin females as described above at 29°C. After mating, males were discarded. Females were kept at room temperature for four days. Vials were checked for the presence of larvae to ensure the first mating had occurred, and females were presented with a virgin three to five-day old *Oregon-R-P2* male. We recorded blinded if females remated within three hours at room temperature. We analyzed the remating data in R (R Core Team 2018) using a Fisher's exact test. (N=23 for female x Dad male, N=22 for female x control male).

The *esg-GAL4* driver is active in secondary cells in the male accessory gland, but *escargot* is also expressed in the testes (Leader et al. 2018). In addition, BMP signaling is important for stem cell maintenance (Kawase et al. 2004). To investigate if Dad males had defects in sperm production, we DAPI-stained nuclei and counted the number of sperm in the seminal vesicles of virgin 5-day old Dad males and control males (N=6 for Dad, N=6 for control). The person counting was blind to the genotypes

of the samples. Sperm counts were compared using a Wilcoxon test in R (R Core Team 2018).

Dissections, RNA extraction, library preparation and sequencing

For RNA-seq samples, we dissected the lower female reproductive tract in sterile 1X PBS on a petri dish filled with dry ice (dry ice was used to keep the 1X PBS cool, but the flies were not frozen during dissections). The digestive system was removed, as was most of the common oviduct and most the fat body, but some oviduct and fat body was still part of the lower reproductive tract sample. Lower reproductive tract samples were stored in groups of ten in 50 ul of Trizol and were mechanically homogenized using a pestle before being flash frozen and then stored at -80°C.

To extract RNA, we pooled all Trizol aliquots of the same sample and brought up the total volume to 1 ml. We extracted RNA using the Direct-zol RNA Microprep Kit according to the manufacturer's instructions (Zymo, CA). For female samples, we extracted RNA from on average 64 female lower reproductive tracts (ranging between 54-70 tracts). For male samples, we extracted RNA from three whole males. We measured the RNA concentration of all samples using Qubit, assessed purity using Nanodrop and verified the integrity of 12 out of 21 samples using the Agilent Bioanalyzer at the Cornell Genomics Facility.

We used the Ovation Universal Drosophila RNA-Seq Kit to sequence all long RNAs, including mRNAs and non-polyadenylated RNAs, and to deplete abundant ribosomal RNAs (Tecan, CA). We prepared the libraries according to the manufacturer's instructions (Tecan, CA). For the optional fragmentation step in the protocol, we

fragmented cDNA using Covaris to obtain a fragment size of 400 bases. Libraries were sequenced on the NextSeq500 platform at the Cornell Genomics Facility, where fastq files were generated using Illumina pipeline software v2.18.

To sequence small RNAs, we used the NEBnext Kit (New England Biolabs, MA), starting from 1 ug of total RNA (the same RNA samples used for long RNA-seq). We adjusted the NEB protocol at particular steps: 1) We ran the 3' ligation reaction overnight at 16°C to improve detection of any piRNAs that could be present in our samples. 2) We modified the protocol to remove abundant small rRNAs. To remove eight abundant small rRNA fragments, we used blocking oligos designed by Fowler et al. (2018). These oligos hybridize with rRNAs and prevent their participation in the library preparation. We incorporated these in the library preparation using a slightly modified method from Wickersheim & Blumenstiel (2013). Specifically, we added 1 ul of an oligo cocktail (each oligo at a concentration of 10 uM) to the 3' ligation reaction. We incubated this reaction for 2 minutes at 70°C and for 5 minutes at 60°C, before proceeding to the reverse transcriptase primer hybridization step. We purified the resulting libraries using the Monarch PCR & DNA cleanup kit (New England Biolabs, MA) and size selected using Pippin Prep (Sage Science, MA), selecting fragments of 120-250 bp. We tried these modifications first on test samples of RNA derived from whole virgin females. After sequencing at a depth of 10 M reads on Illumina's NextSeq500 platform at the Cornell Genomics Facility, we verified that adding rRNA blocking oligos greatly reduced the number of rRNA reads in the final test-library. In the absence of blocking oligos, 88.7% of reads aligned to DNA encoding rRNAs. In the presence of blocking oligos, only 1.5% of reads aligned to DNA encoding rRNAs. The

library preparation and sequencing for small RNAs in the mated and virgin female lower reproductive tract samples are in progress.

Read processing

We analyzed library quality using fastqc (https://www.bioinformatics.babraham.ac.uk/projects/ fastqc/). We used Trimmomatic to remove overrepresented sequences and to filter reads (Bolger et al. 2014). Specifically, bases at the end of a read with a Phred quality score less than 20 were clipped off. A read was also clipped if the average read quality in a window of 5 bases had a Phred score lower than 20. After trimming, we kept only reads with a minimum length of 30 bases. On average, libraries consisted of 17,432,707 reads. Reads were aligned to dm6 using STAR (Dobin et al. 2013) and read counts were obtained using HTSeq (Anders et al. 2015).

Differential expression analysis

We performed differential expression analyses using edgeR on female and male samples separately (Anders et al. 2015; Robinson et al. 2010). A Principal Component Analysis demonstrated that female samples clustered as expected (Fig. S4.1A) and all replicates correlated well with each other based on a Pearson's correlation (correlations ranged from 0.948-0.99). A PCA of male samples indicated some separation between replicates of the same treatment (Fig. S4.1B). Because of this, we excluded outlier samples DM1 (Dad Male) and CM3 (Control Male) from the analysis.

Reads were kept in the dataset if they had a cpm (counts per million) > 1 in at least three samples (or two samples for the male dataset), keeping 9,863 genes for

female samples and 12,813 genes for male samples. For the differential expression analysis of male samples, we made a pairwise comparison between Dad and control males, and these results can be found in the Supplement. For female samples, we set up four pairwise contrasts:

1) Female mated to control male (w^{1118}) vs. virgin female (at room temperature).

2) Female mated to control male (esg^{ts} *F/O* w^{1118}) vs. virgin female (at 29°C).

3) Female mated to BMP-inhibited male (esg^{ts} *F/O UAS-Dad*) vs. virgin female (at 29°C).

4) Female mated to BMP-inhibited male (esg^{ts} *F/O UAS-Dad*) vs. female mated to control male (esg^{ts} *F/O* w^{1118}) (at 29°C).

Contrasts 1-3 inform about mating-responsive transcripts, while contrast 4 informs about differences in female transcript abundance caused by BMP inhibition in the male's secondary cells. Genes were called as differentially expressed based on a Benjamini-Hochberg adjusted *p*-value ≤ 0.05 (Benjamini & Hochberg 1997).

When performing the differential expression analysis on female samples, some mated female samples showed a significant downregulation of ovary-specific genes. Since our samples consisted of the lower reproductive tract only, this suggested that some virgin samples were contaminated with parts of the ovary. To take this into account, we identified 81 genes with an expression bias to the ovary (based on FlyAtlas 2; Leader et al. 2018) and clustered samples based on their expression (cpm) for these 81 genes (Fig. S4.2). This method resulted in two clusters, one containing samples with higher expression of ovary-biased genes (V-29-1, V-29-2, V-29-3, V-RT-1, FxC-29-1 and FxC-29-3) and one containing all other samples with lower expression of ovary-

biased genes. We added an extra variable to the linear model in edgeR, assigning all samples to either one of these two clusters. This approach allowed us to remove the effects of ovary-biased genes from the differential expression analysis.

SNP analysis

To distinguish transcripts originating from females from those originating from males, we used GATK (McKenna et al. 2010) to call SNPs in the virgin female samples (3 replicates collected at 29°C and 3 replicates collected at room temperature) and male samples (3 replicates of Dad males and 3 replicates of control males, all collected at 29°C). Next, we used SNPsplit (Krueger & Andrews 2016) to assign SNP-containing reads to “female origin” or “male origin”. In more detail, we realigned all fastq read files using STAR 2-pass alignment, in which information regarding splice junctions from the first alignment is used to guide a second and final alignment (Dobin et al. 2013). We merged the resulting bam files for the six virgin female samples into one bam file using samtools (Li et al. 2009) and repeated this for the six male samples. We ran the “GATK best practices for RNA-seq” pipeline on these merged female and male bam files (https://software.broadinstitute.org/gatk/documentation/article.php?id=3891). GATK’s HaplotypeCaller by default only outputs SNPs and no reference sites. However, we were also interested in sites that could be reference in female and SNP in male, and vice versa. Thus, in addition to the standard pipeline, we also ran HaplotypCaller using the -ERC BP_RESOLUTION option, which outputs all reference sites. From the resulting vcf files we kept only reference sites supported by at least 3 counts based on the AD field. Further, we only kept homozygous sites, SNPs (no indels) and sites that

had a different base call in female vs. male. Using this method, we obtained 70,162 informative sites. We used bedtools (Quinlan 2014) to mask these sites in the *D. melanogaster* genome (dm6). Next, we realigned fastq files of virgin females and mated females to the masked genome using STAR 2-step alignment (Dobin et al. 2013), removed duplicated reads from the resulting bam files using Picard (http://broadinstitute.github.io/picard/) and ran SNPsplit (Krueger & Andrews 2016). On average 274,640 reads per sample contained a SNP site. Of these, an average of 98% were assigned as "female" and 0.4% were assigned as "male". The remaining reads were unassignable due to conflicting SNP information. SNPsplit outputs for each sample a "female" and "male" bam file with female-specific and male-specific reads respectively. We used HTSeq to map these reads to annotated genes and to retrieve read counts (Anders et al. 2015). In R (R core team 2018), we normalized counts from HTSeq by dividing them by the total number of reads in the respective deduplicated library, multiplying by 10^6 and rounding to the nearest integer. A total of 1,916 genes had a normalized male read count of at least 1 across the mated female samples.

However, during the analysis we noticed that SNPsplit also assigned reads as "male" in virgin female samples. Upon closer inspection we found that at many sites, the *Oregon-R-P2* strain used here was polymorphic, as were the male lines. For example, if one of the alleles in virgin females was sufficiently rare (very few reads carrying this SNP), the site would be called as homozygous by GATK. In certain cases where the rare female allele was common in the male strain, SNPsplit would view those reads as "male". Because of this, we considered all genes to which "male" reads aligned in virgin

females as ambiguous and we omitted those genes from our analysis. After this filtering step we were left with 1,328 genes (out of the 1,916 originally detected) with at least one normalized male read count in at least one mated female sample. From these, we kept only genes for which the summed normalized count across 3 replicates of the same sample was at least 3 (sample referring to: female mated to control male at room temperature, female mated to control male at 29°C or female mated to Dad male at 29°C). This left 110 transcripts with “male” reads in the dataset.

Besides identifying transcripts of male origin on the level of read counts (using HTSeq), we also inspected individual SNP sites. We used bcftools mpileup (http://samtools.github.io/bcftools/bcftools.html) to obtain base calls at the 70,162 SNP sites in virgin and mated female samples. We filtered out 13,182 ambiguous sites that had a “male” base call in virgin female samples. We further removed sites if they were supported by only 1 of the 9 mated female samples. The remaining sites were mapped to the 110 genes identified using HTSeq. We found 518 individual SNP sites that mapped to a total of 108 genes. Thus, 2 of the 110 genes identified using HTSeq were not retrieved using the mpileup analysis, and we omitted these from our final analysis.

Analysis of differentially expressed transcripts and transcripts of male origin

We investigated the functions of differentially expressed transcripts and transcripts of male origin using Flybase (Gramates et al. 2017), using KEGG and GO enrichment analyses in the R package Clusterprofiler (Yu et al. 2012) and using tissue-specific expression data from FlyAtlas 2 (Leader et al. 2018). Figures were generated using the R packages ggplot2 (Wickham 2009) and ComplexHeatmap (Gu et al. 2016).

We used FPKM values from FlyAtlas 2 (Leader et al. 2018) to calculate enrichment scores for each gene in male tissues. Enrichment was calculated by dividing the FPKM value of the gene of interest in the tissue of interest, by the FPKM value for that gene in the whole male body. As described on http://flyatlas.gla.ac.uk/FlyAtlas2/index.html?page=help#, we set whole body FPKM values lower than 2 to 2 for the enrichment calculation.

To identify seminal fluid proteins, we cross-referenced genes against a list of known seminal fluid proteins. This list has been assembled based on published studies (Avila et al. 2015; Ayroles et al. 2011; Begun & Lindfors 2005; Chen et al, 1988; Coleman et al. 1995; Findlay et al. 2009; Findlay et al. 2008; Holloway & Begun 2004; Monsma & Wolfner 1988; Mueller et al. 2005; Ravi Ram et al. 2005; Ravi Ram & Wolfner 2007; Schäfer 1986; Sepil et al. 2019; Simmerl et al. 1995; Swanson et al. 2001; Takemori & Yamamoto 2009; Walker et al. 2006; Wolfner et al. 1997) and is curated by the Wolfner lab (Brown, Sitnik and Wolfner, *unpublished*). We also cross-referenced genes against a list of proteins present in sperm (Wasbrough et al. 2010) and transcripts that had been identified in sperm using a microarray analysis (Fischer et al. 2012) and using single cell RNA-seq of the testes (Witt et al. 2019). From the latter study, only expression data from mature spermatids was used.

Data availability

Supplementary results, figures and table legends can be found in this document. Supplementary tables are available in a separate electronic folder.

Results

To identify the nature of early post-mating changes in transcript abundance, we investigated the lower reproductive tract transcriptomes of virgin females and females collected 10-15 minutes after mating. We next investigated whether BMP-regulated secondary cell contributions to the ejaculate could mediate these changes, by comparing the transcriptomes of females mated to Dad males and females mated to control males.

The female's lower reproductive tract undergoes small-magnitude changes in transcript abundance within 15 min after mating, influencing both coding and noncoding RNAs.

To identify transcripts with an immediate response to mating, we compared the transcript abundance in mated females with that in virgin controls. We found that the transcript levels of 299 genes were altered at 10-15 minutes after the end of mating. The transcript abundance of 45/299 genes was down-regulated after mating, while the transcript abundance of 254/299 genes was up-regulated after mating. Of the 299 genes, 76 underwent at least a 2-fold change in transcript abundance (Fig. 4.1A; Table S4.1). To understand which molecular and biological processes are affected by mating we performed GO and KEGG enrichment analyses on all genes with a q-value ≤ 0.05, regardless of their fold change. Mating-responsive genes were significantly enriched for multiple GO and KEGG terms (Fig. 4.1B-C; Fig. S4.3). To better understand how the GO and KEGG categories fit into the response to mating in the female reproductive tract, we examined the genes that were part of enriched categories (Table S4.2). Out of more than 100 genes that were associated with an enriched GO category, only 11 were

down-regulated after mating, and these were not linked with one particular GO term. This indicates that the processes described below were generally up-regulated in mated relative to virgin females. First, we identified 30 genes with roles in wound healing. Among these were genes with roles in melanization and clotting (Scherfer et al. 2004; Tang 2009), genes with roles in cytoskeleton organization, cell-cell adhesion and cell-matrix interactions, genes involved in (muscle) morphogenesis and genes involved in the JNK signaling pathway, which is known to play a role in tissue regeneration (Rämet et al. 2002). These transcriptome results likely reflect the female's responses to copulatory wounding. The second largest group of mating-responsive genes comprised 15 genes with roles in the nervous system, ranging from functions in learning and memory to neuronal remodeling, synaptic transmission and synaptic growth at neuromuscular junctions. Remarkably, another group of genes whose transcripts were up-regulated after mating, was comprised of 11 genes encoding known seminal fluid proteins. Expression of these genes has generally been thought to be specific to male reproductive tissues. Thus, either females start to express genes encoding male seminal fluid proteins after mating, or these transcripts originated from the male. Using SNPs (see results section below), we were able to confirm that some of these transcripts indeed originated from the male. Further, we identified genes that are components of the Toll and Imd signaling pathways (but no target genes of these signaling pathways). Smaller groups consisted of genes involved in various signaling pathways (e.g. BMP and Egfr), genes involved in the response to ethanol and stress response, seven genes involved in lipid or carbohydrate metabolism and five genes

linked to the regulation of egg laying or sperm storage (juvenile hormone and octopamine signaling).

We further identified 18 noncoding RNAs that responded to mating; 9 of these were up-regulated, while 9 were down-regulated after mating (fig. 4.1D; Table S4.3). The noncoding RNAs included 3 predicted antisense RNAs (asRNAs), 13 long noncoding RNAs (lncRNAs) and 2 snoRNAs. Of the 13 lncRNAs, a function has been described only for *lncRNA:CR42859*, which is involved in spermatogenesis -but its exact mode of action is unknown (Wen et al. 2016). It has been suggested that lncRNA transcription can activate or silence the expression of genes *in cis*, if its transcription modifes the chromatin environment of the locus in which it resides (reviewed in Kornienko et al. 2013). Interestingly, 10/16 lncRNAs or asRNAs overlap with or are in the vicinity of genes that are not differentially expressed in our dataset after mating, but that have similar functions as differentially expressed genes, such as functions in the nervous system and muscle, Wnt signaling and organization of the extracellular matrix (Table S4.3). For example, *asRNA:CR42860* overlaps with the gene region of *sls*, which is involved in myoblast fusion (Zhang et al. 2000) and *lncRNA:CR45345* is in the vicinity of *aru*, which is a regulator of synapse structure and activity (Eddison et al. 2011). It is possible that lncRNA transcription in these loci contributes to the regulation of nearby genes, but this is a process that has not yet been investigated in the context of the female's transcriptional response to mating.

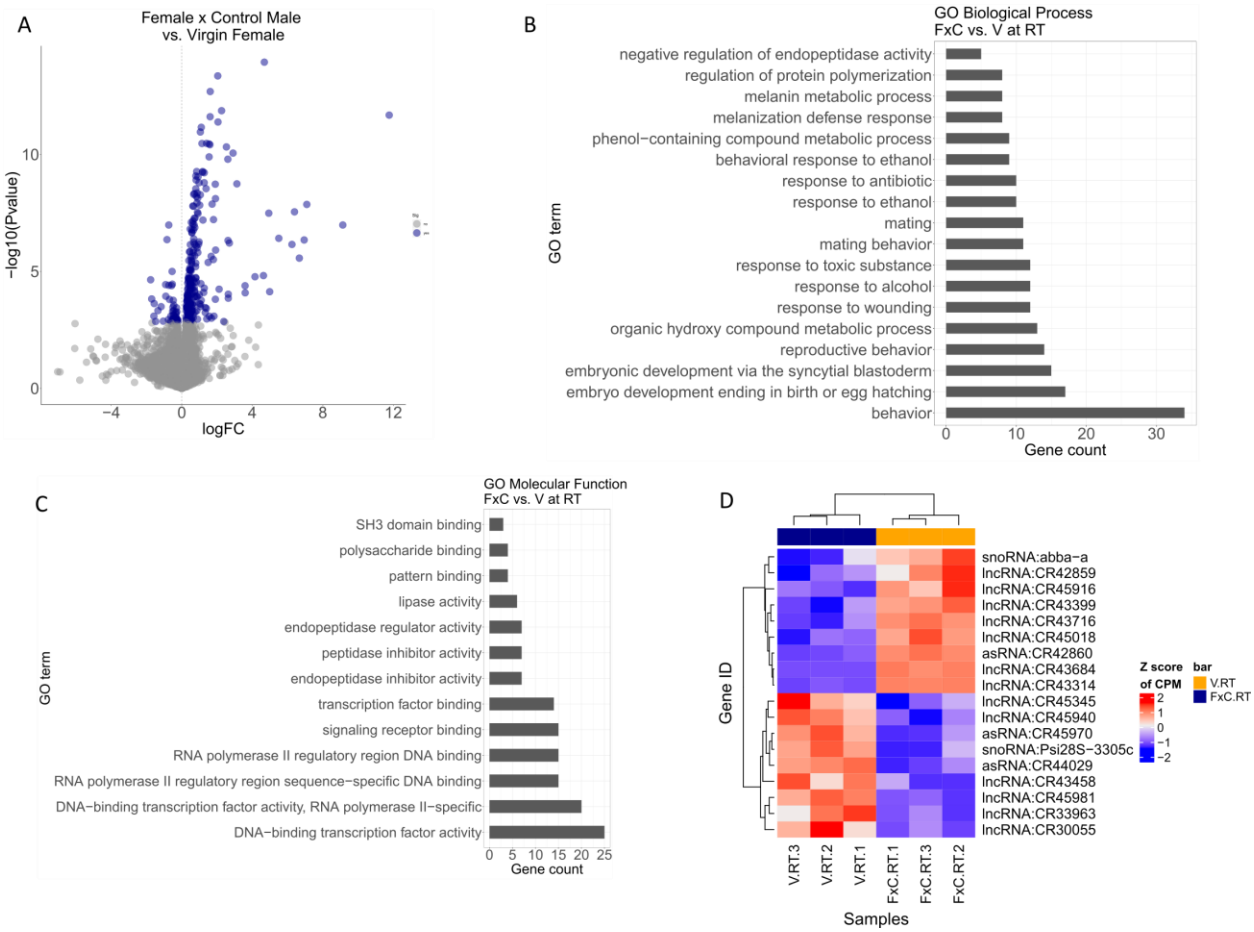

Figure 4.1: Mating alters the transcript abundance of 299 genes in the female lower reproductive tract at 15 min after mating. A: Volcano plot showing transcripts that are differentially expressed between mated and virgin females (in blue; $q \leq 0.05$). B: Enriched GO terms for Biological Process ($q \leq 0.05$). C: Enriched GO terms for Molecular Function ($q \leq 0.05$). D: Z scores of normalized expression values (counts per million; CPM) for 18 noncoding RNAs that respond to mating. V = virgin; FxC = female mated to control male; RT = room temperature.

SNP information reveals the presence of 36 transcripts that were transferred from the male to the female during mating.

Among the transcripts that were up-regulated after mating, we observed transcripts encoding seminal fluid proteins. To investigate if male RNA molecules can be transferred to the female during mating, we called SNPs using GATK (McKenna et al. 2010) and used SNPsplit (Krueger & Andrews 2016) to assign reads to either

“female” or “male” origin. After filtering, we identified 36 genes that had “male” reads aligning to them (Table S4.4). The majority of the 36 genes also had “female” reads aligning to them (Fig. 4.2A). When comparing the total “male” vs. total “female” read count for each gene, we found 3 genes for which more “male” reads than “female” reads were detected (*CG14507 , CG31872* and *Acp53Ea*). However, we discovered that for several of the 36 genes, “female” reads aligned to genes in male RNA samples, indicating uncertainty of the base call in those “female” reads. This suggests that the proportion of “male” versus “female” reads might actually be higher than what is shown in Fig. 4.2A. We used FlyAtlas 2 to identify the potential tissue of origin of the 36 transcripts (Leader et al. 2018). We found that some of the 36 transcripts had a high expression in the male accessory gland or testes, while others had an expression bias to the head and brain (Fig. 4.2B). We found that 4 of the 36 transcripts encode seminal fluid proteins (*Acp53Ea*, *wbl*, *CG15117*, *CG31872*) and 7 encode known sperm proteins (*CG31872*, *ck*, *Dip-B*, *Khc-73*, *CG16758*, *Nost*, *Ziz*). We also detected transcripts of *CG12104*, which encodes a HMG-box containing protein. Such proteins are expressed during the histone-to-protamine transition during spermatogenesis (Gärtner et al. 2015). GO enrichment analysis of the 36 genes detected enrichment for kinase regulators (*CycG*, *I(2)gl*, *mats*), tripeptidyl peptidases (*TppII*, *Dip-B*), translation initiation factor binding components (*eIF3m*, *pix*) and appendage development and imaginal disk morphogenesis (*Wnt6*, *ck*, *px*, *tgo*, *Gug*, *Nost*, *I(2)gl*).

A major question pertaining to the transfer of RNA molecules during mating is whether it is random or whether it is a regulated process. In the case of random transfer, it is possible that abundant transcripts of highly expressed genes are

accidentally secreted from accessory gland cells or are still present in mature sperm after going through spermatogenesis. We investigated this hypothesis using FlyAtlas 2 data (Leader et al. 2018; although RNA stability should ideally be taken into account as well). We plotted the FlyAtlas expression values (FPKM) in the testes and male accessory gland for the 36 genes with transferred transcripts and compared this with the FPKM values of all genes in the FlyAtlas 2 dataset that are expressed in the testes or accessory gland. As seen in Fig. 4.2C, some of the transferred RNAs are very abundant in the accessory gland, but the majority of transferred transcripts originate from genes with average expression levels in the testes or gland.

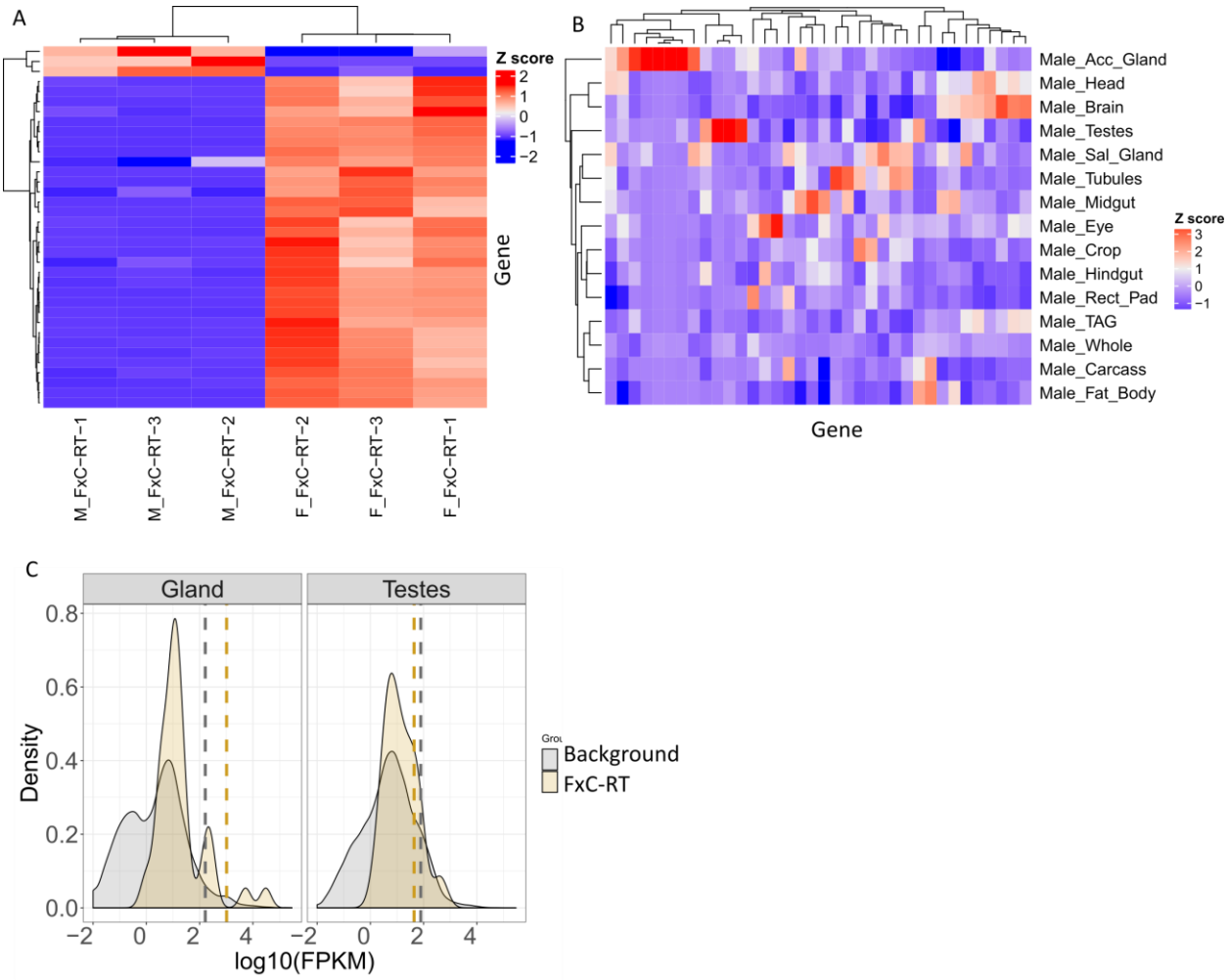

Figure 4.2: Analysis of 36 male-derived transcripts found in females mated to control males, at room temperature. A: Both female and male reads (based on female and male SNPs) align to the 36 genes. Shown are Z scores of normalized read counts. Each row represents one of the 36 genes. M: counts for reads carrying a male SNP; F: counts for reads carrying a female SNP. FxC-RT = female mated to control male at room temperature. B: Z scores of tissue-specific enrichment values across all male tissues available in the FlyAtlas 2 database (Leader et al. 2018). Each column represents one of the 36 genes. Acc_gland=accessory gland, Whole=whole body, Sal_Gland=salivary gland, TAG=thoracico-abdominal ganglion. (A recent report on http://flyatlas.gla.ac.uk/FlyAtlas2/index.html?page=home#mobileTargetG indicated that transcriptome data for the fat body was generated using a different method than what was used for the other tissues, and is therefore not comparable with those other tissues.) C: Distribution of FPKM values of transferred RNAs (yellow) vs. FPKM values of all genes expressed in the testes or accessory gland (grey). Dashed lines mark the average FPKM for each distribution. FPKM values were retrieved from FlyAtlas 2 (Leader et al. 2018).

Early post-mating changes in the female's transcriptome are not significantly affected by BMP-regulated secondary cell contributions to the ejaculate.

We next investigated whether BMP-regulated secondary cell functions influence the mating-induced changes in female transcript abundance. BMP signaling in the secondary cells regulates dense core granule replenishment (Redhai et al. 2016), exosome secretion (Corrigan et al. 2014), the abundance of certain seminal fluid proteins (Hopkins et al. 2019) and we found that it also influences the transcript abundance of 15 genes encoding seminal fluid proteins (Supplemental Results; Fig. S4.6D,E).

In females mated to control males at 29°C (a temperature shift was used to control the timing of BMP inhibition), mating induced changes in the abundance of 504 transcripts (Fig. S4.4A; Table S4.1). In females mated to Dad males, mating induced changes in the abundance of 268 transcripts (Fig. S4.4B; Table S4.1). Most of the transcripts had a smaller than 2-fold change in abundance and the majority of transcripts were up-regulated in mated females vs. virgin females. The total number of mating-responsive transcripts in females mated to Dad or control males differed (Fig. S4.4C). However, when we directly compared the transcript abundance between females mated to control males and females mated to Dad males, we found that these differences were not significant (Fig. 4.3A). This suggests that BMP-regulation in secondary cells does not significantly affect the female's lower reproductive tract transcriptome within 15 min after mating.

More male RNAs are detected in females mated to Dad males than in females mated to control males.

Even though the female's transcriptome was not significantly influenced depending on whether she mated to a Dad or control male, we proceeded to ask whether the transfer of male RNAs differed between Dad and control males. We identified transferred RNAs aligning to 40 genes in females mated to control males, and transferred RNAs aligning to 68 genes in females mated to Dad males (Table S4.4). Only 18 genes were found in both treatments (Fig. 4.3B). We used FlyAtlas 2 to investigate the expression level of genes whose RNAs are transferred, in the male accessory gland and in the testes (Leader et al. 2018). The expression level of genes whose RNAs are transferred by control males is not atypical compared to the expression level of all other genes that are expressed in the gland or testes (Fig. 4.3C). On the other hand, genes whose RNAs are transferred by Dad males have a slightly lower than average expression level in the testes and accessory gland, compared to all other genes expressed in the gland or testes (Fig. 4.3D). This suggests that some differences could exist between the RNAs transferred by control or Dad males. We also compared the transcriptomes of whole virgin Dad and control males (Supplemental Results; Fig. S4.5), but differences in transcript levels between Dad and control males did not directly correlate with differences in RNA transfer. For example, control males transferred a higher number of RNAs aligning to *Lectin-46Ca*, which encodes a seminal fluid protein (Findlay et al. 2008), compared to Dad males (Table S4.4), even though transcript levels of *Lectin-46Ca* were down-regulated in virgin control vs. Dad males (Fig. S4.5E).

We next examined the functions of the genes whose RNAs were detected in the female. Even though we identified a larger number of genes in females mated to Dad vs. control males, the genes we identified fell into similar functional groups. We identified transferred RNAs aligning to housekeeping genes (*RpS4* and *DpI* transferred by controls; *AGO1*, *pAbp* and *Trf2* transferred by Dad), RNAs aligning to genes encoding known sperm or seminal fluid proteins (*CG31872* and *Lectin46Ca* transferred by controls, *NT5E-2* transferred by Dad and *CG15117*, *CG11034*, *tay*, *Dip-B* and *CG16758* transferred by both), and RNAs aligning to neuronal genes (*Arl8*, *cpo, Mob2* transferred by control and *Velo*, *unc-13-4A*, *retn*, *nonC*, *Liprin-beta*, *hang*, *ckn, Ca-alpha1T* transferred by Dad).

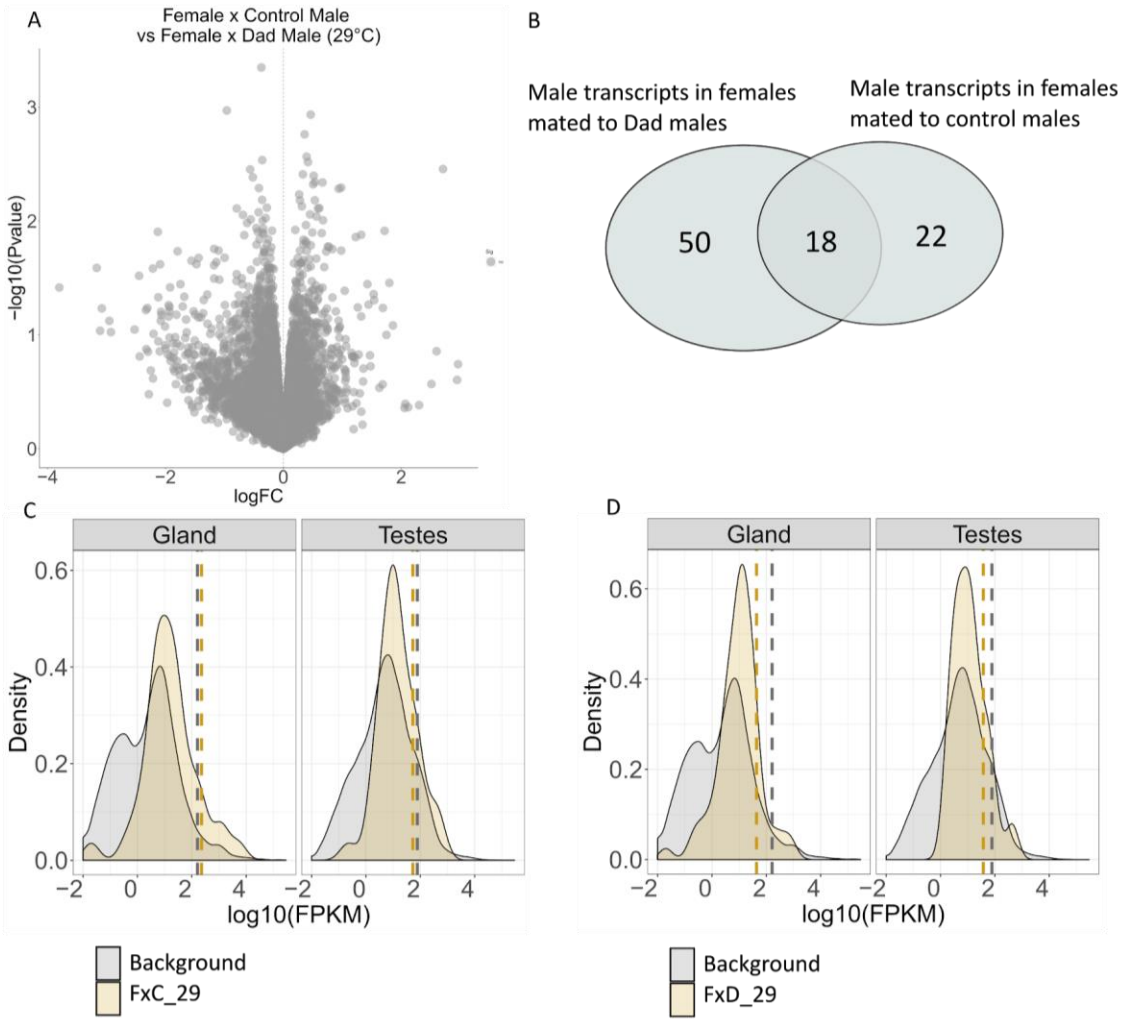

Figure 4.3: Comparison of transcript abundance between females mated to Dad vs. control males. A: Volcano plot showing results of differential expression analysis between females mated to Dad vs. control males (nothing significant). B: Number of male-derived transcripts detected in females mated to Dad vs. control males. C: Distribution of FPKM values of transferred RNAs in females mated to control males (FxC_29; yellow) vs. FPKM values of all genes expressed in the testes or accessory gland (grey). Dashed lines mark the average FPKM for each distribution.
D: Distribution of FPKM values of transferred RNAs in females mated to Dad males (FxD_29; yellow) vs. FPKM values of all genes expressed in the testes or accessory gland (grey). Dashed lines mark the average FPKM for each distribution. FPKM values were retrieved from FlyAtlas 2 (Leader et al. 2018).

Discussion

Changes in transcript abundance measured at an early and well-defined time point elucidate novel responses to mating in D. melanogaster females.

Even though the female lower reproductive tract undergoes essential changes after mating (Avila et al. 2012; Avila & Wolfner 2009; Bloch Qazi et al. 2003; Heifetz et al. 2014; Mattei et al. 2015; Rubinstein & Wolfner 2013), only moderate transcriptional changes have been detected in this tissue within one hour after mating (Mack et al. 2006). Here, immediately after mating, we detected novel transcriptome changes. First, we observed increases in the transcript levels of genes that mediate wound healing and tissue regeneration. Copulatory wounding is known to occur in *Drosophila* (Kamimura 2007; Mattei et al. 2015). In addition, it has been observed that several seminal fluid proteins enter the female's hemolymph during copulation (Lung & Wolfner 1999; Meikle et al. 1990; Monsma et al. 1990) and at least two seminal fluid proteins act on the female's central nervous system (Ding et al. 2003; Haussmann et al. 2013; Yapici et al. 2008), indicating that they have also entered the female's circulation. While it has been suggested that seminal fluid proteins can traverse the posterior vagina because of its thinner lining (Lung & Wolfner 1999), wounding would provide easy access to the female's hemolymph. While this could be advantageous to the male, for females, wounding could result in a great cost, for example if pathogens can enter the hemolymph via the wound. Our data indicate that the female rapidly responds to this potential cost, healing the wound. While we observe a response to wounding on the level of transcription, pre-existing proteins are likely involved in this process as well (De

Gregorio et al. 2002). The post-mating up-regulation of transcripts involved in muscle development and cell adhesion could be part of the response to wounding, but could also reflect a general reorganization of reproductive tract musculature and epithelia to accommodate the conformational changes needed for sperm storage and ovulation (Mattei et al. 2015; Rubinstein & Wolfner, 2013). Using microscopy and immunohistochemistry, Kapelnikov et al. (2008) observed that mating induced an increase in the number of myofibrils, and altered cellular junctions in the epithelium of the oviduct. Our transcriptome data suggest that similar changes occur in the lower reproductive tract as well.

Second, by 20 minutes after the start of mating, neuromodulator dynamics change in the neurons that innervate the reproductive tract and these changes are important to mediate sperm storage and ovulation (Avila et al. 2012; Heifetz et al. 2014; Heifetz & Wolfner 2004; Rubinstein & Wolfner 2013). Further, Kapelnikov et al. (2008) observed a post-mating increase in bouton number of neurons that innervate the oviduct. Our data show that these processes are accompanied by transcriptome changes. In mated females, we detected up-regulated transcripts of four genes whose products regulate synaptic growth at neuromuscular junctions (Cheng et al. 2011; Chen & Ganetzky 2012; Sweeney & Davis 2002; Zhao et al. 2009), and we detected an increase in RNA levels of *Arc2*, which is required for the transfer of RNA molecules between motor neurons and muscles (Ashley et al. 2018). These transcriptome changes could mediate a strengthening of the interaction between the nervous system and reproductive tract muscles in a mated female.

Finally, we observed changes in immune gene transcripts. Transcripts of immune response genes and specifically antimicrobial peptides (AMPs) are upregulated at 1-3 hours and 6-8 hours post-mating (Kapelnikov et al. 2008; McGraw et al. 2008; McGraw et al. 2004; Mueller et al. 2007; Gioti et al. 2012; Domanitskaya et al. 2007). While we did not observe changes in AMP transcripts at 15 minutes after the end of mating, we observed an upregulation of RNAs of components of the Toll and Imd pathways that function upstream of AMP transcription (e.g. *Rel, cact*). This observed upregulation of immune transcripts implies that the reproductive tissue prepares itself for increased infections or prolonged exposure to male seminal fluid proteins, some of which have been shown to be harmful to females (McGraw et al. 2004; McGraw et al. 2008; Morrow & Innocenti 2012; Mueller et al. 2007).

BMP-regulation in the male secondary cells does not influence female transcriptome changes within 15 minutes after mating.

BMP signaling in secondary cells influences the composition of the male's ejaculate (Hopkins et al. 2019; Gligorov et al. 2013; Sitnik et al. 2016; Corrigan et al. 2014; Leiblich et al. 2012; Redhai et al. 2016). We asked if BMP-regulated secondary cell functions influenced the female's transcriptional responses to mating. However, we did not observe significant differences in female transcript abundance depending on the genotype of the male she mated with. It is possible that BMP-regulated secondary cell contributions to the ejaculate have effects only at the protein level or influence small regulatory RNAs in the female tract. Alternatively, 15 minutes after mating might be too early to capture differences in transcript abundance caused by changes in male

secondary cell BMP signaling, although earlier time points are generally favorable to capture direct rather than secondary effects of components in the male ejaculate.

Males transfer RNAs to females during mating.

Using SNP analysis, we identified multiple transcripts that are likely part of the ejaculate of the *D. melanogaster* male and are transferred to females. The transfer of a handful of transcripts encoding seminal fluid proteins has been described in recently-mated mosquitoes (Alfonso-Parra et al. 2016) and in heterospecific matings between *D. mojavensis* and *D. arizonae* (Bono et al. 2011). None of the male-derived transcripts we identified are homologous to transferred seminal fluid protein transcripts identified by Bono et al. (2011). We do observe a significant post-mating up-regulation of *CG17097* transcripts, homologs of which are transferred in *D. mojavensis*, but we did not identify SNPs in *CG17097* that allowed us to distinguish between female or male origin. In addition, we observed the transfer of transcripts encoding proteins with varied functions. This introduces the exciting possibility that next to proteins and sperm, RNAs are a functional part of the male ejaculate in flies, as has been observed for miRNAs in mice (Conine et al. 2018). This would open up the use of *D. melanogaster* to investigate how male-female crosstalk of transcripts influences reproductive success. To this end, several questions beg to be addressed: What is the tissue of origin of these RNAs? Is their release random, or is it regulated? Are the RNAs functional inside the female?

These transcripts could originate from the accessory gland, testes, or other tissues of the male reproductive tract. Once in the ejaculate, they could be inside vesicles, sperm, protected by RNA binding proteins (even though no RNA binding

proteins are known to be part of the seminal fluid), or inside cells. Exosomes in particular are known vehicles for RNAs in seminal fluid, in humans and in mice (Vojtech et al. 2014; Sharma et al. 2018; Conine et al. 2018). In flies, BMP inhibition prevents the secretion of exosomes from secondary cells. Thus, if secondary cell-derived exosomes are required for the transfer of RNAs, we would expect to observe fewer transferred RNAs in females mated to Dad males. However, we detected more RNAs in females mated to Dad males than females mated to control males, suggesting that BMP signaling in the male reproductive tissue has more complicated effects on the transfer of RNAs. In addition, we found no obvious correlation between transferred RNAs and transcriptome differences between Dad and control males.

Fischer et al. (2012) identified many transcripts encoding transmembrane components and translation machinery in mature *D. melanogaster* sperm and showed using YPF tagging and RT-PCR that these transcripts are transferred into the oocyte after fertilization. In line with those findings, we identified some “male” reads aligning to genes encoding translation components. However, the presence of male-derived transcripts encoding housekeeping genes also points to the possibility that whole male cells from secretory tissues in the reproductive tract end up in the ejaculate. The delamination and transfer of intact secondary cells to females has been described by Leiblich et al. (2012), but occurred only in multiply mated males. Hence, the virgin males we used for our experiments are not likely to transfer entire secondary cells to females, but delamination of cells from other reproductive tissues has not been investigated.

Finally, while we observed multiple transferred RNAs, we are still far from a complete identification of ejaculate RNAs. The female and male lines used in this study

were not fully inbred, leading to fewer usable SNP calls. In addition, because we sequenced the entire lower female reproductive tract, the proportion of “male” versus “female” reads is very small, and this can contribute to an increased between-replicate variability. Thus, to improve detection, this experiment could be repeated using different, more homozygous female and male lines, or sequencing could be performed on ejaculates (or isolated ejaculate components such as sperm, cells, vesicles) before they enter the female.

In conclusion, this work has improved our understanding of very early transcriptional responses to mating in the female reproductive tract. At this early time point, the female’s transcriptome was not influenced by BMP-regulated secondary cell contributions to the ejaculate, but BMP-signaling in the male’s secondary cells might influence the transfer of RNAs to the female. The observation that male RNA molecules are transferred during mating, in a system amenable to genetic manipulation, will allow future studies to tease apart any physiological functions these male components might have.

Acknowledgements

We are grateful to A. Jain for help with library preparation and technical aspects of sequencing, E. Cosgrove for help with assembling a SNP calling pipeline, Dr. C. Wilson for sharing the *UAS-Dad* and *esg-GAL4* lines and RO1 HD059060 to AGC and MFW for funding this project.

Supplementary results & discussion

Dad males have an altered transcriptional profile, have fewer sperm in their seminal vesicles and their mates have an increased receptivity to remating.

To ensure that all constructs used to generate Dad males were working as expected, we performed a receptivity assay to reproduce earlier reports that Dad males have a decreased ability to induce refractory mating behavior in their mates (Corrigan et al. 2014; Leiblich et al. 2012). In a receptivity assay we conducted, 21/23 females previously mated to Dad males remated with a wildtype male, while only 6/22 females previously mated to control males remated (Fig. S4.5A; Fisher's exact test $p = 1.3*10^{-5}$), thus confirming previously published results.

Hopkins et al. (2019) reported a proteomics analysis of accessory glands of virgin and mated Dad and control males. Their analysis established altered levels of certain seminal fluid proteins in Dad males (Hopkins et al. 2019). We asked whether the transcriptome of Dad males differed from that of control males. We identified 155 transcripts that differed in abundance between Dad and control males, of which 83 differed with at least a 2-fold change (Table S4.4). Of the 155 transcripts, 95 were down-regulated in Dad males, while 60 were up-regulated relative to control males (Fig. S4.5C). Using expression data from FlyAtlas 2 (Leader et al. 2018), we found that these 155 genes are expressed predominantly in the midgut, testes and accessory gland (Fig. S4.5D). This result fits expectations, since *escargot-GAL4*, the driver used to expressess Dad, is expressed in the midgut, in addition to the testes and secondary cells (Leader et al. 2018). In the midgut, similar as in the testes, BMP signaling is

required for the maintenance of stem cells (Tian & Jiang 2014). Using a GO term enrichment analysis, we identified biological processes that were significantly enriched among either the up- or downregulated genes. Transcripts up-regulated in Dad males were enriched for response to insecticide (*Cyp12d1-p, Cyp6g1, Cyp6a8*) and double stranded break repair and spindle assembly checkpoint (*Blm, ncd, Mps1*). Transcripts down-regulated in Dad males were enriched for the immune response (*TotX, TotA, CG43055, Lectin-GalC1, LysD, LysE, LysP, IM4, Drsl2, Npc2h, CG34215*) and mating (*Gld, CG9029, lectin-46Cb, lectin-46Ca*). The latter four genes encode seminal fluid proteins, and we were able to identify 11 additional genes which encode known seminal fluid proteins, most of which were down-regulated in Dad males vs. control males (Fig. S4.5E). For *Nlaz*, one of the 15 genes that encode seminal proteins, the RNA levels were altered in Dad males in our study, and Hopkins et al. (2019) found that Nlaz protein levels were also altered in Dad vs. control males. In addition, we observed altered RNA levels of *Spn75F*, encoding a serpin in the seminal fluid. Hopkins et al. (2019) did not observe changes in Spn75F, but observed altered protein levels of three other serpins that are part of the seminal fluid. Further, the abundance of 21 noncoding RNAs and the RNAs of multiple genes involved in chitin metabolism and gut lining (such as *Muc30E, Cpr100A, Peritrophin-15a*) differed between Dad and control males.

Finally, because *esg-GAL* is expressed in the testes (Leader et al. 2018) and BMP signaling is important for stem cell function (Kawase et al. 2004), we also compared sperm numbers in the seminal vesicles of Dad and control males. We found a clear, but nonsignificant decrease in sperm numbers in Dad (865 ∓ 134 sperm) vs. control males (2024 ∓ 422 sperm) (Fig. S4.5B; Wilcoxon test $p = 0.07$). After removing

an outlier which contained only 156 sperm, which could be caused by a dissection error, this difference is significant (Wilcoxon test $p = 0.004$). Earlier studies reported that mates of Dad males produced the same number of offspring as mates of control males (Corrigan et al. 2014) and Hopkins et al. (2019) observed no significant decrease in the number of sperm transferred during mating with a Dad male relative to a control male. Additional experiments are needed to determine whether lower sperm number in the seminal vesicles also leads to a significant reduction in sperm transfer to the female in our experimental setup, in which case this could also (partly) be responsible for the receptivity phenotype we observed.

Collectively, our results indicate that the disruption of BMP signaling is effective, has pleiotropic effects, and in particular in the accessory gland, it influences the transcript abundance of certain seminal fluid proteins.

Supplementary table legends

Table S4.1: Mating-responsive genes.
List of genes whose transcript abundance is altered in the female lower reproductive tract at 15 minutes after mating.

Table S4.2: Overview of genes that are part of enriched GO/ KEGG categories.
List of genes and gene functions for transcripts that were differentially expressed 15 minutes after mating to a control male (at room temperature) and that were part of enriched GO or KEGG terms. Green highlight: transcripts are up-regulated post-mating; Red highlight: transcripts are down-regulated post-mating.

Table S4.3: Noncoding RNAs that respond to mating in females mated to control males at room temperature.

Table S4.4: Male transcripts identified using SNPs.

Table S4.5: List of transcripts that differ in abundance between virgin Dad males and control males.

Supplementary Figures

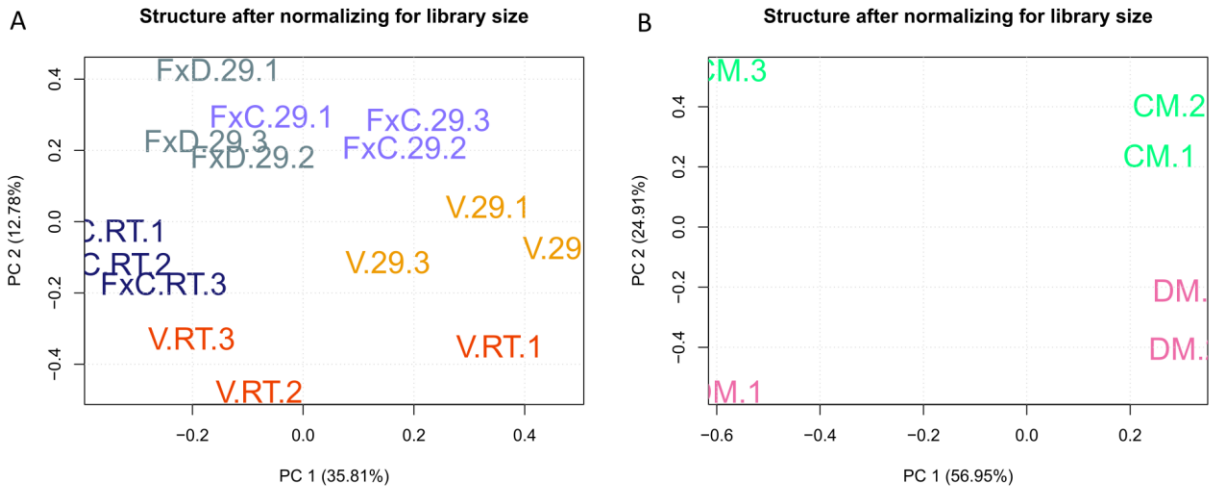

Figure S4.1: Principal Component Analysis of RNA-seq samples. A: PCA of all female samples. FxD = female mated to Dad male; FxC = female mated to control male; V = virgin female; RT = room temperature; 29 = 29°C. B: PCA of all male samples. DM = Dad male; CM = control male. Colors represent different treatments; 3 replicates were analyzed per treatment.

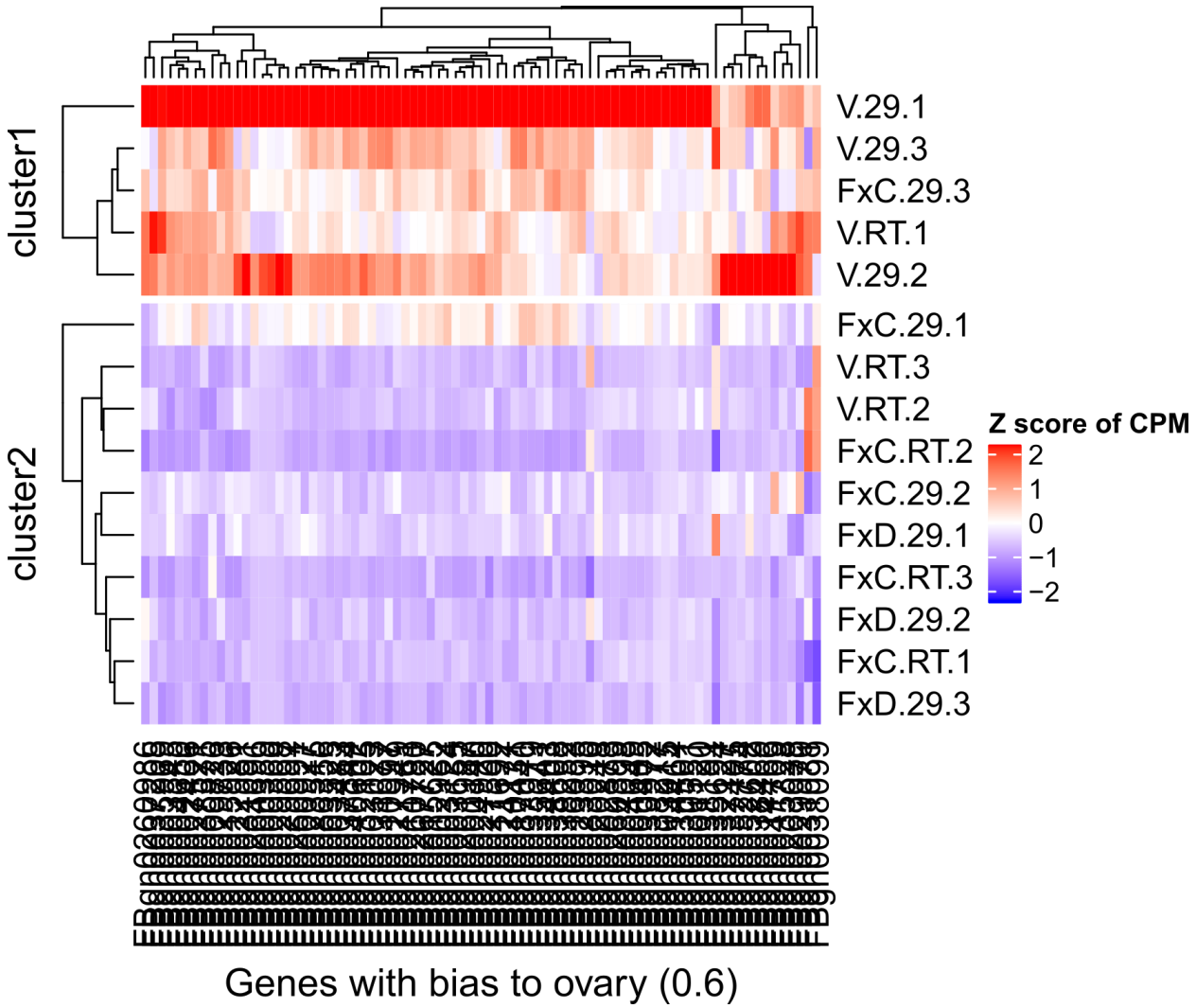

Figure S4.2: Hierarchical clustering of female RNA-seq samples based on their CPM (count per million) for genes with an expression bias to the ovary. Expression bias to the ovary was calculated for each gene as ovary(FPKM) / sum of female tissues(FPKM), which leads to a number between 0 and 1, with 1 indicating a stronger expression bias to the ovary relative to other tissues. The cutoff for ovary expression bias used here was 0.6. Similar clustering of samples was observed with an expression bias cutoff of 0.7 (data not shown). FxC = female mated to control male; FxD = female mated to Dad male; V = virgin female; RT = room temperature; 29 = 29°C.

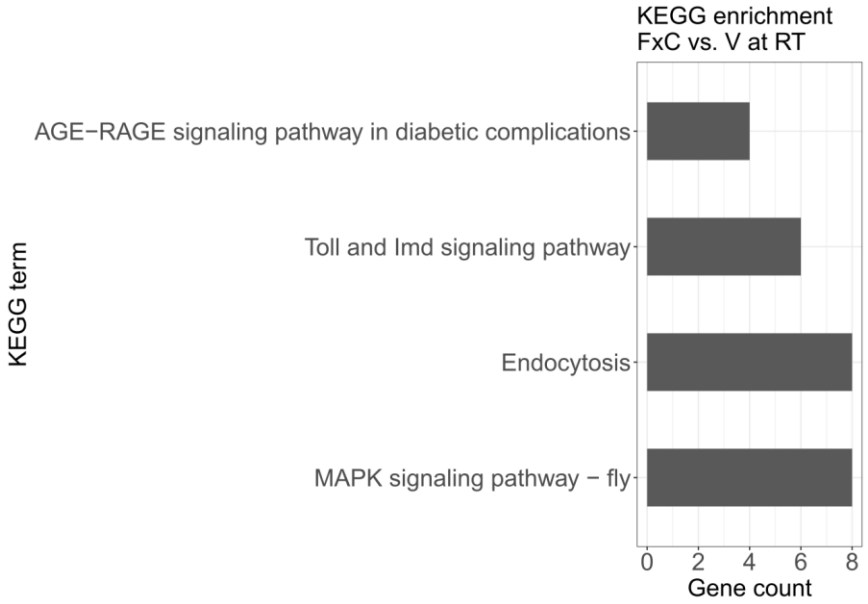

Figure S4.3: KEGG enrichment analysis for differentially abundant transcripts in mated vs. virgin females. V = virgin; FxC = female mated to control male; RT = room temperature.

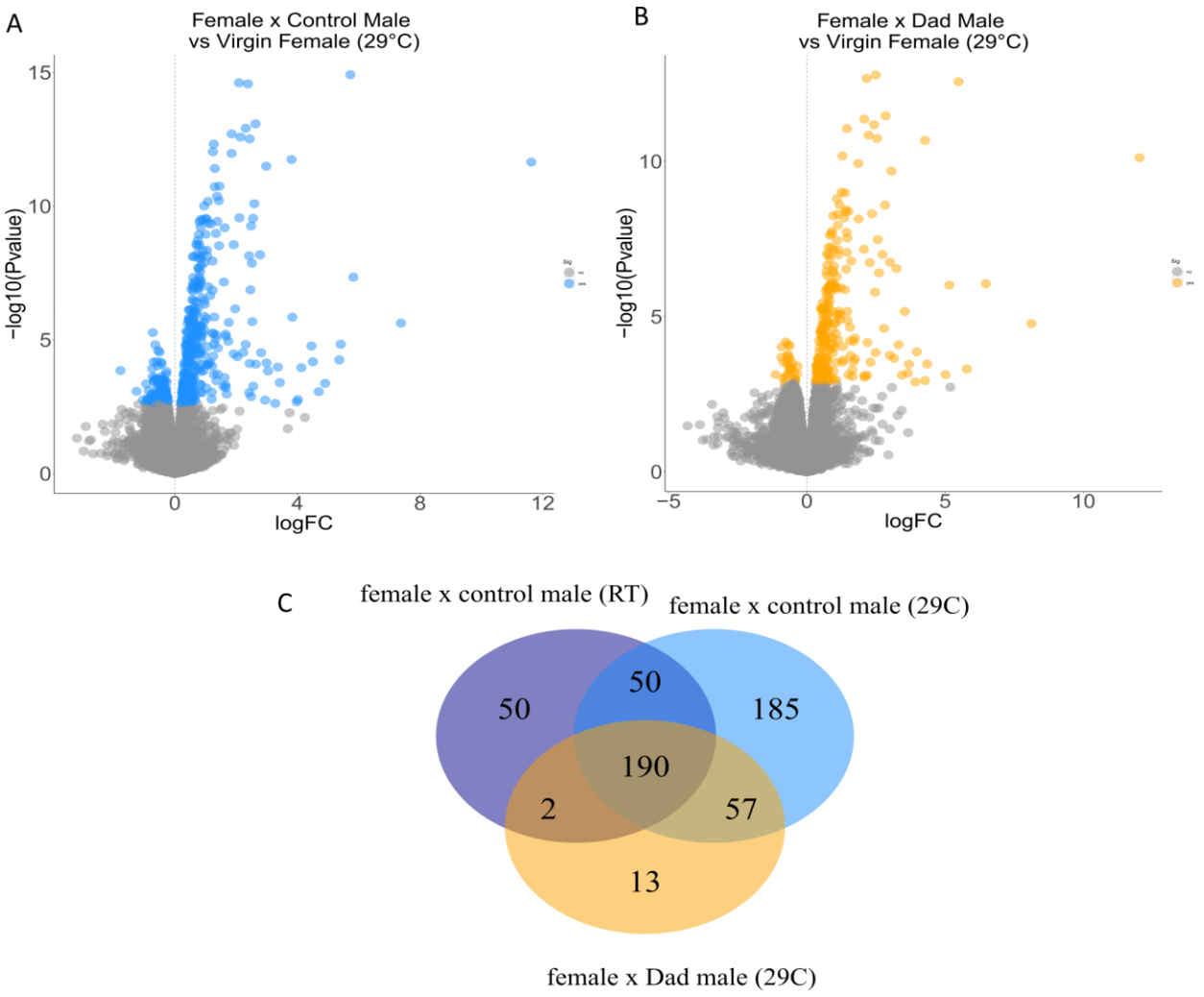

Figure S4.4: Differentially abundant transcripts in mated vs. virgin females. A: Volcano plot showing the results of a differential expression analysis between females mated to control males and virgin females at 29°C. (Blue: genes with $q \leq 0.05$) B: Volcano plot showing the results of a differential expression analysis between females mated to Dad males and virgin females at 29°C. (Orange: genes with $q \leq 0.05$) C: Venn diagram showing intersection and differences in differentially abundant transcripts of females mated to control males at room temperature or 29°C and females mated to Dad males at 29°C.

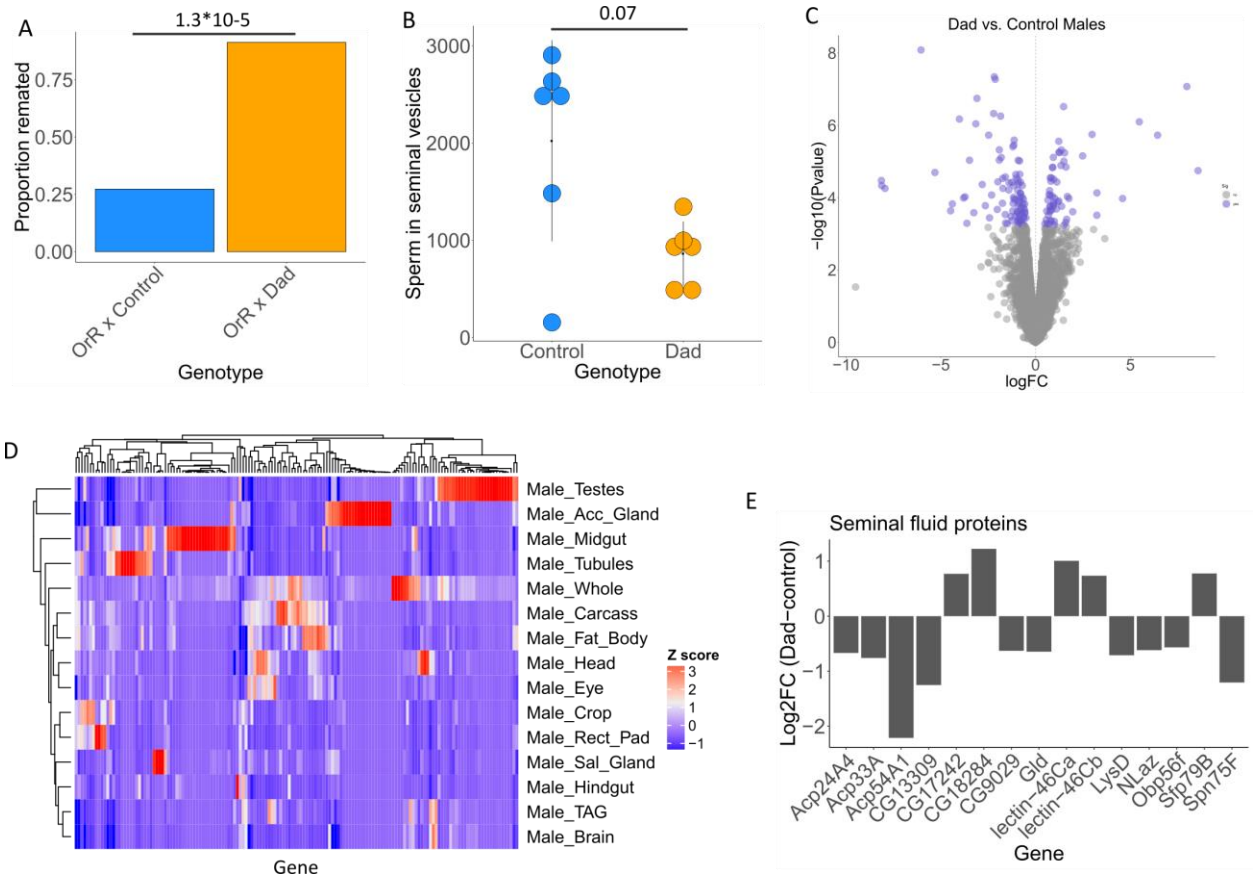

Figure S4.5: Dad males fail to induce refractory mating behavior in their mates, have less sperm in their seminal vesicles and demonstrate transcriptome differences compared to wildtype controls. A: Proportion of remated females in a four-day receptivity assay. N for OrR x Control = 23; N for OrR x Dad = 22. Fisher's test *p*-value = $1.3*10^{-5}$. B: Number of sperm in the seminal vesicles of unmated 5-day old control and dad males. N = 6 for each treatment. Wilcoxon test *p*-value = 0.07 including outlier, 0.004 excluding outlier for control males. C: Volcano plot showing differentially expressed transcripts between Dad and control males ($q \leq 0.05$ in purple). D: Heatmap showing Z scores of tissue enrichment values for all differentially expressed genes across male tissues. Enrichment values were calculated using FPKM data from FlyAtlas 2 (Leader et al. 2018). Acc_gland=accessory gland, Whole=whole body, Sal_Gland=salivary gland, TAG=thoracico-abdominal ganglion. (A recent report on http://flyatlas.gla.ac.uk/FlyAtlas2/index.html?page=home#mobileTargetG indicated that transcriptome data for the fat body was generated using a different method than what was used for the other tissues, and is therefore not comparable with those other tissues.) E: 15 transcripts encoding seminal fluid proteins are differentially expressed in Dad males relative to control males. Log2FC = log2 fold change.

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CHAPTER 5:

FEMALE AND MALE MICROBIOMES INFLUENCE FECUNDITY AND FEMALE TRANSCRIPTOME RESPONSES TO MATING IN *DROSOPHILA MELANOGASTER*[4]

Abstract:

Drosophila melanogaster females undergo a variety of post-mating changes which influence their activity, feeding behavior, metabolism, egg production and gene expression, including an up-regulation of immunity transcripts. These changes are induced by mating itself or by sperm or seminal fluid proteins. In addition, studies in *D. melanogaster* have established that axenic females produce fewer eggs than females with a microbiome and there is evidence that the microbiome of the female's mate influences this process as well. It is not known whether a female or her mate requires a microbiome to enable the female to undergo other post-mating responses. We investigated this by mating axenic and control females to either axenic or control males and measuring fecundity and whole-female transcriptome changes at six hours after mating. We observed interactions between the female's microbiome and her mating status: transcripts of genes involved in reproduction and genes with neuronal functions were differentially abundant depending on whether females had a microbiome, but only in mated females, not in virgin females. In addition, immunity genes showed specific responses to either the microbiome, mating, or a combination of those two factors. We

[4] A modified form of this chapter will be submitted for publication. Delbare SYN and Ahmed-Braimah YH collaborated to collect samples for RNA-seq, perform the egg laying assay and analyze the RNA-seq data. Delbare SYN analyzed the egg laying data, performed qRT-PCRs and wrote the manuscript. Wolfner MF and Clark AG provided advice throughout the project and provided funding.

further observed that the male's microbiome status influenced the fecundity of both control and axenic females, while influencing the transcriptional profile of only axenic females. Our results indicate that the microbiome is vital for females to undergo a post-mating switch in the transcriptome. Failure to do this could underlie the fecundity problems that we and others observed.

Introduction

Reproductive success is determined by the cumulative effects of behavioral and physiological changes that a female undergoes after mating. In *Drosophila*, these post-mating responses include sperm storage, increased oocyte production and ovulation, a decrease in sleep and the female's propensity to remate, alterations to the female's immune system and changes in feeding frequency, gut morphology and physiology (Reiff et al. 2015; Apger-McGlaughon and Wolfner 2013; reviewed in Avila et al. 2011). Changes in metabolism and food uptake are thought to be required to meet the high energetic demands of oocyte production (Reiff et al. 2015). These phenotypic changes are accompanied by extensive transcriptome changes across several female tissues (Innocenti and Morrow 2009; Lawniczak and Begun 2004; McGraw et al. 2008; McGraw et al. 2004; Peng et al. 2005; Short and Lazzaro 2013), including the head (Dalton et al. 2010) and reproductive organs (Kapelnikov et al. 2008; Mack et al. 2006; Prokupek et al. 2009). Transcriptome changes in mated *D. melanogaster* females have been measured as early as zero to three hours after mating and typically reach their highest magnitude by six hours after mating (Mack et al. 2006; McGraw et al. 2008). Functional categories of genes that are affected by mating include genes encoding proteases and

CytP450, genes involved in oogenesis and eggshell formation, metabolism, the immune response and muscle organization (Mack et al. 2006; Kapelnikov et al. 2007; McGraw et al. 2008; Prokupek et al. 2009). It has been well established that these female post-mating responses are influenced by an interplay between the genotypes of the female and her mate (Chow et al. 2010; Clark et al. 1999; Delbare et al. 2017; Reinhart et al. 2015; reviewed by Civetta and Ranz 2019), and that they are induced by male ejaculate components that are transferred to the female during mating (Avila et al. 2011; McGraw et al. 2008; Domanitskaya et al. 2007; McGraw et al. 2004; Peng et al. 2005; Gioti et al. 2012). Some female responses to mating have also been shown to be impacted by environmental factors, including microbiome exposure, as outlined below.

In recent years, *Drosophila melanogaster* has emerged as a valuable model to study fundamental principles of host-microbiome interactions, owing to the availability of genetic resources and a well-characterized and easily-manipulated gut microbiome (Douglas 2018). Removal of the microbiome (bacteria and yeast) from *D. melanogaster* affects a wide range of traits. Among others, the transcriptome is affected, and this reflects regulatory effects of the microbiome on tissue homeostasis, carbohydrate and lipid metabolism, proteolysis and immunity (Bost et al. 2018; Broderick et al. 2014; Combe et al. 2014; Dobson et al. 2016; Elya et al. 2016). In addition, there is evidence that microbiome-induced transcriptome changes underlie a range of phenotypes including larval development time (Bakula 1969; Gould et al. 2017; Newell and Douglas 2013; Ridley et al. 2012), metabolite levels (Ridley et al. 2012; Wong et al. 2014), intestinal stem cell proliferation (Buchon et al. 2009; Fast et al. 2018), behavior (Fischer et al. 2017; Schretter et al. 2018), longevity (Fast et al. 2018; Gould et al. 2017; Obata

et al. 2018) and reproductive capacity (Elgart et al. 2016; Gould et al. 2017; Morimoto et al. 2017; Qiao et al. 2019). Across microbiome studies of *D. melanogaster*, it appears that some effects are robustly observed (e.g. transcriptome changes or changes in metabolite content), while others yield variable results, likely depending on the experimental design or environmental conditions. For example, Schretter et al. (2018) observed a significant increase in the locomotor activity of axenic flies relative to flies with a microbiome, but no significant difference in activity was seen by Selkrig et al. (2018). Further, microbiome-induced changes in courtship were not observed by Selkrig et al. (2018) and Leftwich et al. (2017), while changes in courtship were observed by Qiao et al. (2019) and Sharon et al. (2011). Even egg laying, which was consistently observed to be lower in axenic females compared to females with a microbiome in multiple studies (Elgart et al. 2016; Gould et al. 2017; Morimoto et al. 2017; Qiao et al. 2019; Selkrig et al. 2018), was not observed to be lower in axenic females by Ridley et al. (2012). These varied results could be attributed to variability in food nutrients (Chandler et al. 2012; Wong et al. 2015; Wong et al. 2011), species and strains of microbiota and host, or host age (Obadia et al. 2017; Staubach et al. 2013; Wong et al. 2013), or the requirement for frequent bacterial replenishment to maintain a stable microbiome association (Blum et al. 2013; Douglas 2018; Pais et al. 2018). However, another variable that was not always controlled for is female mating status, i.e. whether a female is virgin or has mated, and if she mated, how long ago and how often she has mated. For example, female mating status was not explicitly taken into account in studies that investigated metabolic changes and activity levels (Ridley et al. 2012; Schretter et al. 2018), even though these are phenotypes that are also influenced as

part of the female post-mating switch in behavior and physiology (Avila et al. 2011). Similarly, Bost et al. (2018), Broderick et al. (2014), Combe et al. (2014) and Elya et al. (2016) measured differences in the transcriptomes of female *D. melanogaster* with or without a microbiome, but the mating status of these females was not explicitly controlled for. Because of this, we were interested in evaluating whether interactions between the female's mating status and the female's microbiome influence her transcriptome. In addition, it is possible that interactions exist with the microbiome of the female's mate. In support of this, Morimoto et al. (2018) observed that gnotobiotic males carrying only *Lactobacillus plantarum* had a longer copulation duration and induced higher short-term egg laying in their mates, while a significant number of females mated to gnotobiotic males carrying only *Acetobacter pomorum* produced very few offspring. Also of particular interest is the post-mating up-regulation of immunity genes that is observed in *D. melanogaster* females. The post-mating up-regulation of some immunity genes was shown to be elicited by sperm and male seminal fluid proteins (McGraw et al. 2008; Domanitskaya et al. 2007; McGraw et al. 2004; Peng et al. 2005), but it is not known whether the microbiome is also involved in this, even though activation of the innate immune system might seem particularly sensitive to prior microbial exposure in females and males.

We performed RNA-sequencing to determine if the *D. melanogaster* female transcriptome is influenced by interactions between female microbiome status (axenic or control) and female mating status (virgin or six hours after mating). We further tested if the male's microbiome status (axenic or control) could influence female fecundity and female transcriptional changes six hours after mating. Our data indicate that the

transcript abundance of genes involved in reproduction, neuronal functions and the immune response, is influenced by interactions between the female's microbiome and her mating status. We further observed interactions between the female's microbiome and the male's microbiome that influenced both fecundity and transcript levels of females.

Materials and methods

Fly stocks, rearing and the generation of axenic and control flies

Canton-S flies were maintained at 25°C on yeast-sucrose-cornmeal food (7 g agar; 12 g yeast; 12 g cornmeal; 40 g sucrose; 1,000 ml water, 26.5 ml Tegosept; 12 ml acid mixture) in a 12 h light/dark cycle. To generate axenic flies and controls, the protocol described by Koyle et al. (2016) was followed. Briefly, population cages of Canton-S flies were set up, and females were allowed to oviposit on grape juice agar plates for 2-3 days until robust egg-laying began. On the 2nd or 3rd day, embryos were collected and treated twice (2.5 min each time) with a 0.6% sodium hypochlorite solution (Clorox) and triple-rinsed in autoclaved distilled water. To ensure a sterile environment, the latter and subsequent procedures were performed in a biosafety cabinet. Axenic embryos were allowed to hatch in 50 ml sterile tubes that contained sterile, autoclaved food with 40 ul of 1X PBS added on top. To generate controls, the same embryo dechorionation procedure was followed, but the tubes for the control samples received 40 ul of Canton-S fly homogenate (prepared in aliquots of 200 ul, at a concentration of 50 flies in 200 ul 1X PBS) on top of the food to add the full set of bacteria found in our lab's Canton-S stock. All food in tubes and subsequent vials also

had autoclaved yeast sprinkled on top. After seven days, pupae from axenic and control tubes were collected and twice treated with 0.6% sodium hypochlorite for 30 sec and subsequently rinsed three times in autoclaved distilled water. Each pupa was isolated in an individual vial with sterile food. Each vial with a 7-day old control pupa received 20 ul of Canton-S fly homogenate on top of the food (on average 2.5 flies ground up for each vial). Each vial with an axenic pupa received 20 ul of sterile 1X PBS.

Confirmation of microbiome status

Two assays were performed to ensure axenic flies were fully germ-free and to ensure the presence of a microbiome in control flies: 1) Individual flies were homogenized in MRS broth and plated on MRS plates (Koyle et al. 2016), which were then incubated at 29˚ C for 2-3 days and checked for the presence of colonies. 2) A PCR assay was performed according to the methods in Ridley et al. (2012). Briefly, genomic DNA was extracted from 3-10 pooled axenic or control larvae, pupae or adult flies. PCR was run using primers designed by Ridley et al. (2012) for a conserved region of the bacterial 16S rRNA gene. PCR products were run on a 1% agarose gel to confirm the absence of bacteria in axenic individuals and the presence of bacteria in controls (Fig. S5.1). We also compared levels of bacteria in 9-12 pooled adults of our control flies with levels of bacteria in 9-12 pooled untreated (not dechorionated) adult Canton-S flies and found an enrichment of bacteria in our control flies (Fig. S5.1). Absence of *Wolbachia pipientis* in our lab's Canton-S stock was confirmed using a PCR assay described by Schneider et al. (2014).

Mating assay and sample collection for RNA-seq and qRT-PCR

Five day old virgin flies were used for the mating assays. Axenic and control females and males were singly mated in a 2x2 full factorial design (Control females x Control males (CC), Control females x Axenic males (CA), Axenic females x Axenic males (AA) and Axenic females x Control males (AC)). Matings were observed and males were removed from the vial after copulation ended. We verified the absence of bacteria in axenic mated females using PCR (as described above). The end time of copulation was recorded and females were flash-frozen for RNA-seq at six hours after mating, at which time virgin axenic and control females were collected as well. For RNA-seq, we collected four replicates for each of the six treatments (four mated treatments and two virgin treatments) on the same day. Around ten females were pooled per replicate. For qRT-PCR, matings were performed using a separate batch of flies that were dechorionated on a different day than those used for RNA-seq. Females were mated to males of the same microbiome status as themselves (no full factorial design) and were flash frozen six hours after mating, at which time virgin females were also frozen. For each treatment for the qRT-PCR, three replicates of 10 pooled females were collected.

RNA extraction, RNA-seq library preparation and qRT-PCR methods

To extract whole RNA, a pool of ~10 frozen females from each sample was homogenized in TRIzol following manufacturer's guidelines (Thermo Fisher Scientific inc., MA). Following liquid phase separation, the RNA-containing upper layer was subjected to column purification and DNase treated using the RNeasy Mini Kit (Qiagen

inc., MD). Purified RNA was quantified and saved at -80˚C for library preparation. RNA-seq libraries were made using the Lexogen 3` FWD kit following the manufacturer's protocol (Lexogen, NH). Libraries were quantified on an Agilent 2100 Bioanalyzer before pooling and cluster generation/sequencing on an Illumina NextSeq platform.

For qRT-PCR, RNA was extracted as above. RNA was DNase treated using RQ1 RNase-Free DNase (Promega, WI) and cDNA was synthesized using SMARTScribeTM Reverse Transcriptase (Clontech, CA). qPCRs were run on three biological replicates, each with three technical replicates, on a Roche LightCylcer 480 Instrument II using LightCycler 480 SYBR GreenI Master (Roche, NJ). Primers were designed using Primer Blast, except for the gene *jhamt*, for which we used primers designed by Schwenke and Lazzaro (2017). We verified that primer efficiency was above 80%. Primer sequences can be found in Table S5.1. *Rp49* or *Nervana* were used as control genes. We verified that these genes were not among the differentially expressed genes for the contrasts of interest. Ct values were analyzed using the Pfaffl method (Pfaffl 2001).

Read processing, alignment and differential expression analysis

Raw reads were processed by trimming 10 bases from the 5` end and quality trimming from the 3` end to a minimum quality PHRED score of 20. Processed reads were mapped to the *D. melanogaster* transcriptome (Flybase r6.23) with bowtie2 and read counts and normalized abundances were extracted using eXpress (Forster et al. 2013; Langmead and Salzberg 2012). All differential expression analyses were performed in R using the packages EdgeR (Robinson et al. 2010) and RUVseq (Risso

et al. 2014). We kept only genes with a cpm >1 in at least 4 samples, leaving 7,649 genes in the dataset. After normalizing counts based on library size, a clear batch effect was visible (Fig. S5.2A). We used RUVseq to identify *k*=3 additional variables that were added to the linear model in EdgeR. These variables were estimated by RUVseq based on the residuals from a linear model fitted with only the variable of interest (i.e. "sample", referring to either A, S, AA, AS, SA or SS). Adjusting for three additional unknown variables resulted in improved clustering of samples in a PCA plot (Fig. S5.2B) and improved Pearsons's correlations between replicates of the same sample (data not shown). Contrasts were set up to find 1) transcripts whose expression depends on the presence or absence of the microbiome, 2) mating-responsive transcripts, and 3) transcripts whose expression in females is influenced by the microbiome status of her mate. Transcripts were considered differentially expressed if they changed at least 2-fold and had a Benjamini-Hochberg corrected *p*-value of less than 0.05 (Benjamini and Hochberg 1997). The package ClusterProfiler (Yu et al. 2012) was used for GO Gene Set Enrichment Analysis (GSEA; based on fold changes for all 7,649 genes in the dataset), GO classification and GO enrichment (based only on differentially expressed transcripts, using the full 7,649 genes as a background set). We called a GO category as significantly enriched if it had an adjusted *p*-value ≤ 0.05, based on a minimal gene set of two genes for GO enrichment, and a minimal gene set of 50 genes and maximal gene set of 500 genes for GSEA. DAVID (Huang et al. 2009a, 2009b) and Flybase (Gramates et al. 2017) were used for further functional annotation of genes.

Tissue enrichment calculation

To determine if differentially abundant transcripts had an expression bias to particular tissues, we used female gene expression data (FPKM values) from FlyAtlas 2 (Leader et al. 2018). Enrichment was calculated by dividing the FPKM value of the gene of interest in the tissue of interest, by the FPKM value for that gene in the whole female body. As described on http://flyatlas.gla.ac.uk/FlyAtlas2/index.html?page=help#, we set whole body FPKM values lower than 2 to 2 for the enrichment calculation.

Fertility assay

A fertility assay was performed by measuring the number of eggs produced by axenic (A) and control females (C) that were mated to axenic (AA, CA) or control males (AC, CC). Matings for the egg laying assay were performed as described above, with sample sizes n=49 for AA, n=47 for AC, n=46 for CA and n=50 for CC. These sample sizes exclude females that did not survive or escaped during the assay. At the end of copulation, which occurred in vial 0 (V0), males were removed and females were transferred into a new vial (V1), in which they were allowed to lay eggs for six hours. After six hours, females were moved to V2 for 24 h, then transferred to V3 for 24 h, after which the females were discarded. Each time females were transferred to a new vial, egg number in the previous vial was recorded. Fly food in V0 was prepared as described above for the RNA-seq assay. V1, V2 and V3 contained the same autoclaved food as described above, but without the addition of yeast or fly homogenate. For each time point, we assessed the presence or absence of bacteria in 2-3 pooled flies using PCR for bacterial 16S rRNA. At each time point, bacteria were absent in axenic flies.

Control flies contained bacteria at each time point, but the amount decreased with each transfer onto sterile food that did not contain fly homogenate (Fig. S5.3). The total number of eggs produced by each female was analyzed in R using a generalized linear mixed model with a Poisson distribution (lme4; Bates et al. 2015), with fixed effects for female microbiome status, male microbiome status and their interaction, an observation-level random effect to account for overdispersion (Harrison 2015) and a random effect to account for the person counting the eggs. The package emmeans (https://cran.r-project.org/web/packages/emmeans/index.html) was used to calculate *p*-values for pairwise comparisons between the four treatments (corrected for multiple testing). Count data are available in Table S5.2.

Data availability

Supplementary figures and supplementary table legends can be found in this document. Supplementary tables are provided in a separate electronic folder.

Results

There are many similarities between phenotypes that are influenced by the microbiome and phenotypes that are influenced by mating in *D. melanogaster* females, but they have not been studied concurrently. We investigated whether the female's transcriptome is influenced by interactions between the female's microbiome and her mating status. In addition, we asked whether interactions exist between the microbiome of the female and that of her mate. To this end, we measured female fecundity and

transcript abundance in females with or without a microbiome, six hours after mating to males with or without a microbiome.

Both female and male microbiome status influence egg laying.

Over the course of 54 hours, control females mated to control males (CC) laid more eggs than axenic females, with an average of 77 eggs for CC (standard deviation *sd*=30; *n*=50) versus 48 eggs (*sd*=34; *n*=49) and 53 eggs (*sd*=38; *n*=47) for AA and AC, respectively (*p*-values are respectively <0.0001 and 0.0004; Fig. 5.1). Interestingly, the CC group laid more eggs than CA, which laid on average 51 eggs (*sd*=30; *n*=46; *p*-value = 0.003; Fig. 5.1).

Fifteen females laid fewer than ten eggs over the course of the entire assay (6 females of AA, 6 of AC and 3 of CA). Because it was not checked whether larvae eclosed from these eggs, we are uncertain whether these females had mated. If we excluded these females from the analysis, we still observed a significantly higher fertility in CC relative to all other mating combinations (*p*-values: AA vs. CC = 0.0009, AC vs. CC = 0.02, CA vs. CC = 0.003, all other pairwise comparisons had $p \geq 0.05$). These data suggest that both male and female microbiomes have an effect on fecundity.

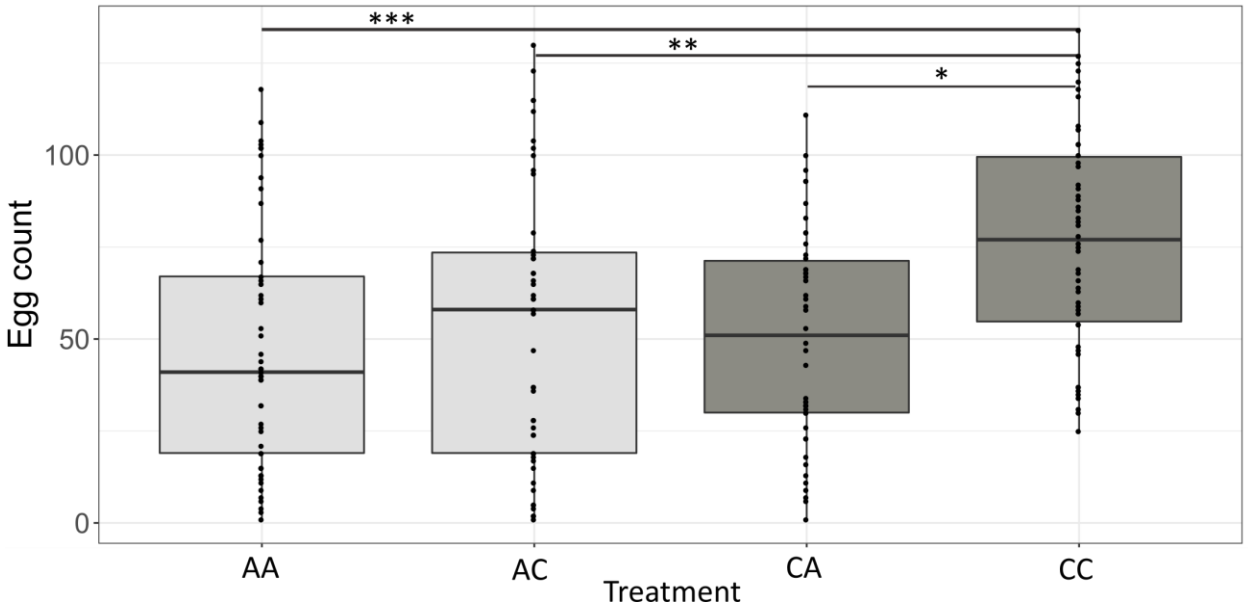


Figure 5.1: Fecundity assay for axenic and control females following a single mating to axenic or control males. Each dot represents eggs laid by one female over the course of 54 h. Boxplots show median eggs produced and 25th and 75th percentiles. A=axenic, C=Control. The first letter refers to the female's microbiome status, the second letter refers to the male's microbiome status. Sample sizes: *n*=49 for AA, *n*=47 for AC, *n*=46 for CA and *n*=50 for CC. Groups were compared using a generalized linear mixed model with a Poisson distribution. *** = $p < 0.0001$; ** = $p < 0.001$; * = $p < 0.01$

Many transcripts differ in abundance between virgin axenic and virgin control females, and the abundance of many additional transcripts is altered once axenic and control females have mated.

We investigated if the microbiome influences a female's transcriptome by directly comparing the transcriptomes of females with a microbiome to those of axenic females. We found 181 transcripts that differed in abundance between virgin females with or without a microbiome (Fig. 5.2A, Table S5.3), while 378 transcripts were differentially abundant between mated females with or without a microbiome, regardless of their mate's microbiome status (Fig. 5.2B, Table S5.3). We will discuss these changes in

transcript abundance in more detail below. The two contrasts described above had 124 genes in common. This suggests that these 124 genes constitute a “core” set of genes that is influenced by the microbiome in all females, regardless of whether they mated or not (Fig. 5.2C). In addition, 57 transcripts were affected by the microbiome only in virgin females, while 254 genes were affected by the microbiome only in mated females (Fig. 5.2C).

Transcripts that are influenced by the microbiome in both virgin and mated females are involved in metabolic processes and the immune response and show a strong expression bias to the midgut.

Among the 124 transcripts that were influenced by the microbiome regardless of whether a female had mated, we found significant enrichment of Biological Process GO terms related to the immune response and carbohydrate, nucleoside, lipid and amino acid metabolism (Fig. S5.4A). Similarly, enriched Molecular Function GO terms included hydrolase, glucosidase, peptidase and lipase and sterol binding activity (Fig. S5.4B). The majority of these transcripts (116/124) were present at higher levels in control females relative to axenic females. These observations are in accordance with studies that showed that axenic flies have altered levels of glucose, trehalose, triglycerides, proteins and insulin-like signaling (Huang and Douglas 2015; Newell et al. 2014; Ridley et al. 2012; Wong et al. 2014; Yamada et al. 2015; Shin et al. 2011). The “core” set of 124 genes had a clear expression bias to the female midgut (Fig. 5.3A) and this result agrees with observations from Broderick et al. (2014), who detected major transcriptome changes in the gut of axenic females, but detected few changes in non-

gut tissues. We tested the difference in RNA levels for two genes, *Mtk* (an antimicrobial peptide) and *Tobi* ("target of brain insulin"; Buch et al. 2008), using qRT-PCR on independently collected samples (with flies bleached and mated at a different time than those used for RNA-seq). For both genes, we were able to validate an up-regulation in control females relative to axenic females, both virgin and mated (Fig. S5.6). Only eight transcripts had a higher abundance in axenic females relative to control females (Fig. 5.2C). Of these, *Pka-R1* caught our attention. *Pka-R1* positively regulates feeding behavior (Hong et al. 2012) and PKA signaling acts downstream of dopamine signals to promote ovarian dormancy (Andreatta et al. 2018).

Finally, 57 transcripts were affected by the presence of the microbiome in virgin females only, but these followed a similar pattern to the 124 "core" microbiome responsive genes (Fig. 5.2C). Specifically, most of the 57 transcripts (51/57) were up-regulated in control virgin females relative to axenic virgin females. These up-regulated transcripts were significantly enriched for peptidases and carbohydrate/ cation transmembrane transporters (Fig. S5.4C). In addition, we identified 10 genes involved in the immune response and three genes involved in reproduction (*tj, Cp36, CG3662*). Only six transcripts were down-regulated in control relative to axenic virgin females. One of these was *takeout*, which links circadian rhythm, starvation and food intake (Sarov-Blat et al. 2000). We tested the transcript levels of *to* using qRT-PCR and verified that it was downregulated in control virgin females relative to axenic virgin females (Fig. S5.6).

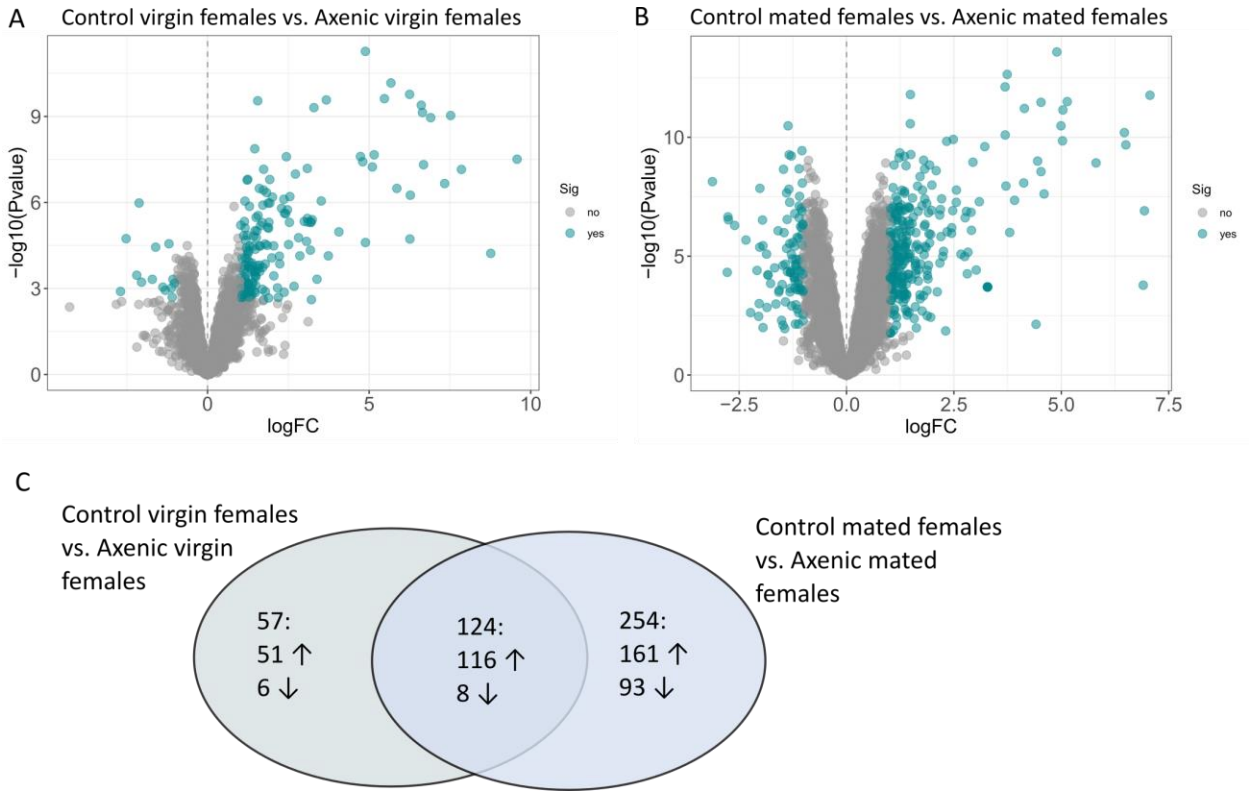


Figure 5.2: Differential expression analysis between females with or without a microbiome. A: Volcano plot showing the results of a differential expression analysis of control virgin females relative to axenic virgin females. B: Volcano plot showing the results of a differential expression analysis of control mated females relative to axenic mated females, at 6h after mating. Females had mated to either a control or axenic male, but male microbiome status was not taken into account for this analysis. Significant genes (in blue) have a *q*-value < 0.05 and at least a 2-fold change in transcript abundance. C: Overlap and differences of genes whose RNA levels are influenced by the microbiome in virgin and mated females. Arrows indicate up- or down-regulation in control vs. axenic females.

Many of the 124 "core" genes influenced by the microbiome in this study were influenced by the microbiome in previously published studies.

The study design we employed is different from the designs used by most published microbiome studies. Specifically, we created control flies with a conventional microbiome by adding homogenate of untreated flies onto the fly food, while most

studies use gnotobiotic flies which carry a limited, curated set of bacterial species that are usually found in the fly gut (Douglas, 2018b). We created control flies to assess the effects of presence/absence of the microbiome, rather than the effects of specific bacteria. In addition, we did not want to omit potential effects of bacteria present in the reproductive tract, which have not been characterized in *D. melanogaster*. Despite the differences in study design, more than half (52%) of our "core" genes were also influenced by the microbiome in at least one other study (Bost et al. 2018; Broderick et al. 2014; Dobson et al. 2016) (Table S5.6). Moreover, these 124 genes were enriched for similar functions, i.e. immune response and metabolic processes (Bost et al. 2018; Broderick et al. 2014; Combe et al. 2014; Dobson et al. 2016). Broderick et al. (2014) also defined a "core" set of 152 genes whose transcript abundance was influenced by the microbiome in the mated female gut, in flies with distinct genotypes (Oregon-R and Canton-S). We found only 11 genes overlapping between our core set and the core set from Broderick et al. (2014). This likely reflects the use of gut versus whole fly, or it could be caused by differences in experimental design. Still, these 11 genes fall into several broad functional classes affected by the microbiome both in this study and in Broderick et al. (2014), including immune and stress response genes (*AttA*, *AttB*, *GstD8*), genes affecting gut structure (*Mur29B*, *CG7017*), metabolism (*Npc2e*, *Acbp6*, *Gba1a*, *CG17192*) and gene expression (*CG15533*).

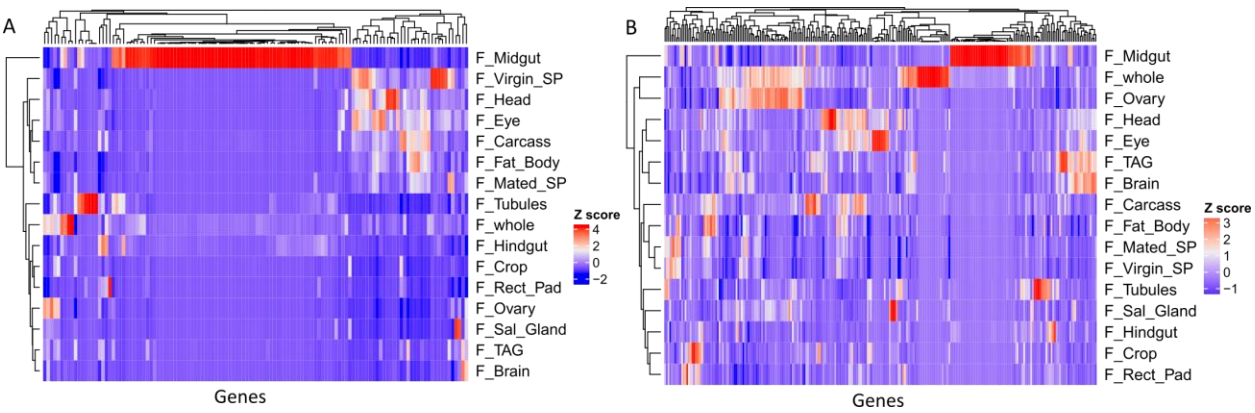


Figure 5.3: Tissue enrichment scores for differentially abundant transcripts across 15 female tissues. A: Heatmap for 124 “core” genes whose RNA levels are influenced by the microbiome in both mated and virgin females. B: Heatmap for 254 genes whose RNA levels are influenced by the microbiome in mated females only. Enrichment scores were calculated using FPKM values from FlyAtlas 2 (Leader et al. 2018). (TAG=thoracico-abdominal ganglion, SP=spermathecae, F_=female, whole=whole body) (A recent report on http://flyatlas.gla.ac.uk/FlyAtlas2/index.html?page=home#mobileTargetG indicated that transcriptome data for the fat body and spermathecae was generated using a different method than what was used for the other tissues, and is therefore not comparable with those other tissues.)

Transcripts of genes involved in reproduction and genes with neuronal functions differ in abundance between mated axenic and mated control females.

We next analyzed the 254 transcripts that differed in abundance only between mated females with or without a microbiome (Fig. 5.2C). These transcripts have an expression bias predominantly to the midgut and the ovary, and to a lesser extent the head, eye and thoracico-abdominal ganglion (Fig. 5.3B). Of the 254 transcripts, 161 transcripts were up-regulated and 93 transcripts were down-regulated in mated control females relative to mated axenic females (Fig. 5.2C).

Among the 161 up-regulated genes we detected GO terms linked with reproduction (29 genes; related GO terms included cell cycle, chromosome segregation, regulation of cell proliferation, stem cell population maintenance and macromolecule

localization). Genes involved in “Chorion-containing eggshell formation” were also significantly enriched based on a Gene Set Enrichment Analysis (Fig. 5.4A). Additional up-regulated genes were involved in metabolism (e.g. the glycogen phosphorylase *GlyP* and the mannosidase *LManV*), the immune response (*Def, PGRP-SC1b, PGRP-SD*) and genes important for the function of (dopaminergic) neurons (*Fer2*, *Catsup, Bx, Atpalpha*)(Fig. 5.4B) and pigment biosynthesis (*yellow-f, bw*). Changes in pigment biosynthesis have been described in axenic flies (Tatum 1939), and this could reflect a sub-optimal metabolism in the absence of bacteria (Broderick and Lemaitre 2012).

Among the 161 genes, the ones with the highest up-regulation had at least a 6-fold higher transcript level in control compared to axenic mated females. These included five members of the histone H4 family (*His4:CG33891, His4:CG33893, His4:CG33895, His4:CG33897 and His4:CG33899*). Histone gene expression occurs during the S phase (reviewed by Kurat et al. 2014). Thus, lower levels of histone transcripts suggest that fewer cells are dividing in axenic females compared to control females. This could reflect a reduction in oogenesis (as described by Elgart et al. 2016) or a reduction in cell proliferation in other tissues, for example in the gut (as described by Broderick et al. 2014, Buchon et al. 2009) in axenic females.

The 93 transcripts with lower abundance in control mated females compared to axenic mated females were significantly enriched for genes involved in sensory perception. These included four genes encoding odorant binding proteins (*Obp8a, Obp44a, Obp56g, Obp57c*)(Fig. 5.4B), genes involved in phototransduction (*Rh5, ninaA and ninaC*) and a cation channel (*trp*). Further, we detected in control mated females a

lower abundance of RNA of three neuropeptides (*Nplp3, Pdf, Capa*)(Fig. 5.4B) and *TpnC4* and *TpnC41C*, which are part of the muscle troponin complex.

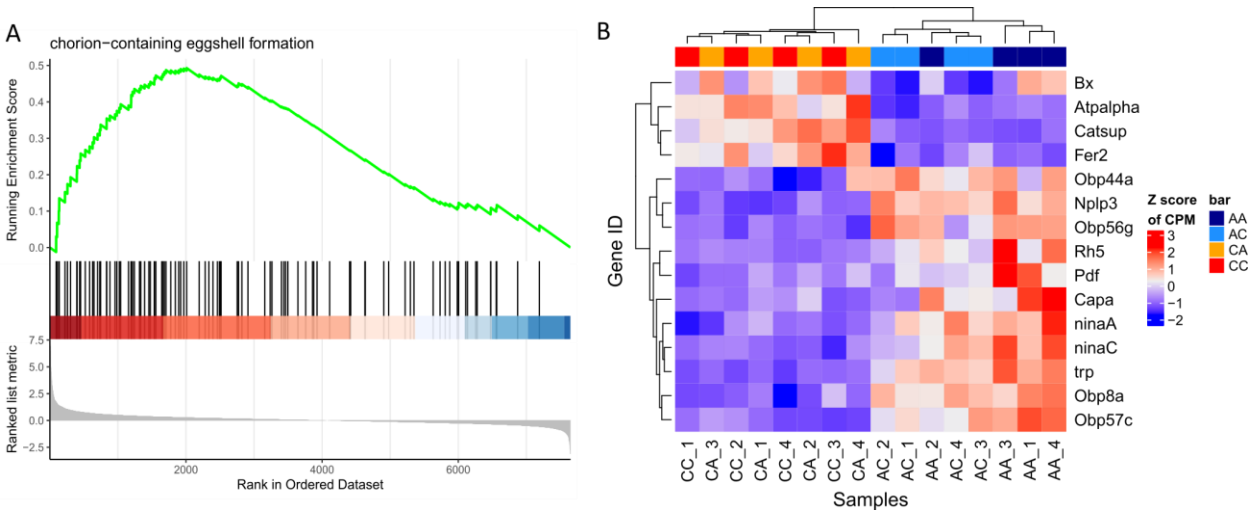


Figure 5.4: Genes whose RNA levels are influenced by the microbiome in mated females only have roles in reproduction and neuronal functions. A: Gene Set Enrichment Analysis plot, showing that transcripts involved in eggshell formation are generally up-regulated in mated females with a microbiome relative to mated females without a microbiome. The top panel of the GSEA plot shows the running enrichment score for genes involved in eggshell formation, moving along the list of ranked genes (genes were ranked based on the $\log_2$ fold change between mated control and mated axenic females, in decreasing order). Each vertical black line represents a single gene involved in eggshell formation. The density of genes involved in eggshell formation is higher among genes with a larger $\log_2$ fold change, as represented by the red bar on the left of the plot, which turns into a blue bar on the right as the density of genes involved in eggshell formation decreases among genes with smaller $\log_2$ fold changes. B: Heatmap showing Z scores of normalized, batch-adjusted counts (CPM; counts per million) for 15 genes with sensory or neuronal functions, whose transcript abundance is altered in mated females depending on whether they have a microbiome or not. A=axenic, C=control. The first letter refers to the female's microbiome status, the second letter refers to the male's microbiome status.

22 genes respond to mating regardless of female microbiome status.

We detected 22 transcripts that were up- or down-regulated after mating in all females, whether axenic or not (Table S5.5). We detected these transcripts by contrasting transcript abundance in mated females with that of the respective virgin females. Mating-responsive genes included three spermathecal serine-type endopeptidases (*Send2*, *CG17239*, *CG17234*), the metallopeptidase *Nep7*, *jhamt*, involved in juvenile hormone synthesis, a maltase, *Mal-B1*, a gene encoding an odorant binding protein *Obp83f*, *wbl*, involved in toll signaling and dorso-ventral patterning, the antimicrobial peptide *Listericin* and *CG14191*, involved in sarcomere function. Using qRT-PCR on independently collected samples, we confirmed the post-mating up-regulation of *jhamt* and down-regulation of *Mal-B1* in both control and axenic females (Fig. S5.6).

Male microbiome status does not impact transcript abundance in females with a microbiome, but impacts the abundance of 136 transcripts in axenic females.

We next studied contrasts that reveal the effect of male microbiome status on the female's transcriptome. When we directly compared transcript abundance in control females mated to control males with that of control females mated to axenic males, we did not detect any differentially-expressed transcripts (Fig. 5.5A). This suggests that the male's microbiome does not affect RNA levels of females that have a microbiome, at six hours after mating. We then contrasted transcript abundance directly between axenic females mated to axenic males (AA) and axenic females mated to control males (AC) and found 136 transcripts that were differentially expressed (Fig. 5.5B, Table S5.4).

Hierarchical clustering of all samples based on the normalized counts for these 136 genes showed that the transcript abundance of these genes was similar across virgin females and mated control females. Mated axenic females formed separate clusters depending on the male they mated with, showing opposite patterns of transcript abundance (Fig. 5.5C). Only 14 transcripts were detected at a higher level in AC vs. AA. These included three immune effectors (*IM18*, *Dro* and *Listericin* (Fig. 5.5D)). The majority (122/136) of the transcripts were present at a lower level in AC vs. AA. These genes had an expression bias to the ovary and midgut, and to a lesser extent the malpighian tubules and head (Fig. S5.5). GO term classification and Gene Set Enrichment Analysis indicated that male microbiome status altered the abundance of transcripts involved in proteolysis, the stress and immune response (17 genes), reproduction (8 genes, among which are *vas* and *jhamt*; Fig. 5.5E and 5.5D) and neuronal functions, in particular ones that have been linked with vision (*Atx2, NinaE*) and motor neurons (*Dsk, TBPH, Bx).*

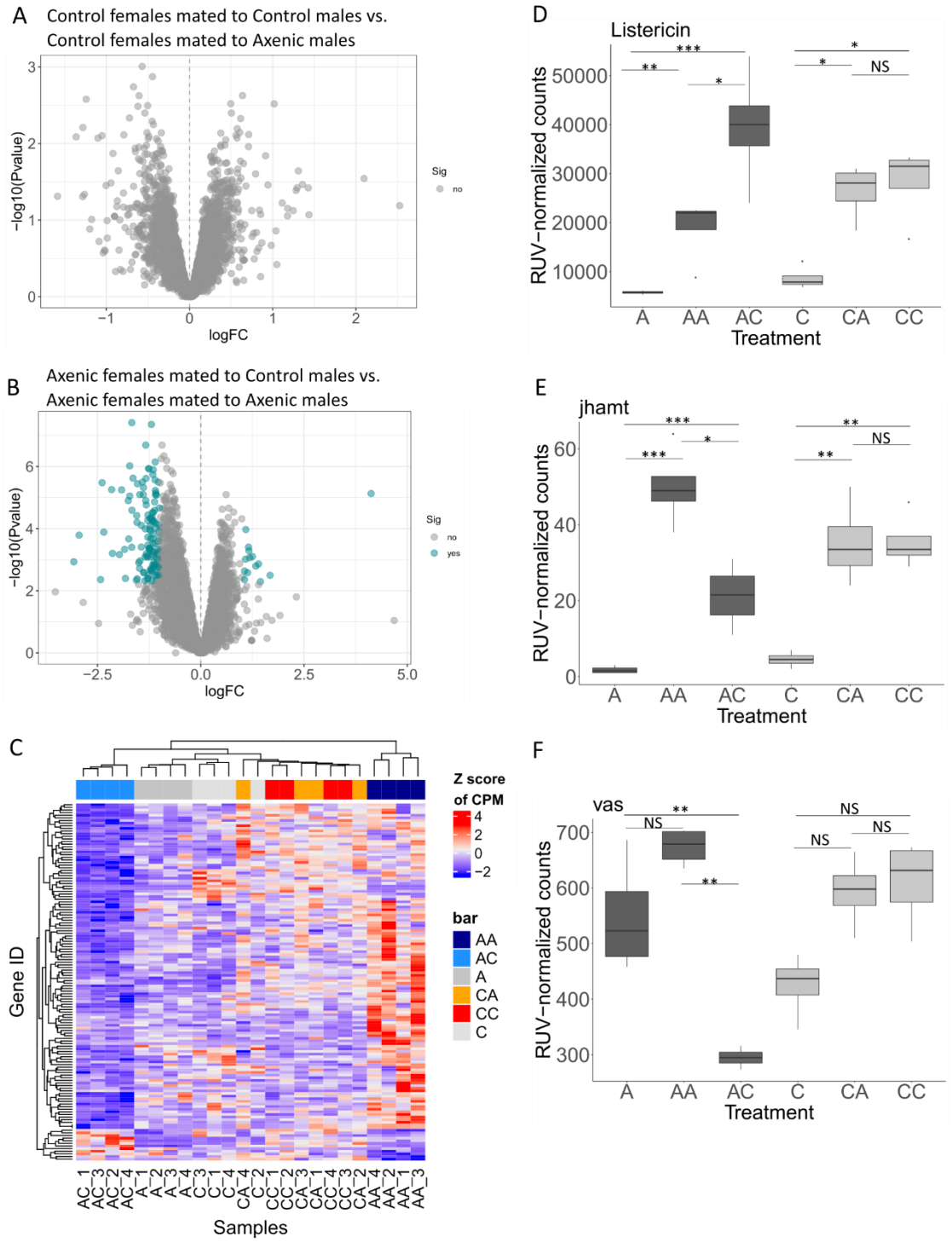


Figure 5.5: Analysis of female transcriptome changes that are influenced by the male's microbiome status. A: Volcano plot showing the results of a differential expression analysis assessing the effect of male microbiome status on control females (nothing significant). B: Volcano plot showing the results of a differential expression analysis assessing the effect of male microbiome status on axenic females (preponderance of

down-regulated genes). C: Heatmap showing Z scores of normalized, batch-adjusted expression values (CPM; counts per million) for genes whose transcript abundance is influenced in axenic females depending on the microbiome status of their mate. D-E-F: Boxplots for three genes, *Listericin, jhamt* and *vas*, whose transcript abundance (shown as normalized, batch-adjusted counts from four replicates) is influenced by the male's microbiome in axenic mated females. *** = $p \leq 0.0001$; ** = $p \leq 0.001$; * = $p \leq 0.01$; A=axenic virgin female, C=control virgin female, AA=axenic female mated to axenic male, AC=axenic female mated to control male, CA=control female mated to axenic male, CC=control female mated to control male.

Female effects versus female x male interaction effects.

Since we observed a strong effect of male microbiome status on the RNA levels of axenic females (influencing transcripts of 136 genes), we went back to our previous analysis in which we compared RNA abundance between control and axenic mated females. For that analysis, we had disregarded male microbiome status and found 378 transcripts that were differentially abundant between control and axenic mated females. We wondered if any of the 378 significant hits were driven by an axenic female x male effect, rather than an overall effect of axenic female, regardless of her mate's microbiome status. We compared the 378 genes with the 136 genes whose RNA levels are influenced by male microbiome status, and found only 20 genes present in both datasets. This indicates that 20 of the 378 genes are subject to a female x male interaction effect (one of the 20 genes is *Bx*, see Fig. 5.4B). The remaining 358 genes have the same RNA levels in axenic females, regardless of their mate's microbiome status.

Discussion

We addressed two main questions in this study: Is a female's transcriptome influenced by interactions between her microbiome and her mating status? And can interactions between the female's microbiome and the male's microbiome influence the female's transcriptome and fecundity? We found evidence for the existence of such interactions and below, we discuss their implications for the female's reproduction and metabolism, neuronal functions and immune gene expression.

The decreased fecundity of axenic females relative to control females is accompanied by a lower abundance of transcripts encoding proteins involved in egg production and is associated with altered transcript levels of metabolic genes.

Using a fecundity assay, we observed that females without a microbiome laid fewer eggs than females with a microbiome. This observation confirms published results (Elgart et al. 2016; Gould et al. 2017; Qiao et al. 2019). Accompanying this decrease in egg laying, we observed a lower RNA abundance of genes involved in egg production in axenic females. This was apparent already in axenic virgin females, which had, compared to control virgin females, lower RNA levels of *Cp36*, which encodes a chorion protein (Cernilogar et al. 2001), and *tj*, which is involved in gonad morphogenesis (Li et al. 2003). After mating, we detected differential abundance of many additional transcripts involved in reproduction. This is likely because mating, and specifically seminal fluid proteins, kickstart egg production (Avila et al. 2011). But where do things go awry after mating in the absence of a microbiome? Clues can be found in observations from both our study and previously published results. First, we observed

an up-regulation of *jhamt* after mating in all females, whether axenic or control. JHAMT is essential for the synthesis of juvenile hormone (JH) (Shinoda and Itoyama 2003). JH is an endocrine factor that among others stimulates the production of vitellogenin and yolk proteins (Bownes 1982). JH production is stimulated by the male seminal fluid protein Sex Peptide (Moshitzky et al. 1996; Bontonou et al. 2015). The observed up-regulation of *jhamt* RNAs suggests that in the absence of a microbiome, signals received during mating still elicit an attempt to initiate oogenesis via JH, but somehow oogenesis is curtailed in the absence of a microbiome. One factor that likely contributes is an altered metabolism in axenic vs. control mated females, which was observed both in our study (using transcript abundance) and previously published studies (using transcript abundance or metabolite measurements) (Bost et al. 2018; Broderick et al. 2014; Combe et al. 2014; Dobson et al. 2016; Elya et al. 2016; Ridley et al. 2012; Wong et al. 2014). Our study took these observations further, by showing that the abundance of RNAs of metabolic genes is lower in the absence of the microbiome both before and after mating. Studies of post-mating responses in *D. melanogaster* females have shown that mating induces metabolic changes and changes in feeding behavior, likely to accommodate the high energy demands of oogenesis (Lee et al. 2013; Uchizono et al. 2017; Reiff et al. 2015; Apger-McGlaughon and Wolfner 2013; Cognigni et al. 2011; McMillan et al. 2018). Without a microbiome, females might be unable to manage the metabolic changes needed to sustain egg production, since even as virgins, their metabolism is severely altered.

The RNA levels of genes involved in olfaction, vision and locomotion are altered in mated axenic females relative to mated control females.

Our data show that the microbiome influences the RNA abundance of genes with neuronal functions. This was also reported by Dobson et al. (2016), but they did not control for female mating status. Our data show that this phenomenon specifically occurred in mated females and not in virgin females. We will discuss below why the RNA levels of these genes could be sensitive to interactions between the female's microbiome and her mating status.

We observed an up-regulation of genes encoding odorant binding proteins and genes encoding components needed for phototransduction in axenic mated females relative to control mated females. One the one hand, several studies have shown that fly olfactory behavior changes in the absence of a microbiome or upon changes in microbial composition (Leitão-Gonçalves et al. 2017; Wong et al. 2017; Fischer et al. 2017; Qiao et al. 2019). (But changes in vision due to the microbiome have to our knowledge not been investigated.) On the other hand, the transcript levels of genes involved in olfaction and phototransduction also change after mating, in *Drosophila* (Dalton et al. 2010; Delbare et al. 2017; McGraw et al. 2008; McGraw et al. 2004), and in honeybees, after exposure to seminal fluid (Liberti et al. 2019). In flies, such changes in sensory genes post-mating could mediate changes in female receptivity to other males (Harada 2008; Matsuo 2007) or aid her in finding suitable sites for egg laying (Gioti et al. 2012; Zhu et al. 2014). Thus, it is not unlikely that sensory post-mating responses are altered depending on whether the female has a microbiome or not. An alternative explanation for the observed changes in sensory genes lies in our

experimental design: to keep flies virgin before the mating assay, we isolated all pupae, axenic or control, in individual vials. The food in vials with control pupae contained whole fly homogenate (to add back a microbiome to these pupae), while the food in vials with axenic pupae contained only sterile PBS. Thus, isolated control adults could have been exposed to other flies' pheromones that were present in the fly homogenate. Isolated axenic adults would have been exposed to other flies' pheromones for the first time when a male was introduced for the mating assay. That could lead to the observed upregulation of odorant binding proteins.

We further observed changes in the RNA levels of two genes with functions in circadian and locomotor behavior (*Bx, Pdf*; Renn et al. 1999; Tsai et al. 2004). In addition, two troponins required for muscle contraction had higher RNA levels in mated axenic females relative to mated control females. *D. melanogaster* female activity levels increase after mating (Isaac et al. 2010) and in the absence of a microbiome (Schretter et al. 2018). Thus, the transcript changes we observed could reflect those changes in locomotion on a molecular level. In addition, we observed changes in the RNA levels of two genes involved in dopamine signaling (*Fer2, Catsup*; Tas et al. 2018; Wang et al. 2011). Dopamine has many effects on fly behavior (Yamamoto and Seto 2014) and the causes and consequences of changes in dopamine signaling cannot be determined based on the current study. But, these results further support that interactions between the female's microbiome and her mating status have a significant impact on RNA levels of neuronal genes.

The transcript abundance of immune genes is influenced by the microbiome and by mating.

Overall, transcripts of immunity genes were down-regulated in axenic females relative to control females, confirming results from other studies (Bost et al. 2018; Broderick et al. 2014; Combe et al. 2014; Dobson et al. 2016; Elya et al. 2016). In addition to this, the transcript levels of some immunity genes were influenced by significant interactions between the female's microbiome and her mating status. *Def, PGRP-SC1b* and *PGRP-SD* were represented by higher RNA levels in control mated females relative to axenic mated females, while this was not the case when comparing virgin females. This indicates that mating elevated the RNA levels of these genes only in the presence of a microbiome in the female. On the other hand, transcripts of the antimicrobial peptide *Listericin* were up-regulated by mating in all females, regardless of whether they or their mate had a microbiome. This observation is particularly interesting because it indicates that some aspect of mating, without the need for microbiota, can increase the RNA levels of this antimicrobial peptide. This aspect of mating could be copulatory wounding (Kamimura et al. 2010; Mattei et al. 2015), or exposure to sperm or seminal fluid proteins (Domanitskaya et al. 2007; Gioti et al. 2012; McGraw et al. 2008; McGraw et al. 2004; Morrow and Innocenti 2012; Peng et al. 2005; Short and Lazzaro 2013). If an axenic female mated to a control male, additional immune transcripts were up-regulated (*IM18*, *Dro*, *Listericin*) relative to when an axenic female mated to an axenic male, indicating that exposure to microbiota during courtship or copulation stimulates an additional up-regulation of immune gene transcripts. *Anopheles* mosquitoes (Segata et al. 2016) have female- and male-specific

reproductive tract microbiomes, but whether *D. melanogaster* have reproductive-tract specific microbiomes that can influence the post-mating up-regulation of immune transcripts, is not yet known.

The female's transcriptome and fecundity are influenced by interactions between female and male microbiomes.

Using RNA-seq data, we did not observe effects of male microbiome status on the transcriptome of control females, but we observed significant effects on the transcriptome of axenic females. We wondered whether exposure to bacteria on the male's cuticle during courtship or mating, or exposure to male excreta, could make the RNA levels of an axenic female more similar to those of a control female. However, at six hours after mating, that does not appear to be the case. The genes whose RNA levels were influenced by the male's microbiome had various functions (including egg production) and were mostly down-regulated after mating with a control male. Perhaps a sudden exposure to bacteria during mating does not make axenic female RNA levels more similar to those of control females because resources are used to initiate an immune response rather than oogenesis. Additional experiments at multiple time points would be necessary to investigate this hypothesis.

Aside from transcript abundance, female fecundity was also influenced by the male's microbiome. Fecundity was lower not only in axenic females, but also in control females that had mated with an axenic male. This indicates that the absence of a microbiome impacts a male's reproductive success. Interactions between a male's reproductive success and his microbiome were also observed by Morimoto et al. (2017),

using gnotobiotic males. Axenic males could differ from control males in pheromone production, or in the production, transfer or quality of seminal fluid proteins or sperm. The reduced fecundity in control females mated to axenic males was not accompanied by transcript level changes in our dataset, perhaps because of the time point measured. It is also possible that egg production is unaffected in control females mated to axenic males, but that they differ from control females mated to control males in how often they ovulate.

To conclude, we have shown, using *D. melanogaster*, that a female's transcriptome is influenced by interactions between her microbiome and her mating status, and that both the transcriptome and fecundity are influenced by interactions between the female's microbiome and that of her mate. Specifically, genes involved in reproduction, neuronal signaling and the immune response were influenced by interactions. Our results demonstrate that it is important to consider whether a female is virgin or whether and when she has mated to better understand and interpret how microbiota are tied into their hosts' fitness.

Acknowledgements

We thank the Cornell Genomics Facility, the Feschotte and Johnson labs at Cornell for sharing equipment, E. Cosgrove and A-M Jaksic for advice regarding RNA-seq analysis with batch effects, J. Bubnell for help with *Wolbachia* detection, A. Douglas for advice regarding rearing of axenic flies and NIH RO1 HD059060 to MFW and AGC for funding.

Supplementary table legends

Table S5.1: Primers used for qRT-PCR assay.

Table S5.2: Count data for the egg laying assay.
V1, V2, V3= vial 1, vial 2, vial 3. OLRE= Observation Level Random Effect. AA= axenic female mated to axenic male; AC= axenic female mated to control male; CA= control female mated to axenic male; CC= control female mated to control male.

Table S5.3: Genes whose transcript abundance is influenced by the microbiome in virgin and mated females.
AA= axenic female mated to axenic male; AC= axenic female mated to control male; CA= control female mated to axenic male; CC= control female mated to control male; A= axenic virgin female; C=control virgin female.

Table S5.4: Genes whose transcript abundance is influenced by male microbiome status, in axenic females.
AA= axenic female mated to axenic male; AC= axenic female mated to control male.

Table S5.5: Genes whose transcripts are responsive to mating in all females, regardless of microbiome status.
Log_2 fold changes represent difference in transcript abundance between mated and resp. virgin females. AA= axenic female mated to axenic male; AC= axenic female mated to control male; CA= control female mated to axenic male; CC= control female mated to control male; A= axenic virgin female; C=control virgin female.

Table S5.6: “Core” set of genes whose expression levels are influenced by the microbiome, and that were found to be affected by the microbiome in previous studies.

Supplementary figures

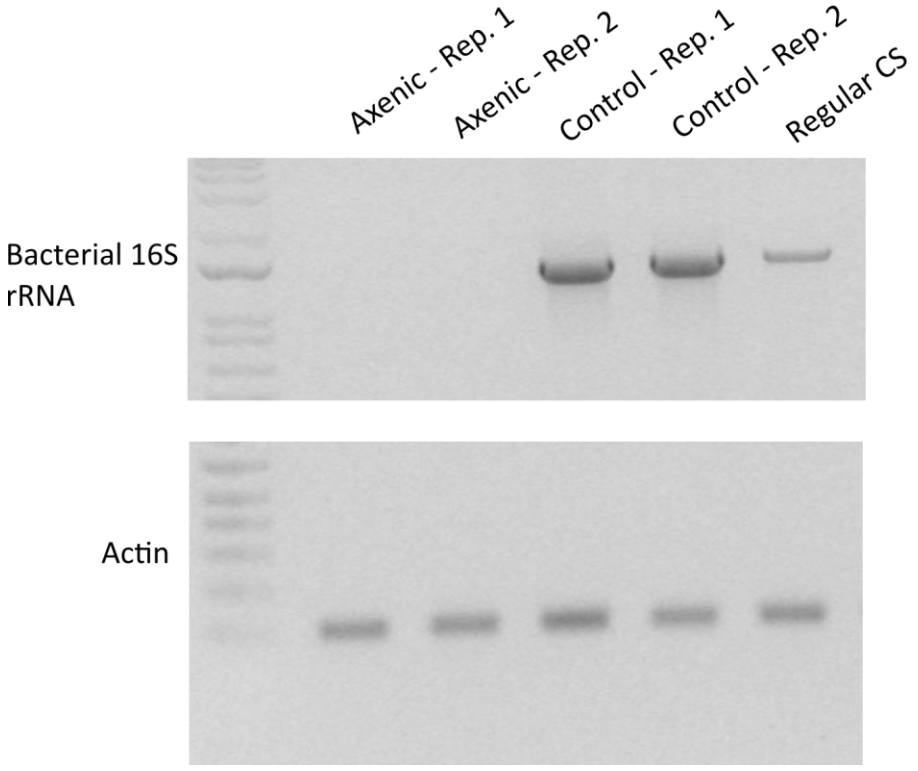


Figure S5.1: PCR assay to verify absence of bacteria in axenic flies and presence of bacteria in control controls. A mix of 9-12 pooled adult females and males was used for each sample on this gel. Axenic = dechorionated Canton-S flies with sterile 1X PBS added to sterile food. Control = dechorionated Canton-S flies with fly homogenate added to sterile fly food. Regular = untreated Canton-S flies. Rep. = Replicate.

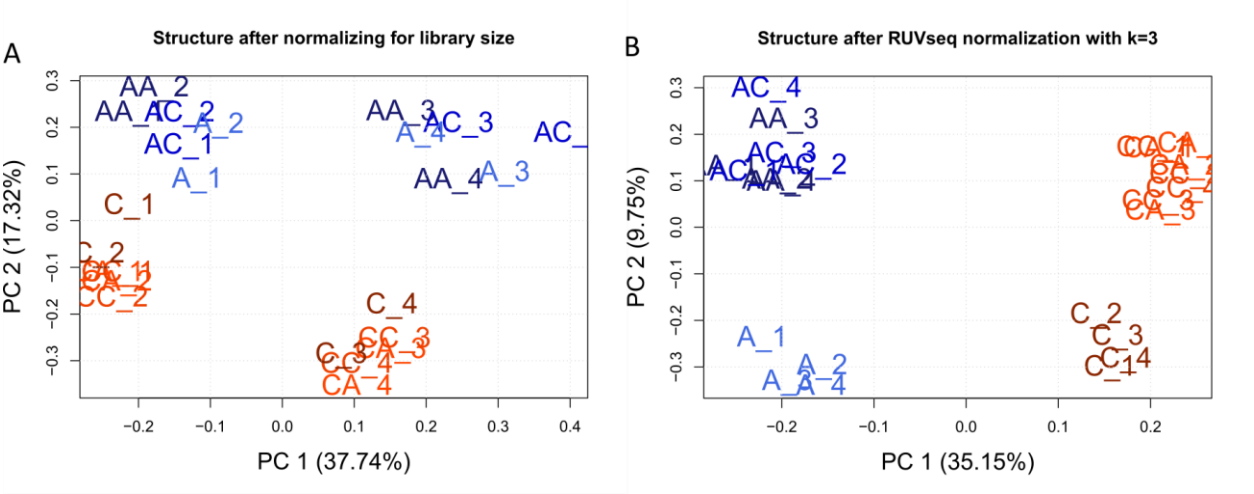


Figure S5.2: Principal Component Analysis of RNA-seq samples. A: Before adjustment for batch effect using RUVseq. B: After adjusting for batch effect using RUVseq. AA= axenic female mated to axenic male; AC= axenic female mated to control male; CA= control female mated to axenic male; CC= control female mated to control male, C=control virgin female, A=axenic virgin female.

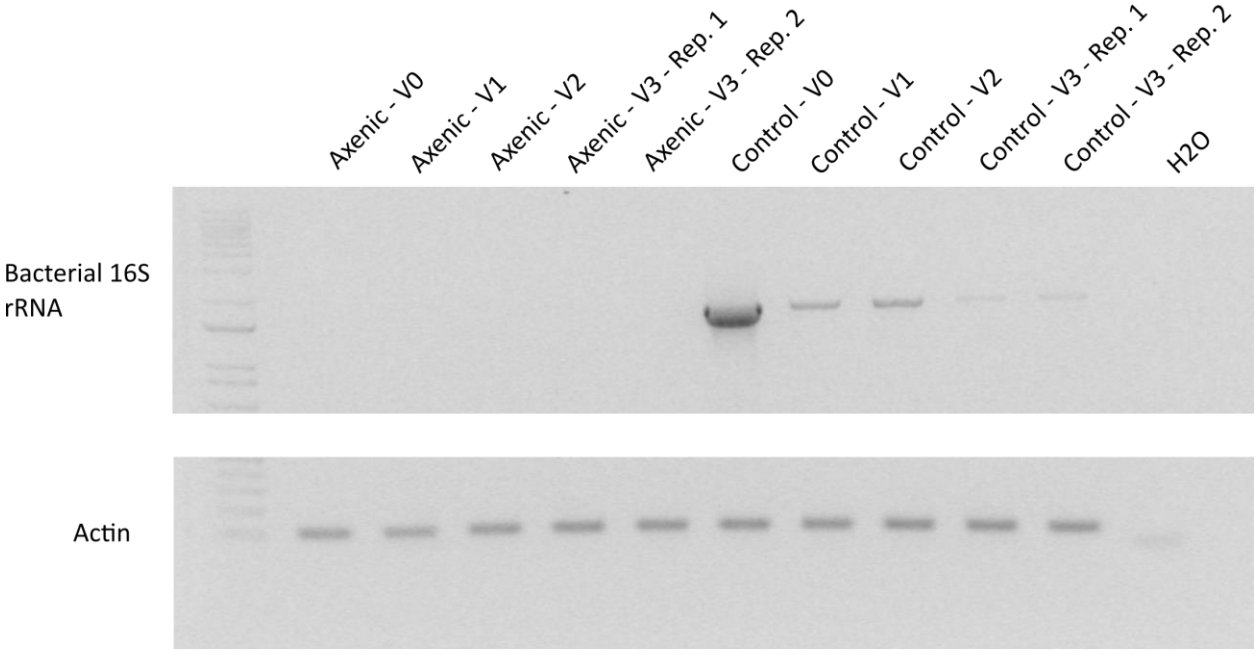
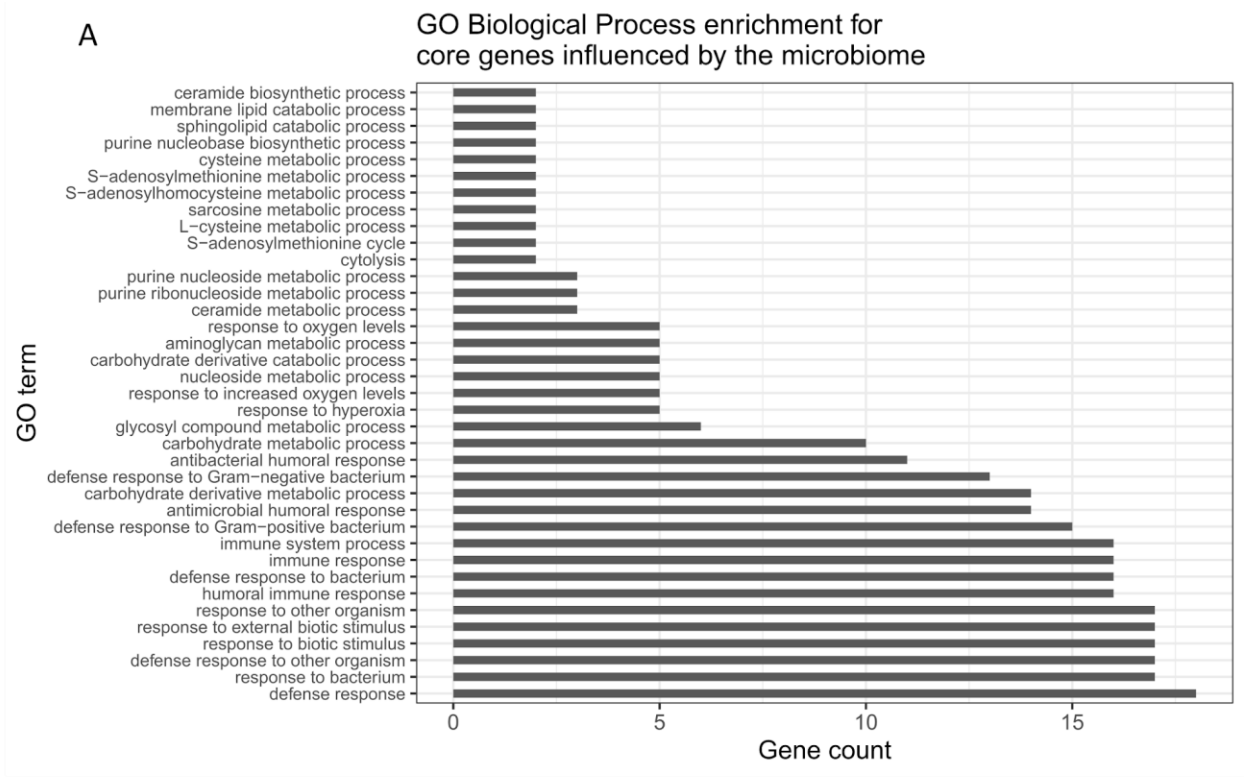
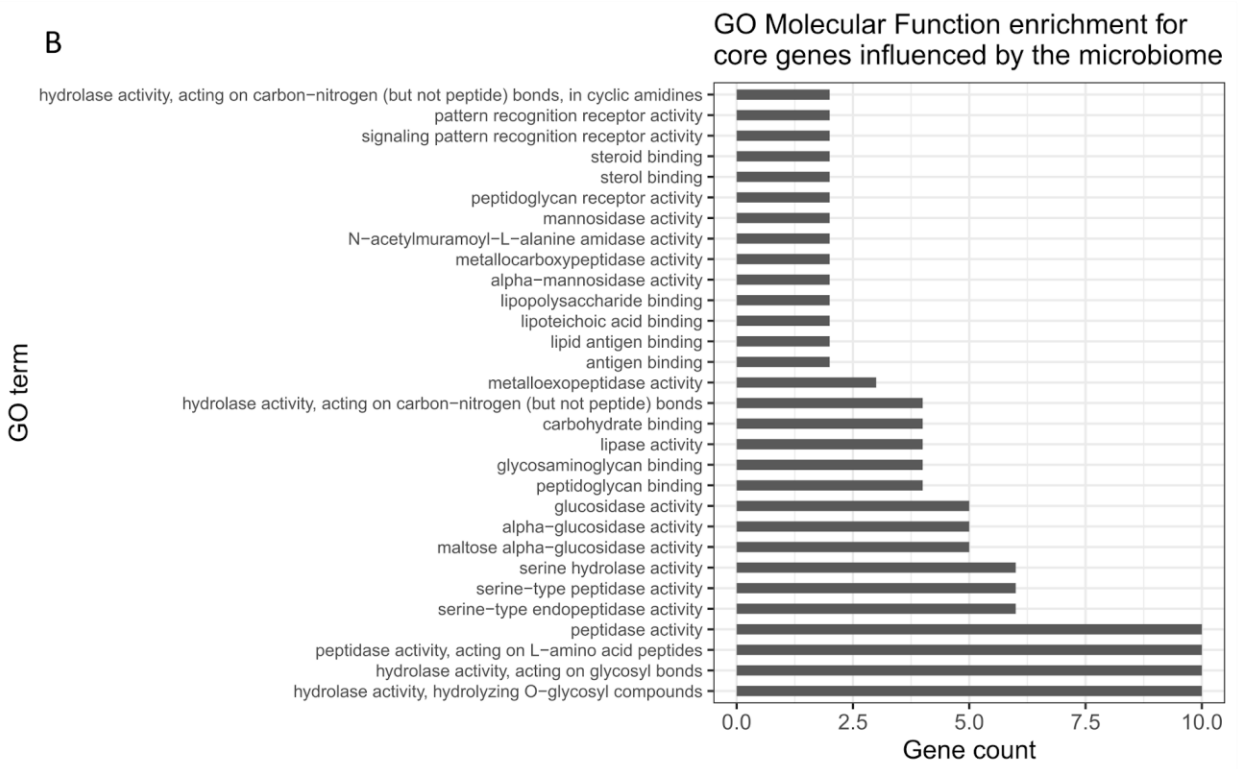


Figure S5.3: PCR assay to verify the presence of bacteria in control flies and absence of bacteria in axenic flies used for the egg laying assay. 2-3 flies were pooled at each time point. V0-3 = vial 0 to 3. Rep. = Replicate.


A
GO Biological Process enrichment for
core genes influenced by the microbiome
GO term
ceramide biosynthetic process
membrane lipid catabolic process
sphingolipid catabolic process
purine nucleobase biosynthetic process
cysteine metabolic process
S−adenosylmethionine metabolic process
S−adenosylhomocysteine metabolic process
sarcosine metabolic process
L−cysteine metabolic process
S−adenosylmethionine cycle
cytolysis
purine nucleoside metabolic process
purine ribonucleoside metabolic process
ceramide metabolic process
response to oxygen levels
aminoglycan metabolic process
carbohydrate derivative catabolic process
nucleoside metabolic process
response to increased oxygen levels
response to hyperoxia
glycosyl compound metabolic process
carbohydrate metabolic process
antibacterial humoral response
defense response to Gram−negative bacterium
carbohydrate derivative metabolic process
antimicrobial humoral response
defense response to Gram−positive bacterium
immune system process
immune response
defense response to bacterium
humoral immune response
response to other organism
response to external biotic stimulus
response to biotic stimulus
defense response to other organism
response to bacterium
defense response
0
5
10
15
Gene count


B
GO Molecular Function enrichment for
core genes influenced by the microbiome
GO term
hydrolase activity, acting on carbon−nitrogen (but not peptide) bonds, in cyclic amidines
pattern recognition receptor activity
signaling pattern recognition receptor activity
steroid binding
sterol binding
peptidoglycan receptor activity
mannosidase activity
N−acetylmuramoyl−L−alanine amidase activity
metallocarboxypeptidase activity
alpha−mannosidase activity
lipopolysaccharide binding
lipoteichoic acid binding
lipid antigen binding
antigen binding
metalloexopeptidase activity
hydrolase activity, acting on carbon−nitrogen (but not peptide) bonds
carbohydrate binding
lipase activity
glycosaminoglycan binding
peptidoglycan binding
glucosidase activity
alpha−glucosidase activity
maltose alpha−glucosidase activity
serine hydrolase activity
serine−type peptidase activity
serine−type endopeptidase activity
peptidase activity
peptidase activity, acting on L−amino acid peptides
hydrolase activity, acting on glycosyl bonds
hydrolase activity, hydrolyzing O−glycosyl compounds
0.0
2.5
5.0
7.5
10.0
Gene count

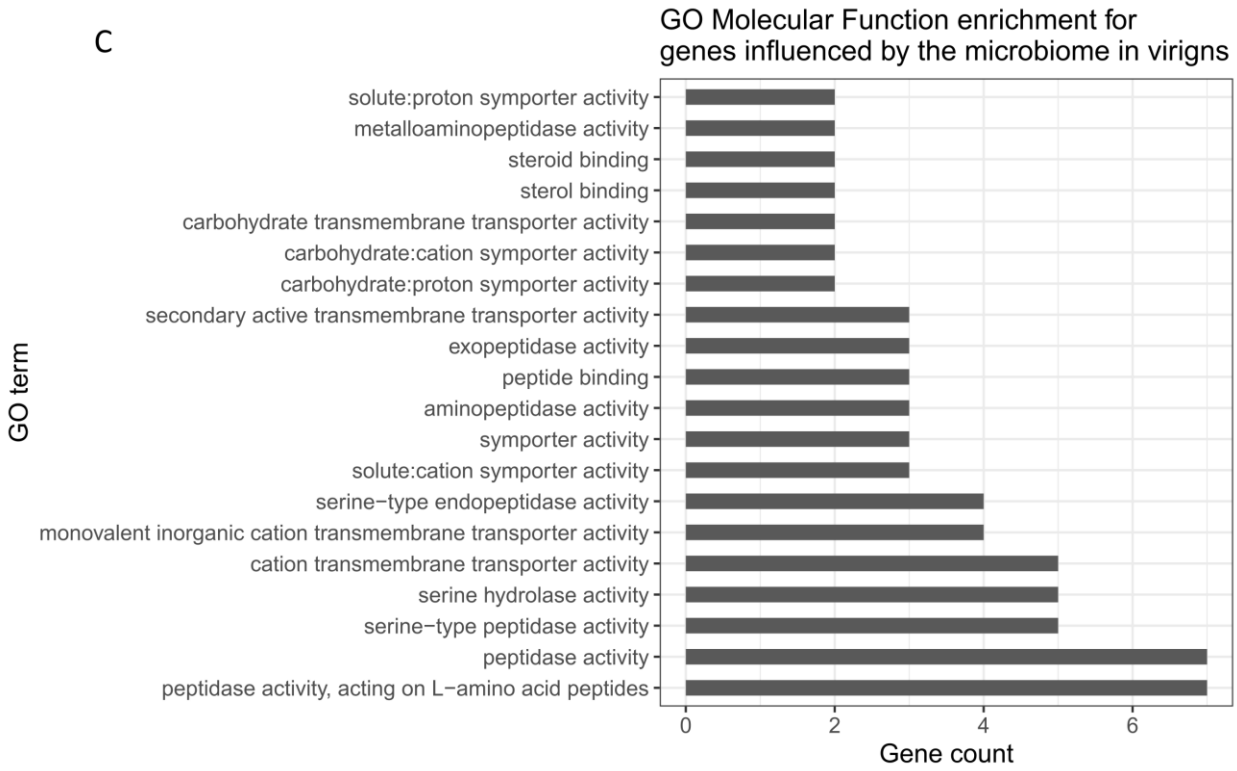


Figure S5.4: GO term enrichment analysis for differentially abundant transcripts. A-B: Enriched GO terms for the 124 “core” genes whose RNA levels are influenced by the microbiome in both virgin and mated females. C: Enriched GO terms for 57 genes whose RNA levels are influenced by the microbiome in virgin females only.

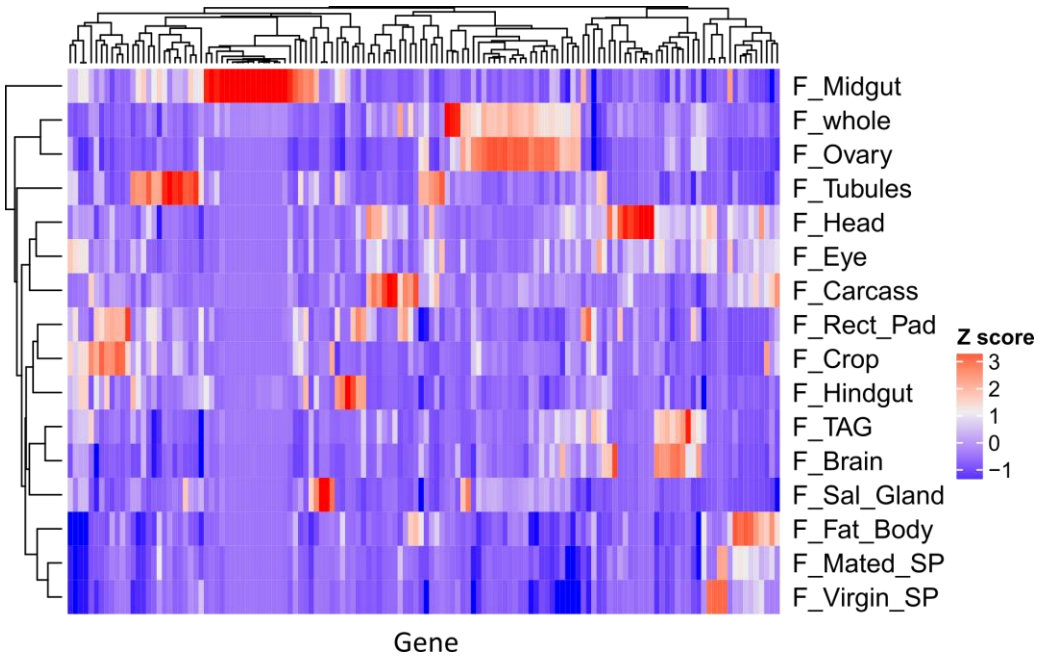


Figure S5.5: Enrichment values across 15 tissues for genes whose transcript abundance is impacted in axenic females by the male's microbiome.Enrichment scores were calculated using FPKM values from FlyAtlas 2 (Leader et al. 2018). (TAG=thoracico-abdominal ganglion, SP=spermathecae, F_=female, whole=whole body) (A recent report on http://flyatlas.gla.ac.uk/FlyAtlas2/index.html?page=home#mobileTargetG indicated that transcriptome data for the fat body and spermathecae was generated using a different method than what was used for the other tissues, and is therefore not comparable with those other tissues.)

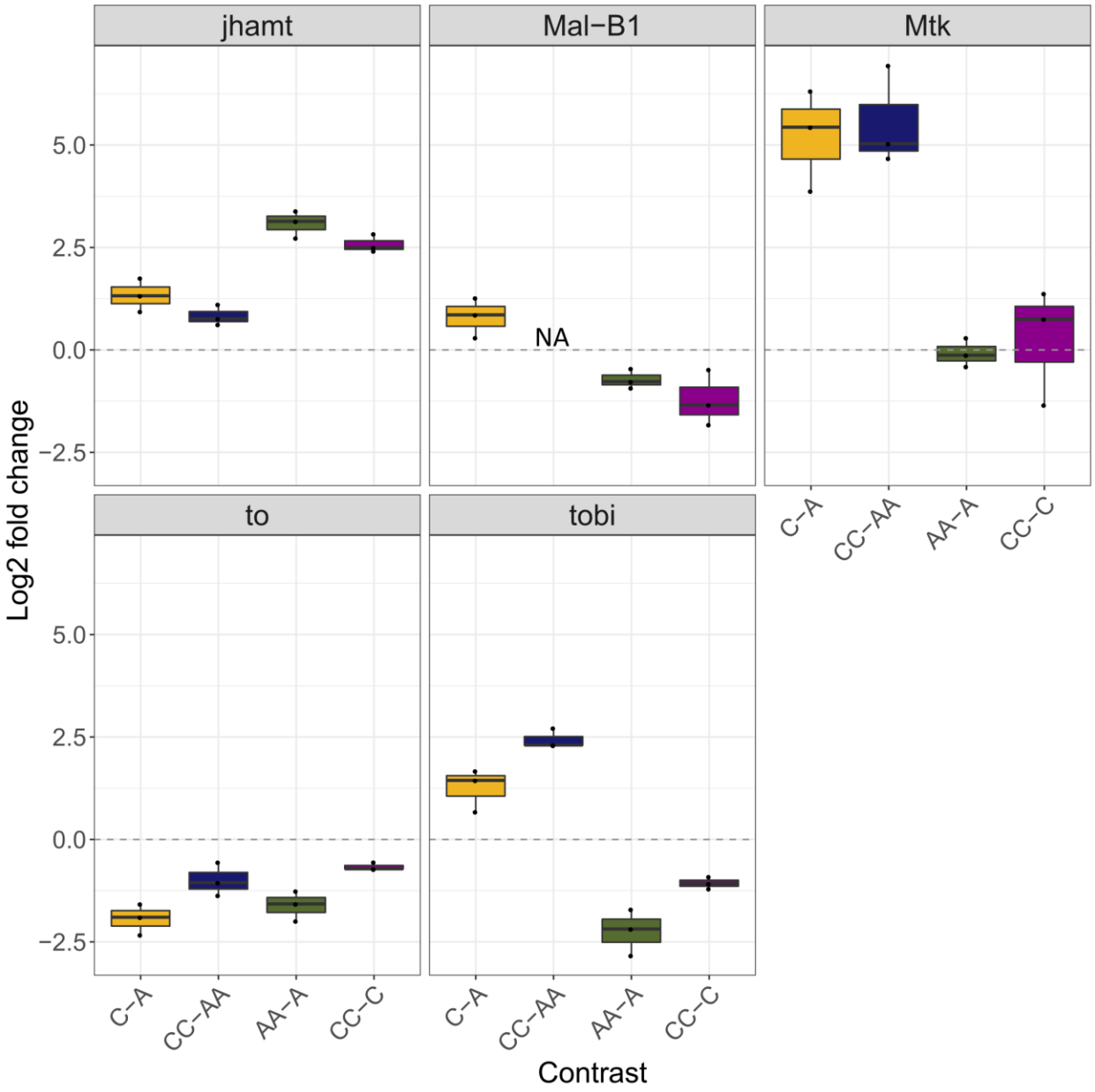


Figure S5.6: qRT-PCR validation of changes in transcript abundance observed in the RNA-seq analysis. C-A= axenic virgin females vs. control virgin females. CC-AA= axenic females mated to axenic males vs. control females mated to control males. A-AA= axenic virgin females vs. axenic females mated to axenic males. C-CC= control virgin females vs. control females mated to control males. Each dot per boxplot represents one biological replicate (10 pooled females), for which we averaged three technical replicates. All expression values were normalized to the housekeeping gene *Nervana*, except for *Mal-B1*, which was normalized against *Rp49*. Since *Rp49* was differentially expressed between mated axenic and control females, we could not use our qPCR data to assess changes in this contrast (NA).

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CHAPTER 6:

DISCUSSION

Studies using *Drosophila melanogaster* have shown that interactions between the genotypes of the female and her mate influence reproductive success, measured in terms of phenotypes such as egg laying, female receptivity to remating and the outcome of sperm competition (Arbuthnott et al. 2014; Chow et al. 2010; Civetta and Clark 2000; Fiumera et al. 2006; Hollis et al. 2019; Pischedda et al. 2012; Zhang et al. 2013; Reinhart et al. 2015; Bjork et al. 2007; Chow et al. 2013; Clark 1999; Clark et al. 1995; Fiumera et al. 2005; Lüpold et al. 2013). Male contributions to these interactions have been well studied, indicating roles for seminal fluid proteins (SFPs) and sperm (Chow et al. 2010; Clark et al. 1995; Fiumera et al. 2005; Reinhart et al. 2015; Yeh et al. 2012; Zhang et al. 2013). Females also contribute to these interactions. This has been demonstrated using isofemale or chromosome extraction lines and GWAS, and using functional studies of SFPs (Laflamme et al. 2014; Findlay et al. 2014; LaFlamme et al. 2012; Ram and Wolfner 2009; Singh et al. 2018; Clark, 1999; Lüpold et al. 2013; Lüpold et al. 2019; Chow et al. 2013). However, research has only scratched the surface when it comes to the female's contributions and molecular responses to female x male interactions that influence reproductive success. The goal of this thesis was to expand our understanding of the female's side of female x male interactions on a molecular level. Below, I will discuss three themes that came forward in the data chapters and that are promising subjects for further study.

A role for the up-regulation of immune gene transcripts in female x male interactions.

Multiple studies have shown that females up-regulate immune genes after mating (Gioti et al. 2012; Kapelnikov et al. 2008a; Mack et al. 2006; McGraw et al. 2008; McGraw et al. 2004; Short and Lazzaro, 2013). This is caused by both sperm and SFPs, and among the SFPs especially expsoure to Sex Peptide and Acp62F increases the RNA levels of immune genes in females (Domanitskaya et al. 2007; Gioti et al. 2012; McGraw et al. 2008; McGraw et al. 2004; Peng et al. 2005). In chapter 2 of this thesis, my colleagues and I showed that some combinations of female and male genotype elicit a stronger post-mating up-regulation of certain immune transcripts (Delbare et al. 2017). In chapter 4 of this thesis, I further showed that shortly after mating, the female's transcriptome reflects a response to copulatory wounding. In chapter 5 of this thesis, my collaborator and I showed that *Listericin* transcripts are up-regulated post-mating even in the absence of a microbiome in both female and male. However, the post-mating abundance of immune transcripts was higher in females with a microbiome compared to germ-free females. Taken together, these results indicate that male ejaculate components are not the only contributors to the post-mating up-regulation of immune gene RNAs. The extent of copulatory wounding can play a role, as can the female's sensitivity to wounding or her sensitivity to the male's ejaculate components, or prior exposure to microorganisms in the environment. Although we have a more complete view of factors that can influence the post-mating up-regulation of immune transcripts, a lingering question is of course whether this response has any fitness consequences for the female and her mate. The work described in this thesis

does not answer that question. However, our findings can be used to design experiments to investigate this question.

In chapter 2, I observed no correlation between the strength of the post-mating up-regulation of immune transcripts at 6 hours after mating, and 5-day egg laying or 4-day female receptivity to remating (Delbare et al. 2017). For future experiments, measuring a more subtle phenotype around 6 hours after mating (e.g. sperm storage or sperm viability) could be more informative. Radhakrishnan and Fedorka (2012) showed that females exposed to peptidoglycan stored less sperm and had more dead sperm in storage, compared to unexposed controls. This indicates that the female's immune system impacts sperm storage and survival, but whether female x male interactions on the female's post-mating up-regulation of immune RNAs can influence these processes is now known. To investigate this, flies from the Global Diversity Lines (Grenier et al. 2015) could be used, especially combinations of females and males that were shown to elicit a stronger or less strong post-mating up-regulation of immune transcripts, to assess if correlations exist between post-mating immune gene RNA levels and sperm storage or sperm viability. Alternatively, heterospecific mating combinations could be used. If such correlations exist, CRISPR could be used to modify the expression of immune genes in those backgrounds, to test if that influences sperm storage or survival.

A role for the female's nervous system in female x male interactions.

A second pattern that came forward in the chapters presented here is that the female's nervous system, neuronal genes and genes involved in sensory perception are particularly sensitive to female x male interactions. Mating is known to influence the

transcript levels of genes encoding certain odorant binding proteins in females (Lawniczak and Begun 2004; McGraw et al., 2008; McGraw et al. 2004). In chapter 2, my colleague and I showed that the magnitude of mating-induced changes in RNA levels of genes involved in sensory perception (e.g. encoding odorant binding proteins and an olfactory receptor) differs depending on female x male genotype interactions (Delbare et al. 2017). Whether these changes in RNA levels are due to mating itself or exposure to pheromones during courtship is not known.

Further, many studies have established the involvement of neurons in regulating female receptivity, egg laying, sperm storage and sperm competition (Avila et al. 2012; Chow et al. 2013; Häsemeyer et al. 2009; Rezával et al. 2014; Rezával et al. 2012; Rubinstein and Wolfner 2013; Yapici et al. 2008). Specifically the action of three genes expressed in female *ppk*$^{+}$ neurons was shown to alter the paternity success of two competing males (Chow et al. 2013). As described in chapter 3, my colleagues and I built upon results from Chow et al. (2013) to identify five additional genes that influence the outcome of sperm competition when knocked down in the female nervous system, and one of these influenced sperm competition when knocked down in *tdc2*$^{+}$ neurons (and two additional candidates had time-specific effects when knocked down in *tdc2*$^{+}$ neurons; Chen, Delbare, White et al. 2019). Together with earlier results, the results presented in this thesis indicate that the female's nervous system is an important interface between signals from the male and post-mating responses. But, many aspects of this interaction call for further investigation.

First, it is not known whether the genes identified in Chow et al. (2013) and in chapter 3 are themselves directly involved in sperm competition, or whether the neurons

in which they act are involved. This could be tested by silencing or activating (subsets of) *ppk*$^{+}$ and *tdc2*$^{+}$ neurons in the female and measuring the effects on P_1. Second, we do not know the mechanisms underlying changes in P_1. Counting sperm inside the reproductive tract of females with impaired function of these genes or neurons, at multiple time points after single and double mating, can inform on changes in sperm storage, ejection, displacement or use. Third, we do not know whether the action of these genes or neurons influences P_1 due to the genotypes of the males or due to the order in which males mate. It is possible that variable interactions between the female's nervous system and components of the male's ejaculate influence the intensity of signaling in the female nervous system, which could in turn influence (for example) the intensity of muscle contractions that mediate sperm movements in the female reproductive tract. On the other hand, the genes and neurons identified by Chen, Delbare, White et al. (2019) and Chow et al. (2013) could influence paternity success depending on whether a male is 1st vs. 2nd to mate, regardless of his own or his competitor's genotype. For most genes tested, gene knockdown in females lowered P_1 (Chow et al. 2013; Chen, Delbare, White et al. 2019). It is possible that gene knockdown lowered the signaling capacities of neurons, leading to a weaker response to the 1st mating, and a stronger response to the 2nd mating, which exposes the female to a 2nd dose of male ejaculate components. Related to this, it has not been investigated whether the nervous system of a virgin female responds differently to mating than the nervous system of a previously mated female. This is not unlikely, since there is evidence, based on transcript changes described in chapter 4 and in Prokupek et al. (2009), and based on studies by Heifetz et al. (2014), Heifetz and Wolfner (2004),

Rubinstein and Wolfner (2013) and Kapelnikov et al. (2008b) that mating induces remodeling of the neurons that innervate the female's reproductive tract. To determine if the genes and neurons we and Chow et al. (2013) identified influence P_1 based on male genotype or male mating order, sperm competition experiments can be performed in which competing males are tested in both the defense and offense positions. (Defense being when the male is first to mate and must resists sperm displacement, offense being when the male is second to mate and must displace stored sperm.) Ideally, these experiments are performed using females and males with diverged genotypes, which are more likely to manifest female x male interactions. Whether male genotype or mating order are responsible for the observed differences in P_1, both scenarios offer the opportunity for further study of interesting biology, including the study of post-mating pre-zygotic mechanisms involved in reproductive isolation (Castillo and Moyle 2014; Manier et al. 2013).

A potential role for male-derived RNAs in female x male interactions.

In chapter 4 I described evidence that *D. melanogaster* males transfer RNAs to females during mating. These results could have implications for female x male interactions, if any noncoding male RNAs have the potential to regulate the female's gene expression, or if male coding RNAs are translated inside the female. These interactions could occur within female tissues if male RNAs are taken up into female somatic cells, but, based on results from Fischer et al. (2012), they could also occur between female and male gametes. However, the results presented in this thesis are preliminary and different experimental approaches are needed i) to fully characterize the

RNAs that are present in the male's ejaculate, ii) to determine if they are inside cells, vesicles or sperm in the ejaculate (e.g. are the RNAs in the ejaculate sensitive to RNases alone, or to RNases and detergent? - Similar to experiments done in human seminal fluid by Vojtech et al. (2014)), iii) to determine from which tissue in the male reproductive tract the RNAs originate (e.g. by using males that do not transfer sperm or have ablated accessory gland cells, as in McGraw et a. 2004), and iv) to determine where they localize in the female after mating (e.g. by tagging candidate RNAs and performing PCR on female somatic tissues or very young embryos, as in Fischer et al. (2012)). Since Corrigan et al. (2014) observed that *D. melanogaster* secondary cell-derived exosomes fuse with sperm inside the female reproductive tract, and since research in mice showed that the fusion of male somatic vesicles with sperm changes the sperm's RNA content (which is important for embryo implantation) (Conine et al. 2018; Sharma et al. 2018), it would be especially interesting to test if the RNA content of *D. melanogaster* sperm changes between what is observed in sperm from male seminal vesicles, vs. sperm that has been transferred to the female reproductive tract.

The study of tissues and molecules involved in female x male interactions can help in the identification of cryptic female choice.

Many female post-mating responses in *D. melanogaster* can be an element of sexual conflict (Sirot et al. 2015). Post-copulatory sexual selection is expected to act on genes in males to optimize the storage and survival of their sperm, and minimize competition, while selection is expected to act on genes in females to let them exert control over these processes (Sirot et al. 2015; Birkhead and Pizzari 2002; Eberhard

1996; Eberhard 2009). *D. melanogaster* females with a masculinized nervous system have sperm storage defects (Arthur et al. 1998). In addition, females with different genotypes differ in their timing of sperm ejection, which in turn influences the number of sperm stored, or displaced (Lüpold et al. 2013; Lüpold et al. 2019). This suggests that females have the means to control sperm storage in *D. melanogaster*. However, to validate that this is a form of cryptic female choice, it is necessary to establish a molecular mechanism that females use to assess males (typically in a competitive context), and that this mechanism determines, for example, the timing of sperm ejection, or differences in sperm storage (Eberhard, 2009; Firman et al. 2017). Complete evidence for cryptic female choice has not been presented in *D. melanogaster*, but the identification of genes and neurons involved in female x male interactions will be important in future work to shed light on the role of cryptic female choice in reproduction.

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APPENDIX:

CONSPECIFIC SPERM PRECEDENCE

Background

Crosstalk between female and male molecules is crucial for reproduction. Because of this, it has been hypothesized that the divergence of reproductive molecules, due to sexual selection or drift, can contribute to reproductive isolation (Kirkpatrick et al. 2002). Reproductive isolation can act on several levels. Males and females can be reproductively isolated through pre-mating isolation, for example when pheromones or courtship behavior are incompatible (reviewed in Marie Curie SPECIATION Network et al. 2012). Reproductive isolation can also manifest itself post-mating on the level of gamete interactions and zygote viability (Marie Curie SPECIATION Network et al. 2012). A final type of reproductive isolation acts at the level of processes that occur post-mating, but before a zygote is formed (post-mating pre-zygotic, or PMPZ). A type of PMPZ reproductive isolation is conspecific sperm precedence (CSP). CSP occurs in a variety of species (many insects but also some vertebrates, e.g. rabbits; Howard et al. 1998). When pre-mating isolation is incomplete, females who mate multiply can mate with both males of their own species ("conspecifics") and males of a different species ("heterospecifics"; Howard et al. 1998). If this happens, the conspecific male will always be more successful than the heterospecific male, regardless of the order of mating (Howard et al. 1998). In *Drosophila*, processes that are act during sperm competition between males of the same species (e.g. sperm displacement and timing of sperm ejection by the female;

Manier et al. 2010; Manier et al., 2013a) also act during conspecific sperm precedence (Manier et al., 2013b). Besides mechanisms, molecules are also shared between sperm competition and conspecific sperm precedence, supporting the hypothesis that divergence in reproductive molecules can contribute to reproductive isolation. Several seminal fluid proteins in *D. melanogaster* were shown to be important for a male to be a successful competitor both against a male of this own species and against a male of a different species (Castillo & Moyle 2014; Civetta & Finn 2014; Levesque et al. 2010). However, the role of female molecules involved in this process is undetermined.

We hypothesize that female molecules that influence the outcome of sperm competition can also influence the outcome of CSP. We focus specifically on CSP between *D. melanogaster* females and males and *D. simulans* males. One candidate gene was tested here, *Rim. Rim* encodes a Rab-interacting molecule required for efficient synaptic transmission in the nervous system (Graf et al. 2012). Chow et al. (2013) previously demonstrated that knockdown of *Rim* in *D. melanogaster* female ppk^{+} neurons led to a decrease in the proportion of offspring sired by the first male to mate (P_1) in sperm competition assays.

Methods

Fly husbandry

To knock down *Rim* (“KD”) in the female ppk^{+} neurons, a Rim UAS-RNAi line (VDRC ID #39384) was crossed to a *ppk-GAL4* line. Since the UAS-RNAi line was made in a w^{1118} background, a w^{1118} line was crossed to *ppk-GAL4* to obtain control females for the CSP experiment. *D. melanogaster* males used for the CSP experiment

carried a protamine-B-RFP marker in their sperm (Manier et al. 2010). The *D. simulans* males used for this experiment carried a protamine-B-GFP marker in the sperm and expressed GFP in the ocelli and pseudopupil (Manier et al. 2013b).

Next to a CSP assay, we also conducted an assay to compare the heterospecific mating rate between two *D. simulans* lines. *D. simulans* lines used for this assay were a mutant *ppk23* line from Seeholzer et al. (2018) and a wildtype *D. simulans* line from Castillo and Moyle (2014). The *D. melanogaster* females used in this assay were either from the w^{1118} or *AttP* background. All flies were kept in a 12h light/dark cycle at room temperature or 25°C, on glucose-yeast food.

CSP experimental setup

On the first day of the experiment, 3-5 day old KD and control females were mated to *D. simulans* males in mass matings overnight, in vials with 10 females and 20 males. On the morning of day 2, *D. simulans* males were removed and *D. melanogaster* females were isolated in individual vials (“V0”). In the afternoon of day 3, each female was transferred to a new vial (“V1”), which contained two 3-5 day old *D. melanogaster* males. Females were given time overnight for a second mating. The morning of day 4, both *D. melanogaster* males were removed. On day 5, females were transferred to V2, and then were transferred every other day up until V5. After spending 48 hours in V5, females were flash frozen for sperm counts. Once the offspring had eclosed, females offspring were separated from male offspring (since male hybrid offspring are not viable). Female offspring that were sired by the *D. simulans* male have a GFP marker in

the ocelli and pseudopupil. Based on this marker, the number of hybrid vs. conspecific female offspring was recorded. All counting was performed blinded.

Sperm counts

To count sperm, the female reproductive tract was dissected on ice in ice cold 1X PBS. Spermathecae and seminal receptacle were separated from the reproductive tract and mounted in 1X PBS on a microscope slide to count GFP- and RFP-labeled sperm in both sperm storage organs.

Comparison of heterospecific mating rates between D. simulans strains.

We compared how likely *D. melanogaster* females were to mate with either mutant *ppk23 D. simulans* males (Seeholzer et al. 2018) or wildtype *D. simulans* males (Castillo and Moyle 2014). Virgin *D. melanogaster* females were 3-5 days old, *D. simulans* males were 3-6 days old. Mass matings were set up at a female:male ratio of 1:2, for 24h. After 24h, females were isolated in individual vials which were checked for larvae after two days, to determine if a heterospecific mating had taken place or not.

Statistical Analysis

To analyze the results of the CSP experiment, P_1 was calculated by dividing the number of female offspring sired by the *D. simulans* male by the total number of female offspring sired by both males, in vials 2, 3, 4, and 5. We omitted earlier vials from the analysis, since V2 is the first vial in which the female resides for 48h after both matings had taken place. P_1 was compared between control and KD females using a linear

model (lme4; Bates et al. 2015), in which “vial” (V2-V5), “treatment” (female KD or control) and their interaction were used as fixed effects. Female ID was added as a random effect.

To analyze sperm counts, we used four Wilcoxon tests, to assess if the genotype of the female (KD or control) influenced 1) RFP sperm counts in the spermathecae, 2) GFP sperm counts in the spermathecae, 3) RFP sperm counts in the seminal receptacle and 4) GFP sperm counts in the seminal receptacle. We corrected the four resulting *p*-values for multiple testing using a Benjamini Hochberg correction (Benjamini and Hochberg 1997). No statistical test was done on the results of the heterospecific mating rate assay.

Results & Discussion

The paternity success of the first male to mate is lower when mating to a Rim knockdown female vs. a control female.

For the CSP experiment, we retrieved 39 doubly mated KD females and 37 doubly mated control females. We observed a significant effect of female genotype across vials 2-5, with a significantly lower P_1 for KD females compared to control females ($p = 0.0008$; Fig. 1). These results show that *Rim* knockdown in the female lowers the paternity success of the heterospecific *D. simulans* male. The change in P_1 observed in the CSP experiment is in the same direction as what was observed in the sperm competition experiment (Chow et al. 2013). This suggests that *Rim* knockdown always affects the first male’s paternity success, regardless of that male’s genotype. It is

unlikely that *Rim* function in the *ppk*$^{+}$ neurons is required for the recognition of a compatible male. Otherwise, we would expect the heterospecific P_1 to increase upon *Rim* KD.

At the end of the CSP assay, Rim knockdown females have more 2nd male sperm in their spermathecae compared to control females.

We counted *D. simulans* (1st male, GFP-labeled) and *D. melanogaster* (2nd male, RFP-labeled) sperm in the female's sperm storage organs, 10 days after the second mating took place (at the end of the CSP experiment). We found significantly more RFP-labeled sperm in the spermathecae of *Rim* KD females vs. control females (*p*-value corrected for multiple testing = 0.008), but observed no differences in the seminal receptacle, and observed no differences for GFP-labeled sperm (Fig. 2). Thus, while *Rim* KD females produced more offspring from the 2nd male over the course of 8 days, they still had more 2nd male sperm in storage after those 8 days, compared to control females.

Comparison of heterospecific mating rate between D. simulans strains.

To determine if the heterospecific mating rate differed between wildtype *D. simulans* males and mutant *ppk23 D. simulans* males, we set up heterospecific matings at a ratio of one *D. melanogaster* female to two *D. simulans* males. After 24 hours, 16/52 *w^{1118}* females had mated with a mutant *ppk23 D. simulans* male and 11/79 *AttP* females had mated with a mutant *ppk23 D. simulans male,* while 0 females had mated to a wildtype *D. simulans* male. Seeholzer et al. (2018) reported that mutant *ppk23 D.*

simulans males court *D. melanogaster* females more than wildtype *D. simulans* males. Our experiment indicates that this increased courtship also translates into a higher heterospecific mating rate. Based on this result, future CSP experiments will benefit from using the mutant *ppk23 D. simulans* male as heterospecific male.

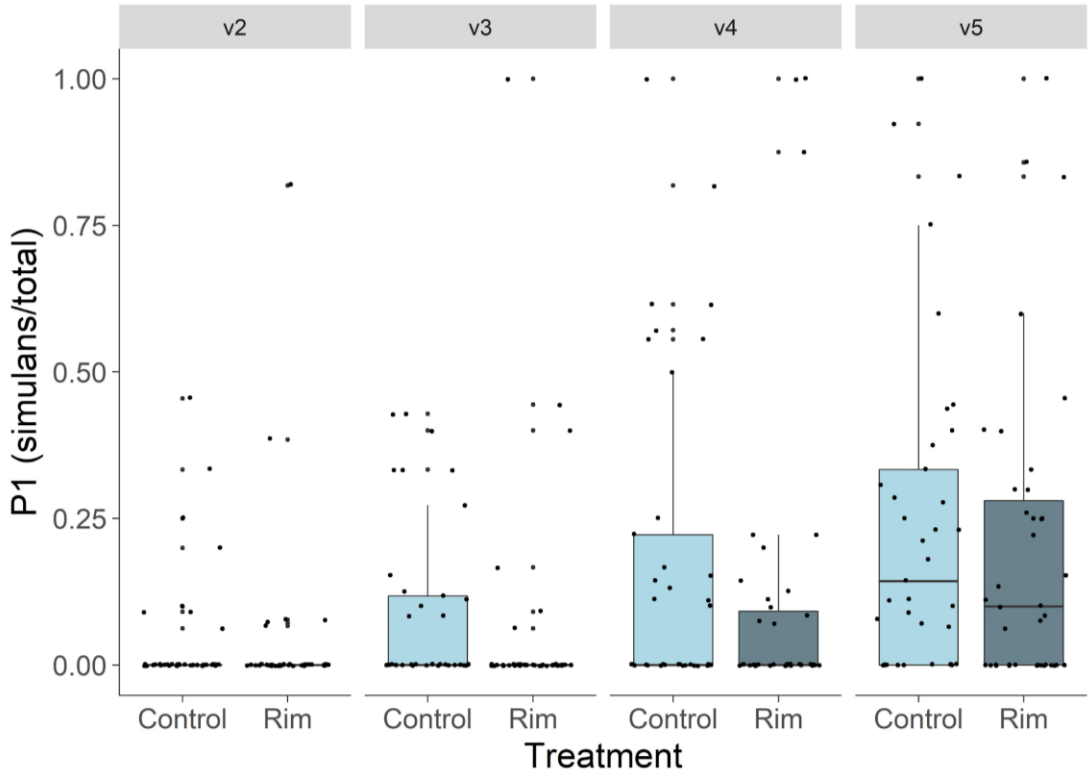

Figure 1: Conspecific Sperm Precedence experiment using *Rim* knockdown and control females. *Rim* knockdown and control *D. melanogaster* females were mated first to a *D. simulans* male, second to a *D. melanogaster male.* Female offspring was counted over the course of 8 days (vial v2 to vial v5). P_1, the proportion of heterospecific offspring, was analyzed using a linear mixed model. This model indicated that P_1 differed significantly, across all vials, depending on female genotype (*p*-value = 0.0008).

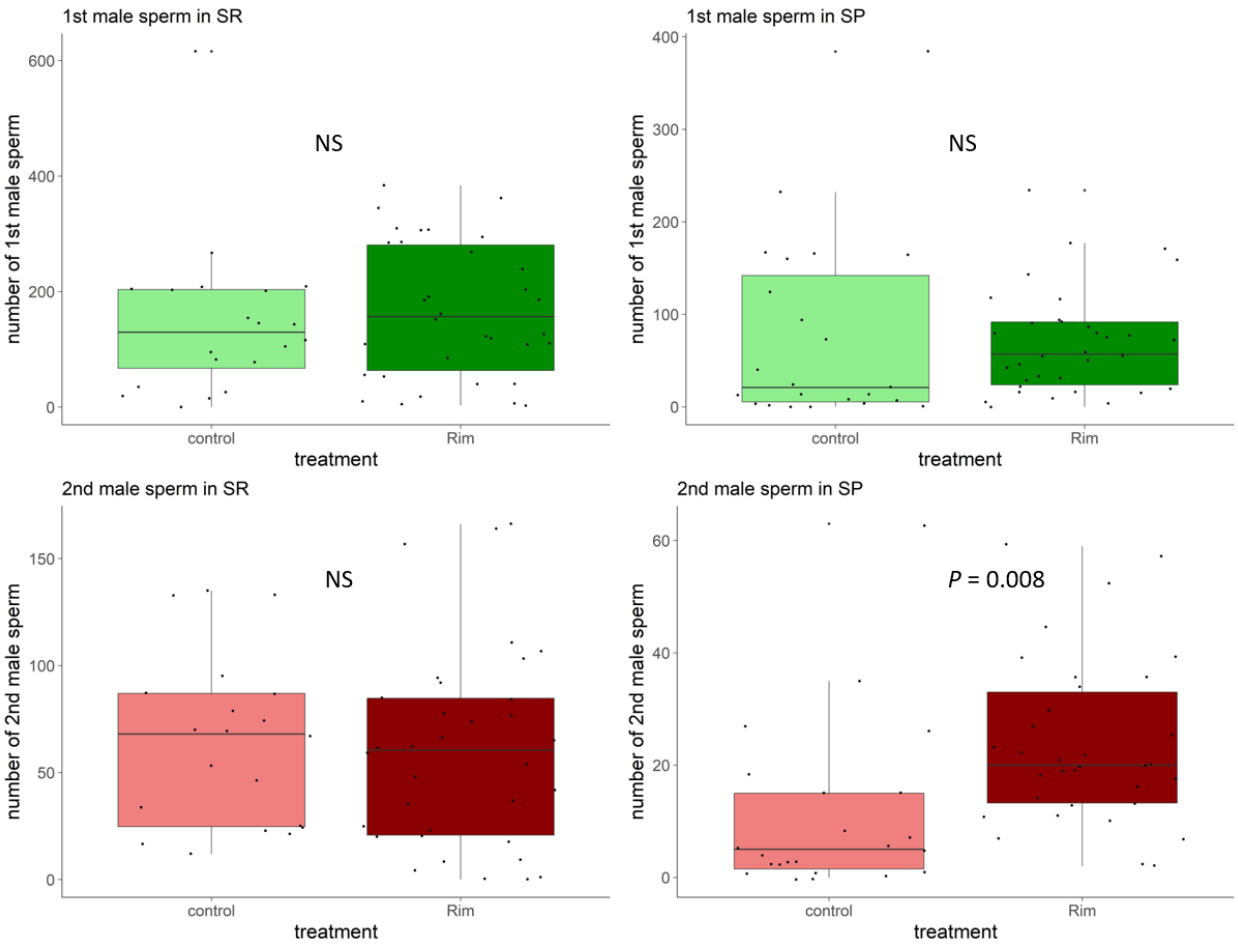

Figure 2: Heterospecific and conspecific sperm counts in the female seminal receptacle (SR) and spermathecae (SP). Sperm of the first male to mate (the heterospecific *D. simulans* male) was labeled with GFP. Sperm of the second male to mate (the conspecific *D. melanogaster* male) was labeled with RFP. Using four Wilcoxon tests, and after correcting for multiple testing, we found that *Rim* knockdown females had significantly more 2nd male sperm in their spermathecae than control females (*p*-value=0.008).

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