Experimental sexual selection reveals rapid divergence in male and female reproductive transcriptomes and their interactions

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Abstract

Mating causes substantial changes in females, altering male and female reproductive fitness. Some postmating effects are hypothesized to be at least partially mediated by gene expression changes, driven by postcopulatory sexual selection, which results in population divergence of reproductive proteins that could generate reproductive isolation. However, understanding of the direct role of sexual selection on gene expression divergence along with the subsequent molecular mismatches that could occur between diverging populations is limited. Here, we analyze gene expression divergence following over 150 generations of experimental evolution in which *Drosophila pseudoobscura* evolved under either elevated polyandry or enforced monogamy. We find that sexual selection rapidly impacted sex-, tissue-, and mating-specific responses, and not always in the predicted direction. Postmating female responses are either unique to each sexual selection treatment or exhibit asymmetric non-congruence, in which monogamy females upregulate and polyandry females downregulate the same genes following mating. This substantial population divergence of gene expression also gives rise to either unique or mismatched gene expression patterns in crosses between treatments. Many of these genes are involved in immune and stress responses, and non-congruent responses are particularly prevalent in the female reproductive tract, the main arena for postcopulatory sexual selection. In summary, we show that sexual selection has pervasive impacts on gene expression divergence acting both differentially between reproductive tissues of the same sex and asymmetrically in postmating female responses, and this divergence is highest in the female reproductive tract, the main arena for postcopulatory sexual selection.

Keywords: postmating response, postmating prezygotic reproductive isolation, RNA-seq, testes, accessory glands, female reproductive tract.

Introduction

Sexual reproduction involves pre-ejaculatory and post-ejaculatory interactions between the sexes with sexual selection influencing male and female traits that mediate the fitness outcome of these interactions. While aspects of reproduction can be cooperative, the sexes also diverge over the optima of reproductive traits, such as courtship signals, fertilization and offspring production. The intensity of sexual selection is linked to the extent to which reproductive fitness optima differ between the sexes and can generate sexual antagonism, in which selection acts in opposing directions on the sexes (Rice, 1996; Holland and Rice, 1999). As the sexes share the majority of their genome, intra-locus sexual conflict can occur over phenotypes that are influenced by these shared genes. Resolving this conflict can occur by the evolution of sexual dimorphism, mediated by changes in the shared genome, such as sex-specific regulation of gene expression (Mank 2017). Comparative genomic studies have found that genes with rapid divergence and that show stronger signatures of positive divergent selection are often sex-biased or sex-limited in expression (e.g. Cheng *et al.* 2016; Pröschel *et al.*, 2006; Ellegren & Parsch, 2007; Zhang *et al.*, 2007). This pattern is sometimes stronger in species that have traits associated with indicators of sexual selection, such as increased sexual dimorphism (Harrison et al. 2015; Wright *et al.* 2019).

In internally fertilizing species, the main arena for post-ejaculatory molecular interactions is the female reproductive tract which includes sites of sperm transfer, storage and subsequent fertilization of eggs transiting from the ovaries. Postmating female responses are extensive, influencing female behaviour, morphology and physiology. These responses are mediated by interactions between components of the male ejaculate, including sperm produced in the testes, rapidly evolving seminal fluid proteins (Sfps) produced by accessory glands, and female reproductive proteins in the female reproductive tract and ovaries (Sirot *et al.* 2015; Oku *et al.* 2019; Wolfner 2009). Postmating changes in females include altering investment in oogenesis (Wolfner 2009), remating propensity (Chapman *et al.* 2003), and sperm storage and usage (Avila *et al.* 2010; Avila *et al.* 2015). Other aspects of physiology are also altered, for example, female hunger (Carvalho *et al.* 2006), female aggression (Bath *et al.* 2017), and homeostasis (Ribeiro and Dickson 2010; Cognigni *et al.* 2011). Genes associated with immunity and stress have altered gene expression upon mating in females, which may impact susceptibility or resistance to pathogens and/or parasites (Oku *et al.* 2019; Zhong *et al.* 2013).

An increasing number of studies have characterized transcriptomic postmating changes and used gene ontology to determine the associated gene functions, although many studies typically examine either whole bodies or abdomens of females (Lawniczak and Begun 2004; McGraw *et al.* 2004, 2008, Innocenti and Morrow 2009; Innocenti *et al.* 2014; Hollis *et al.* 2014, 2016; Delbare *et al.* 2017; Fowler *et al.* 2019) or examine only one component of the female reproductive tract ("lower" reproductive tract, defined by the female sperm storage organs, Mack *et al.* 2006,

Prokupek *et al.* 2008; or the "upper" reproductive tract, defined by the oviducts, Kapelnikov *et al.* 2008). No study has simultaneously assessed postmating effects on the sperm receiving components of the female reproductive tract (the uterus and sperm storage organs) and the egg production components (oviducts and ovaries). Receipt of the male ejaculate effects sperm storage dynamics, oogenesis and oviposition (Sirot *et al.* 2015) so all are subject to sexual selection (and sexual conflict). Likewise, for males, the main focus on the role of sexual selection and sexual conflict has been on Sfp evolution given that they are among the most rapidly evolved proteins known (Ahmed-Braimah *et al.* 2017; Ellegren and Parsch 2007), although sperm and the cellular architecture of the testes also can be subject to rapid morphological evolution and sexual selection (Lüpold *et al.* 2009).

The reproductive interactions between the sexes, by generating strong postmating sexual selection and sexual conflict, also cause rapid evolutionary changes between lineages which may lead to reproductive isolation (Markow 1997; Manier *et al.* 2013; Ahmed-Braimah *et al.* 2020). In particular, postmating prezygotic (PMPZ) reproductive isolation in which gametes do not interact properly prior to fertilization (Ahmed-Braimah *et al.* 2017; Garlovsky *et al.* 2020) or affect egg production (Matute and Coyne 2010) may evolve due to mismatches between lineages in male ejaculate-female reproductive tissue interactions (Ahmed-Braimah *et al.* 2020). Direct evidence of any consequences of rapid evolutionary divergence between male and female reproductive processes and the generation of PMPZ isolation is lacking. Some studies have examined the postmating gene expression responses in females mating to either con- or hetero- specific males to assess evidence for mismatches to understand how PMPZ isolation occurs (Bono *et al.* 2011; Ahmed-Braimah *et al.* 2020). However, in most cases, these lineages are quite distinct, representing different species, and the sexual selection history is unknown.

Experimental sexual selection, in which populations are subjected to either polyandrous conditions that facilitates sexual selection or enforced monandrous conditions that eliminate sexual selection, has been used to examine the evolution of gene expression responses to sexual selection, and link these to macroevolutionary patterns of sex-biased and sex-limited gene expression evolution (Hollis *et al.* 2014, 2016; Immonen *et al.* 2014; Veltsos *et al.* 2017). Previous studies supported the role of sexual selection in divergent sex-biased gene expression, but whether male- or female-biased genes responded the most depended on species (Hollis *et al.* 2014; Immonen *et al.* 2014; Veltsos *et al.* 2017; Parker *et al.* 2019; Hollis *et al.* 2019). Additionally, these studies were limited in that postmating responses and/or sex-specific tissue responses were rarely examined. Consequently, the understanding of how sexual selection impacts sex-limited and reproductive tissue-specific gene expression, the consequences of this divergence on postmating responses, and the opportunity for this divergence to trigger barriers to reproduction is limited.

In this study, we use replicate populations of *D. pseudoobscura* following over 150 generations of experimental evolution in which either monandry is enforced (referred to as M), eliminating sexual selection and conflict, or elevating the opportunity for polyandry (referred to as E). We take a quantitative transcriptomics approach to investigate the impact of sexual selection on gene expression divergence. We separate the effects between different reproductive tissues of males (testes and accessory glands) and females (female reproductive tract and ovaries), and examine the postmating response within the separate female reproductive tissues to determine whether sexual selection acts on these similarly or differently. This approach also allows us to polarize the direction of postmating gene expression changes arising from variation in sexual selection intensity to then compare the female postmating response when mating occurs between individuals of the different sexual selection treatments. Previous work on these populations has documented the action of sexual selection and sexual conflict on phenotypic reproductive interactions between the sexes (Crudgington *et al.* 2005; 2009; 2010; Debelle *et al.* 2014; Debelle *et al.* 2016; Snook *et al.* 2005), including those that could influence postmating responses. For example, changes in accessory gland investment (Crudgington *et al.* 2009) of males and ovariole number and subsequent offspring production in females (Crudgington *et al.* 2010; Immonen *et al.* 2014). Sex-biased gene expression evolution has also occurred but previous studies of expression divergence either examined whole bodies of only one sex (Immonen *et al.* 2014) or in heads and abdomens of each sex, but not following mating (Veltsos *et al.* 2017).

Results

Gene expression divergence in male tissues

Previous work has hypothesized that monandry selects for downregulation and polyandry selects for upregulation of genes expressed in male reproductive tissues in the abdomen of *D. melanogaster* (Hollis *et al.* 2016). We found that virgin male testes had 359 differentially expressed genes, of which 161 were upregulated in polyandry (or downregulated in monandry) and 198 upregulated in monandry (or downregulated in polyandry; Figure 1a). While marginally significantly different (χ^2 = 3.81, df = 1, p = 0.051) the gene expression pattern is in the opposite direction to that predicted. The genes upregulated under polyandry were enriched for biological processes (BP) related to stress responses, such as double strand break repair, cellular response to UV, and terpenoid metabolic process (Supplementary File 1). In contrast, genes upregulated in monandry males were related to biological processes (BP) of proteolysis, digestion and the innate immune response (Supplementary File 1).

In male accessory glands, 80 genes were differentially expressed between sexual selection treatments, with 34 genes upregulated in polyandry males (Figure 1a) and 46 upregulated in monandrous males. While the number of DE genes did not differ between sexual selection treatments (χ^2=1.8, p=0.18), the proportion of DE genes was higher among all genes expressed in

accessory glands than all genes expressed in testes (0.031 vs 0.007; x2=179.32, df=1, p<0.001). The 34 accessory gland genes with higher expression under polyandry were enriched for BP terms related to eggshell chorion assembly, development, and neuropeptide signaling (Supplementary File 1). The 46 genes upregulated in monandry males were enriched for the BP term "detection of chemical stimulus involved in sensory perception" (Supplementary File 1).

Sfps are some of the most rapidly evolving proteins, hypothesized to be in response to sexual selection. To examine whether Sfps were more likely to be upregulated in polyandrous males (Hollis *et al.* 2016), we compared expression levels between the sexual selection treatments of different sets of accessory gland proteins recently described for *D. pseudoobscura* (Karr *et al.* 2019). Karr and colleagues identified 3281 proteins produced in the accessory gland (the accessory gland "proteome"), of which 528 had protein secretory signals (the "secretome") and 163 were orthologous to *D. melanogaster* seminal fluid proteins (putative Sfps; also referred to as the "exoproteome"). In contrast to the prediction, we found that monandry males had higher overall expression of secretome proteins compared to polyandry males (W=21345000, p<0.001; Figure 2a) and there were no significant treatment effects on the proteome or Sfps. However, of the 80 accessory gland genes that were differentially expressed between treatments, we found a non-significant trend towards higher expression in polyandry males (Figure 2b).

Overall, while relatively more gene expression changes were found in the accessory glands than testes, we found a weak trend for monandry males to have higher expression in both the testes and accessory glands relative to polyandry males, including accessory gland genes with secretory function.

Gene expression divergence in virgin female tissues

Virgin female reproductive tracts had 120 differentially expressed genes, 66 (54) of which were upregulated in polyandry (monoandry) females (Figure 1b), a nonsignificant difference (χ^2 = 1.2, df = 1, p = 0.27). The 66 genes upregulated under polyandry were enriched in GO terms associated with the immune system and egg production, such as egg activation whereas the 54 genes upregulated under monandry were enriched for multicellular reproduction biological processes (Supplementary File 1).

Virgin ovaries had 417 differentially expressed genes between the sexual selection treatments (Figure 1b), with significantly more genes upregulated in polyandry (299) than in monandry (118) (χ^2 = 78.6, df = 1, p < 0.001). The proportion of DE genes in ovaries was significantly greater than that in FRT (0.040 vs 0.014; x2=120.1, df=1, p<0.001). Upregulated polyandry genes were enriched in BP terms associated with eggshell chorion assembly whereas upregulated genes in monandry ovaries were enriched for negative regulation of BMP signaling pathway (Supplementary File 1).

Thus, we find that the female reproductive tissues do not respond similarly to the sexual selection treatment, with the ovaries showing more gene regulation divergence in response to sexual selection treatment than the reproductive tract. The biological processes associated with differentially expressed genes also differed between sexual selection treatment and tissues.

Sexual selection causes divergence in the female postmating response

Sexual selection is thought to result in rapid divergence of the postmating female response. Above, we show that, in virgins, different male and female reproductive tissues have different responses to sexual selection which sets the stage for divergence in postmating responses. Sexual selection treatment did not affect the total number of genes involved in the female postmating response in the reproductive tract (n= 400 for polyandry females, n=357 for monandry females; χ^2=2.4425, p=0.12) (Figure S1). However, polyandry females had significantly more postmating responsive genes showing downregulation (n=246) than upregulation (n=154) after mating (χ^2=21.16, p<0.001), whereas monandry females showed the opposite pattern, with significantly more genes upregulated (n=233) than downregulated (n=124) after mating (χ^2=33.28, p<0.001) (Figure S1). In contrast to the large number of genes involved in the postmating response of the female reproductive tract, the ovaries had relatively few genes showing expression divergence upon mating (n=23; Figure S1). Polyandry females showed significantly more differentially expressed genes (n=22) than monandry females (n=1) (χ^2=19.174, p<0.001). Of the 22 genes involved in postmating divergence of polyandry ovaries, 14 were upregulated after mating and 8 downregulated, not significantly different (χ^2=1.64, p=0.2; Supplementary File 1).

For the reproductive tract, genes downregulated in polyandry females after mating were significantly enriched for metabolic related processes, eggshell chorion assembly, and immune response, whereas downregulated genes in postmating monandry females were related to terms associated with development (Supplementary File 1). Postmating upregulated genes in the polyandry female reproductive tract were significantly enriched for immune and stress responses, whereas monandry females upregulated genes related to immune response and chorion-related functions (Supplementary File 1). For the ovaries, downregulated genes in polyandry females after mating were significantly enriched for cold acclimation and synaptic target inhibition whereas the one monandry downregulated gene was related to synaptic target inhibition and motor neuron axon guidance (Supplementary File 1). Postmating upregulated genes in the polyandry female ovaries were significantly enriched for processes involved with egg production and monandry females did not significantly upregulate any ovarian genes after mating (Supplementary File 1).

Unique and shared postmating responses between sexual selection treatments

We compared shared and divergent postmating responses between the selection lines in the female reproductive tract (Figure 3a) and the ovaries (Figure 3d). In the female reproductive tract,

many of the genes whose expression was altered upon mating were unique to each treatment (blue (E) and yellow (M) dots, Figure 3a). However, there was a subset of genes that altered expression in both sexual selection treatments (green diamonds, Figure 3a). 86 of these genes responded congruently; that is mating resulted in both monandry and polyandry females either upregulating (n = 67) or downregulating (n = 19) those genes (purple congruent line, Fig. 3a). Shared upregulated genes were enriched for defense responses whereas downregulated genes were enriched for fatty acid elongation and chitin-based cuticle development (Supplementary File 1). Intriguingly, 57 genes responding to mating were regulated non-congruently between the sexual selection treatments; that is, gene expression was altered upon mating in both monandry and polyandry females but the direction of the expression change was opposite in the sexual selection treatments (red non-congruent line, Fig. 3a). Moreover, this non-congruence was asymmetrical; only genes that were significantly upregulated after mating in monandry females, but were downregulated after mating in polyandry females, were observed. These non-congruent genes were related to chitin and melanin catabolic processes, chorion related processes, and immune responses (Supplementary File 1). In contrast, the postmating response in ovaries predominantly showed the upregulation of genes only in polyandry females and, while monandry females did not significantly alter these genes, these E genes are congruent (Figure 3d).

Matings with non-coevolved individuals affected different genes than those of the coevolved female postmating response

Same female, different male

We use the coevolved female postmating response contrasts (Figure 3a and 3d) to polarize the direction of change and assess whether gene expression differs in non-coevolved crosses. We first contrast the effect of mating on females within a sexual treatment when mated to a coevolved vs non-coevolved male (“same female, different male contrast”: MM v ME and EE v EM). In the female reproductive tract, only monogamy females show differential regulation of gene expression dependent on male partner (number of DE genes = 19; Figure 3b, blue crosses; Figure S2). 18 of these genes are downregulated after monandry females mate with polyandry males. However, 17 of these genes are on the non-congruent axis (Figure 3b), indicating that these genes are normally relatively downregulated after EE mating but upregulated after an MM mating (compare blue pluses on Fig. 3b with data in the lower right quadrant of Fig. 3a). Thus, a M female mating with an E male downregulates postmating response genes, which is similar to the effect of E male mating with E females. Even though this is the female reproductive tract, these genes are significantly enriched for egg production (Supplementary file 1).

The ovarian gene expression pattern as a result of mating with a non-coevolved male (Figure 3e) differs from the response in the female reproductive tract (Figure 3b). In the ovaries, gene expression changes when mated to non-coevolved males occur only for E females (n=88 genes; Figure S2). Nearly 100% of these genes were not differentially expressed upon mating in EE crosses (n=87; compare Figure 3d with Figure 3e). 12 genes are downregulated (Figure 3e, purple triangles), and 76 genes are upregulated (Figure 3e, yellow triangles), after an E female mates with a non-coevolved M male. Most of the genes that are upregulated in E females after mating with a M male are non-congruent. This means that the coevolved postmating response tends towards upregulation in MM and downregulation in EE. Thus, mating with M males, makes E females more M-, and less E-like. Non-coevolved upregulated genes are enriched for a variety of disparate biological processes with molecular functions of DNA and mRNA binding (Supplementary file 1). Non-coevolved downregulated genes are significantly enriched for rRNA transcription and purine ribonucleoside biosynthetic processes, both of which are involved in cell growth and proliferation (Supplementary file 1). The enriched molecular functions of these genes are associated with structural constituents of chorion (Supplementary file 1).

Different female, same male

We then contrast the impact of male sexual selection treatment on the postmating female response ("different female, same male contrast"; here we compare the effect of a M female mating with an M male (MM) with a E female mating with an M male (EM); likewise we compare a E female mating with an E male (EE) with a M female mating with an E male (ME)). A large number of genes are differentially regulated in these contrasts but the effect of sexual selection treatment of the male varies across female tissues. In the female reproductive tract, mating with monandry males led to nearly 8x as many genes changing expression than mating with polyandry males (431 vs. 58; χ^2=284.52, p<0.001; Figure S3). Ovarian responses were in the opposite direction such that mating with polyandry males changed expression of about 2x more genes than mating with monandry males (310 vs. 148; χ^2=57.3, p<0.001; Fig. S3). Regardless of tissue or male sexual selection treatment, the majority of genes that changed in expression following a non-coevolved mating were not differentially expressed in coevolved crosses.

In the female reproductive tract contrast between a coevolved MM and non-coevolved EM mating, 106 of 196 genes that showed downregulation when a E female mated to a M male (Figure 3c, purple pluses) were not DE in coevolved postmating responses (Figure 3a). These downregulated genes were significantly enriched for various metabolic processes, proteolysis and antimicrobial humoral response. 213 of the 235 genes that showed relatively higher expression in E females after mating with M males (Figure 3c, orange crosses) did not change expression in coevolved crosses (Figure 3a). These upregulated genes were enriched for 68 different BP terms, and

subsequent analysis of cellular components and molecular functions suggest changes in genes related to DNA replication. In the opposite cross, comparing the effects of a M female mating to an E male relative to a EE mating, 31 genes were downregulated and 27 genes were upregulated. Upregulated genes are enriched for immune responses and downregulated genes are enriched for ecdysone biosynthetic process and midgut development.

In the ovaries, because there were so few coevolved changes (Figure 3d), nearly all the ovarian gene expression changes following a non-coevolved mating with a different sexual selection line male are uniquely altered (Figure 3f). Genes downregulated in the ovary after a E female mates with a M male are enriched for redox process, vitellogeneis and tracheal system whereas genes upregulated are enriched for BP processes that have cellular components associated with the P granule and chorion. The majority of change in the ovaries were following a noncoevolved mating between a M female and E male. The 196 genes downregulated after mating are enriched for a small number of BP terms (eggshell chorion assembly, male meiosis 1, and rRNA transcription) whereas the 114 genes upregulated after mating are enriched for immunity and stress related terms.

While most non-coevolved gene expression changes were unique, the direction of change was sometimes along the congruent axis of coevolved responses and for other genes differential expression occurred along the non-congruent axis (Figure 3c; Figure 3f). In the female reproductive tract, significantly more genes are DE along the non-congruent axis than the congruent axis when M males mate with E females ($X2 = 44.73$, $df = 1$, $P < 0.001$) whereas there is no difference between non-congruent and congruent responses in the female reproductive tract when M females mate with E males. For the ovaries, non-coevolved matings with either a M or E male result in more genes with differential expression that are along the congruent axis compared to the non-congruent axis (M: $x2=10.28$, $df=1$, $p<0.001$; E: $x2=10.4$, $df=1$, $p=0.0012$).

Because non-congruent changes may be more likely to generate incompatibilities, we note enrichment for DE genes in that axis. In the female reproductive tract, non-congruent changes when M males mated with E females were the large number of BP terms related to DNA replication that were upregulated after mating and for E males mated with M females associated with immune responses, also upregulated after mating (Supplementary file 1). In the ovaries, non-congruent DE genes when mating with M males were enriched for only two seemingly disparate biological processes and two cellular components, 3 of which were unique terms not seen in other contrasts with EM data. Non-congruent DE genes when mating with E males are enriched for eggshell chorion assembly and rRNA transcription which were downregulated after mating.

Discussion

Understanding how sexual selection impacts the molecular basis of sexual interactions is important because it can have profound effects on sex-specific fitness and is predicted to influence the evolution of postmating prezygotic reproductive isolation. Here we combined transcriptomics with experimental evolution to determine how sexual selection affects gene regulation simultaneously in multiple sex-specific tissues in male and female virgins and in the postmating female response when mating with either males that evolved in the same or different intensity of sexual selection as the females. Our results reveal substantial gene expression divergence after only 150 generations of altered sexual selection intensity, with sexual selection affecting components of male and female reproductive tissues differentially, as well as alterations in gene expression specific to mating with non-coevolved partners.

An evolutionary history of polyandry previously has been suggested to select for higher expression of genes affecting sperm competition and/or showing antagonistic effects on female reproductive behavior. This hypothesis has been examined in male abdomens of *D. melanogaster* testing the effects of experimental sexual selection on gene expression of 138 Sfps (Hollis *et al.* 2016). That study found an overall lower gene expression across all Sfps in monandry-selected males, while no individual Sfp was differentially expressed. Here we separate the effects of sexual selection on evolutionary divergence in both accessory gland and testes. Testes are also subject to sexual selection via increased sperm number for male-male competition and/or in testicular architecture to support rapid sperm production (Lüpold *et al.* 2009; Pitnick *et al.* 2008). Experimental sexual selection can alter relative testes size and accessory gland size, although this varies between species, between studies of the same species, and has different fitness outcomes (Hosken *et al*. 2001; Hosken and Ward 2001; Pitnick *et al*. 2001; Wigby and Chapman 2004; Linklater *et al*. 2007; Reuter *et al*. 2008; Crudgington *et al.* 2009; Hollis *et al.* 2019). Previously we found that *D. pseudoobscura* polyandry males had evolved larger accessory glands but not testes, and polyandry males had greater mating capacity (Crudgington *et al.* 2009). Although we found that accessory glands had proportionally more DE genes than testes, contrary to predictions, virgin monandry males had more upregulated genes both in the accessory glands and testes than polyandry males. A similar result was observed when we limited our analysis to putative *D. pseudoobscura* Sfps (either the secretome or Sfps with *D. melanogaster* orthologs; Karr *et al.* 2019).

The male genes that changed in expression in response to sexual selection treatment were associated with different biological processes. In the accessory glands, polyandry males had higher expression of neuropeptides, which are diverse neuronal signaling molecules that regulate physiological processes and behaviour in animals (Elphick *et al*. 2018). Given that postmating female responses frequently involve changes in female reproductive physiology and behavior (Sirot *et al*. 2015), polyandry may generally increase investment by males in the expression of genes that profoundly alter female reproductive fitness. In contrast, monandry male accessory

gland genes were enriched for "detection of chemical stimulus involved in sensory perception" and, while genes encompassed by this term can evolve rapidly, they have weak fitness effects (Librado and Rozas 2016). Thus, for male responses, we see that monandry males have increased gene expression in testes and accessory glands, including putative Sfps, although distinctly enriched gene functions between sexual selection treatments suggests differences in the potential consequences on female mates.

In females, the reproductive tract is considered to be the main arena for postmating sexual interactions. However, the ovaries may also be subject to sexual antagonism given that they are the egg-producing structure, and the sexes can be in conflict over fecundity schedules (Arnqvist and Rowe 2005). We have previously shown that polyandry *D. pseudoobscura* females have more ovarioles than monandry females (Immonen *et al.* 2014). Using whole body microarrays, we found that genes upregulated in polyandry females are enriched in the ovary and associated with oogenesis whereas monandry females upregulated genes associated with somatic tissues and metabolism (Immonen *et al.* 2014). In virgin abdomens, RNAseq data showed sexual selection treatment affected sex-biased gene expression (Veltsos *et al.* 2017). Here we decompose responses between the female reproductive tract and the ovaries in virgin and mated monandry and polyandry females. The reproductive tract showed a similar number of differentially expressed genes between virgin polyandry and monandry females while in the ovaries more genes were upregulated under polyandry than monandry. Genes overexpressed in virgin polyandry reproductive tissues were associated with late stages of egg production (eggshell chorion assembly, egg activation), whereas the genes over-expressed in monandry reproductive tissues were associated with earlier egg production (BMP signalling pathway, involved in patterning the *Drosophila* eggshell; Niepielko *et al*. 2012). Thus polyandry females appear ready ("primed") for fast reproduction.

Related to this, previous work in *D. melanogaster* based on whole body gene expression has suggested that polyandry females are poised for receipt of a manipulative ejaculate (Heifetz and Wolfner 2004; McGraw *et al.* 2004), and therefore do not have as elevated a postmating response as monandry females (Hollis *et al.* 2016). Our results partially support this prediction, dependent on female reproductive tissue. In the female reproductive tract, polyandry females downregulated more genes following mating than were upregulated, whereas monandry females showed the opposite pattern. However, in the ovaries, only polyandry females changed expression with more upregulated, than downregulated, genes and enriched for genes associated with egg production. Overall our results support an interpretation in which polyandry females are poised to receive the ejaculate and are more reproductively mature with respect to egg production.

Many of the postmating differentially expressed genes in the female reproductive tract are distinct between the polyandry and monandry treatments. However, some were shared across sexual

selection treatments. Differential expression of these genes could be either congruent (both sexual selection treatments upregulated or downregulated these genes) or non-congruent, in which monandry upregulated the postmating gene expression, whereas the same gene in polyandry females was downregulated. Both upregulated and downregulated congruent genes were related to fatty acid elongation and some upregulated congruent genes were related to stress/immune responses. Fatty acids are critical to signaling pathways and have been associated with sex pheromone synthesis and with maturation of egg follicles in *D. melanogaster* (Vrablik and Watts 2013). The shared responses between sexual selection treatments suggests a shared critical postmating reproductive function. Non-congruent genes were enriched for immune response (see later) and egg production.

In polyandrous species, interactions between the sexes that impact fertility are thought to be particularly dynamic and coevolve, perhaps antagonistically. In turn, such coevolution may generate postmating prezygotic (PMPZ) incompatibilities between populations, contributing to reproductive isolation. Standard approaches in speciation research to identify reproductive isolation use crosses between males and females within and between populations. Several studies have compared postmating transcriptomic responses to test the hypothesis that misregulation between components in the male ejaculate and female reproductive tissues could generate PMPZ incompatibilities (Bono *et al.* 2011; Ahmed-Braimah *et al.* 2020). However, it is very difficult to infer the historical role of different evolutionary processes from patterns of contemporary divergence between species. Experimental evolution introduces fewer confounding variables but lacks the full realism of natural conditions. Previous experimental evolution work has been criticized for crossing different populations to infer the direction of sexual conflict (Pizzari and Snook, 2005; Rowe *et al.* 2005). Here we can use known coevolved population differentiation in gene expression to examine the consequences on mismatches in gene expression when crossed with a non-coevolved individual. The effect of mating with a non-coevolved individual depended on whether we were contrasting the effect of either the male or female treatment, what female reproductive tissue was being analyzed, and the sexual selection treatment itself. Regardless of these factors, most of the postmating genes that were differentially expressed in non-coevolved crosses were distinct from the postmating differentially expressed genes within a coevolved cross. This pattern indicates much of the non-coevolved postmating response is unique, supporting substantial and rapid coevolution between the male ejaculate and female reproductive tissues within each treatment that differs between sexual selection treatments. Such novel mismatches may have negative fitness consequences that could potentially contribute to the evolution of PMPZ reproductive isolation.

When contrasting the effect of the same female treatment when mated to different males (MMvME and EEvEM), differential expression in the female reproductive tract only occurred with M females mated to E males whereas in the ovaries, differential expression only occurred with E females mated to M males. In both cases, the differentially expressed genes were mainly non-congruent

and in such a way that the postmating response was more similar to one of the co-evolved crosses. We have previously found that when M females mate with E males, they increase the number of eggs laid earlier in their lifespan relative to E females mated to E males (Crudgington *et al.* 2010). Whether the gene expression changes in the female reproductive tract at least partially explains sexual conflict over oviposition patterns remains to be determined. In the ovaries, mismatched gene expression was related to a variety of different biological processes, including oocyte development and regulation of antimicrobial peptide biosynthesis. To get a better idea of the overall impact of this diverse set of responses, we examined the cellular component enrichment, which included P body which is involved in mRNA metabolism and in Drosophila oogenesis (Lin *et al.,* 2008). Enrichment of molecular function was of mRNA 3'-UTR binding which may be involved in post-transcriptional gene expression regulation mediated by *trans*-acting factors and in which microRNAs guide-associated proteins towards the 3' UTRs of mRNAs to repress expression (Mayya and Duchaine 2019). miRNAs have been implicated in regulation of male and female fertility and ovary morphology (Chen *et al.,* 2014) and were recently shown to be involved in a number of postmating responses in *D. melanogaster* females (Fowler *et al.,* 2019). That these show mismatched expression changes in the ovary, potentially affecting female fertility, suggests that these alterations could negatively impact fitness resulting in postmating prezygotic reproductive isolation.

When contrasting the effect of the sexual selection treatment of the male-on-female postmating responses (MMvEM; EEvME) we again found differences between the female tissues. In the female reproductive tract, the main arena for postcopulatory sexual selection, the number of mismatched differentially expressed genes was 8x greater after mating with monandry males than polyandry males whereas in the ovaries, polyandry males caused 2x more differentially expressed genes than monandry males. In the female reproductive tract, more genes were upregulated after non-coevolved mating (M males with E females) than downregulated and in ovaries more genes were downregulated after a non-coevolved mating (E males with M females) than upregulated. However, in both the female reproductive tract and ovaries, genes upregulated when a E female mates with a M male are related to a large number of biological processes influencing DNA replication, and in the female reproductive tract these genes are non-congruent. This indicates that, while these responses are unique and mismatched in non-coevolved crosses, M males tend to cause postmating responses that makes the E female more M-like, although many of these genes do not show extreme changes in gene expression. In the ovaries, while E males mated to M females resulted in a large number of differentially expressed genes, the number of biological processes was limited, impacting eggshell assembly and immunity.

We often found changes to immune-related genes. Differential expression of Drosophila immune-related genes in response to mating in *D. melanogaster* have been shown previously (Lawniczak and Begun 2004; McGraw *et al.* 2004; 2008; Mack *et al.* 2006). Innoncenti and Morrow (2009)

have suggested that upregulation of immune genes after mating indicates males are immunogenic, arising from sexually antagonistic sexual interactions. Here we found that some of these genes are upregulated after mating, no matter what the sexual selection treatment. We also found that polyandry females had significant upregulation of some immune genes when virgin which were downregulated after mating. The upregulation of immune genes in virgin polyandry females may be in preparation for a manipulative or pathogenic ejaculate, and upregulation of some genes during courtship provides immunogenic benefits (Zhong et al. 2013). However, such priming does not always appear to benefit *D. melanogaster* females (McKean and Nunney 2005). In contrast, monandry females, who do not experience sexual conflict, upregulated immune genes only after mating. Thus some postmating expression changes in immune related genes are not immunogenic *sensu* Innocenti and Morrow (2009). Previous work examining the postmating response of females mated to heterospecific males identified immunogenic effects (Ahmed-Braimah *et al.* 2020). We found a consistent pattern in which gene expression of immune and stress related genes were either uniquely altered in non-coevolved matings or showed non-congruent changes. While we show immune-related genes frequently change expression during mating, alter expression patterns in response to sexual selection, and can be mismatched during non-coevolved crosses, how these gene expression changes affect fitness remains to be determined (Oku *et al.* 2019).

Changes in gene expression we identified here and in sex-biased gene expression in response to sexual selection (Veltsos et al. 2017) have recently been shown to associate with genomic divergence in these lines (Wiberg *et al.* in review). Sexual selection has been implicated in gene expression and genomic divergence in natural populations and our experimental evolution work supports rapid molecular evolution in response to sexual selection. Rapid coevolution of the molecular interactions, particularly in the female reproductive tract, as we see here, and the subsequent effect on the postmating response is thought to generate mismatches between male and female proteins necessary for successful fertilization (e.g., Ahmed-Braimah *et al.* 2020). Consequently, we found gene expression mismatches in matings between diverged populations, with the female reproductive tract being particularly sensitive. Such mismatches may form the basis of subsequent postmating prezygotic reproductive isolation. Our results, simultaneously comparing gene expression responses in multiple reproductive tissues and under mating system manipulation, highlight the complexity and rapid evolution of ejaculate-female interactions and their potential to influence population divergence.

Material and methods

Experimental evolution lines

The origin, establishment, and maintenance of the selection lines are described in detail elsewhere (Crudgington *et al.* 2005). Briefly, 50 wild-caught females of *D. pseudoobscura* from a population in Tucson, AZ USA were brought into the laboratory and reared for three generations, then four replicate lines of two different sexual selection treatments were established. We modified the opportunity for sexual selection by manipulating the adult sex ratio in food vials (2.5 x 80 mm) by either confining one female with a single male (enforced monogamy treatment; M) or one female with six males ("elevated" polyandry treatment; E). This species is naturally polyandrous with wild-caught females frequently being inseminated by at least two males at any given time (Anderson 1974). We successfully equalized effective population sizes between the treatments (Snook *et al.* 2009). At each generation, offspring were collected and pooled together within each replicate line for each treatment, and a sample from this pool was used to start the next non-overlapping generation in the appropriate sex ratios. Thus, this proportionally reflected the differential offspring production across families within a replicate and treatment. Generation time was 28 days and all populations were kept at 22°C on a 12L;12D cycle, with standard food media and added live yeast. Note that 'monandry' versus 'polyandry' as used here refers to the evolutionary history under which the individuals have evolved, not their current reproductive status.

Sample preparation

To generate experimental males and females, parents were collected from each replicate at generation 157-158. We standardized for maternal and larval environments as previously described (Crudgington *et al.* 2010). Briefly, parents were mated en masse in food bottles, transferred to containers with oviposition plates, allowed to oviposit for 24 h, and then 48 hr later, 100 first instar larvae were seeded in standard food vials (Crudgington *et al.* 2010). Virgin males and females were collected under light CO_2 anesthesia on the day of eclosion and kept in vials of 10 individuals for 5 days to ensure reproductive maturity (Snook and Markow 2001). On Day 5, within a 2 h window after lights turned on, one virgin female was placed in a food vial with one virgin male that was from either the same experimental replicate ("coevolved"; MM, EE where the first letter is the female) or the other treatment ("non-coevolved"; ME, EM). We dissected age- and circadian rhythm-matched virgin males and females from the same collections. Each treatment used 100 individuals, the tissues of which were equally split into 4 separate tubes, for easy pooling. For the mating treatments, males were put first in individual vials with fly food and allowed to settle. Females were then added, and were dissected 6 h after the first couple mated, in the order of mating, within a 2 h block. Dissections were performed under ether anaesthesia in RNA later (Ambion) on ice blocks. We separated the ovaries and the remainder of the female reproductive tract, including the sperm storage organs (seminal receptacle and spermathecae). We refer to these different female tissue sets as ovaries and the female reproductive tract. The male accessory glands and testes were dissected separately (ejaculatory bulbs were not included). All tissues were left at 4°C in RNA later for one day and then transferred to -80°C until RNA

549 extraction. Pools of 4 replicates of the E and M treatments, each containing tissues from 25
550 individuals per replicate, were processed for RNA extraction using Trizol (Ambion) following the
551 manufacturer's instructions. RNA extractions were cleaned up in Qiagen RNeasy kit columns
552 according to the manufacturer's protocol, including the 15 min DNase treatment. The quality of
553 RNA extractions was checked with Nanodrop and Bioanalyser.

554 **Sequencing and mapping**

555 The sample libraries were sequenced using an Illumina HiSeq 2000. Reads were mapped to the *D.*
556 *pseudoobscura* genome v3.1, and indexed using bowtie2 (Langmead *et al.* 2009). Paired-end
557 reads were aligned using option "-g 1 –library-type fr-secondstrand" with TopHat2.0.8b (which calls
558 bowtie2.1.0; Kim *et al.* 2013). The option "-g 1" instructs TopHat2 to report the best alignment to
559 the reference for a given read. Exon features were counted using HTSeq-count (Anders *et al.*
560 2015) and the reads of all exons of each gene were combined to provide overall measures of gene
561 expression.

562 **Statistical analyses**

563 We analysed the count data using edgeR v3.18.1 (Robinson *et al.* 2010) running in R v.3.4.0 (R
564 Development Core Team, 2007). Libraries were normalised with the default edgeR normalisation
565 procedure (TMM) and only genes with at least 3 counts per million across all libraries used in each
566 analysis were retained. Dispersion was measured with default parameters using a negative
567 binomial model using only the directly contrasted libraries. We performed different contrasts, but
568 within each we tested for mean expression differences between the significantly upregulated and
569 downregulated genes using Mann-Whitney rank tests using R v3.4.0 (R Development Core Team
570 2007). We considered genes to be differentially expressed if they were below the 5% false
571 discovery rate (FDR) threshold (Benjamini and Hochberg, 1995). We did not employ a $\log_2$FC
572 threshold because allometry is unlikely to influence results obtained from specific tissues
573 (Montgomery and Mank 2016).

574 We performed several different contrasts including between the lines for divergence in virgin gene
575 expression in testes, accessory glands, ovaries, and female reproductive tract separately. Note
576 that differential gene expression results are relative. For simplicity, figures report upregulated
577 genes next to the name of each contrast; they can also be interpreted as downregulated genes for
578 the opposite contrast. For example, 161 genes were upregulated in the testes of E males, which
579 could also be interpreted as 161 genes downregulated in M testes. We also assessed divergence
580 in the female postmating response by contrasting, within each sexual selection treatment,
581 differential expression between virgin and mated ovaries and the female reproductive tract,
582 separately. We consider the virgin gene expression status to be the baseline, so we can categorize
583 changes as either upregulated after mating (gene expression is higher in mated compared to

584 virgin) or downregulated after mating (gene expression is higher in virgin compared to mated). We
585 also investigated the effect of non-coevolved matings relative to coevolved matings, either focusing
586 on the same female treatment but different male treatment (e.g. a contrast between either MM v
587 ME or EE v EM, where the focal sex is unscored and females are always listed first) or on the
588 same male treatment but different female treatment (i.e. MM v EM or EE v ME). These were
589 conducted for both components of the female reproductive tissue. Here we consider the coevolved
590 mating as a baseline, so we can categorize changes in gene expression in non-coevolved matings
591 relative to the coevolved. Notation then is either that the non-coevolved postmating response has
592 relatively higher or lower expression (the former indicated by, e.g., “EM up” and the later indicated
593 by, e.g., “EM down”).

594 We identified gene subsets based on congruent and non-congruent response to sexual selection
595 between the selection lines using venn diagrams, constructed using Venny 2.1 (Oliveros 2015).
596 We performed GO enrichment analysis for all DE genes from all contrasts, and those udentified
597 from the venn diagrams, using topGO v2.22.0 with the weight01 algorithm option to account for
598 GO topology (Alexa and Rahnenfuhrer 2010). Results with $p < 0.05$ on Fisher’s exact tests,
599 corrected for topology, were retained (Supplementary File 1).

600 For analysis of Sfps, we contrasted the distribution of the change in expression (log_2FC) of all
601 genes, and differentially expressed genes between the selection lines from accessory glands using
602 density plots. We made contrasts to the distribution of 3 Sfp-related gene subsets, identified from
603 *D. pseudoobscura* proteomics (Karr *et al.* 2019). The largest subset was 3281 proteins produced in
604 the accessory gland (“proteome”). Of these, 528 had protein secretory signals (“secretome”) and
605 163 were also orthologous to *D. melanogaster* seminal fluid proteins (putative Sfps or
606 “exoproteome”). Differences in the median expression differences between these sets were
607 analysed with Wilcoxon tests, conducted in R v3.4.0 (R Development Core Team 2007).

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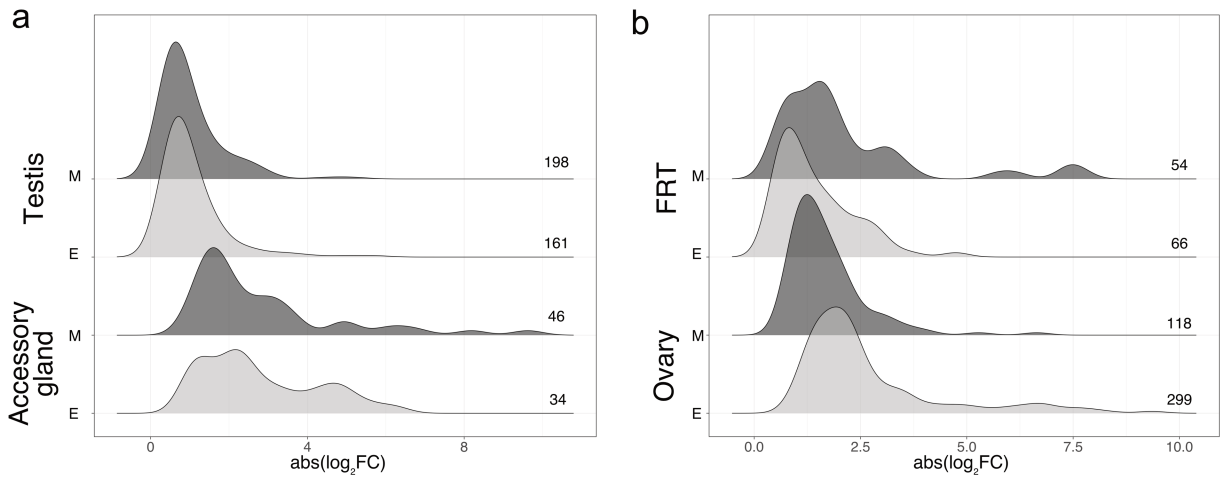
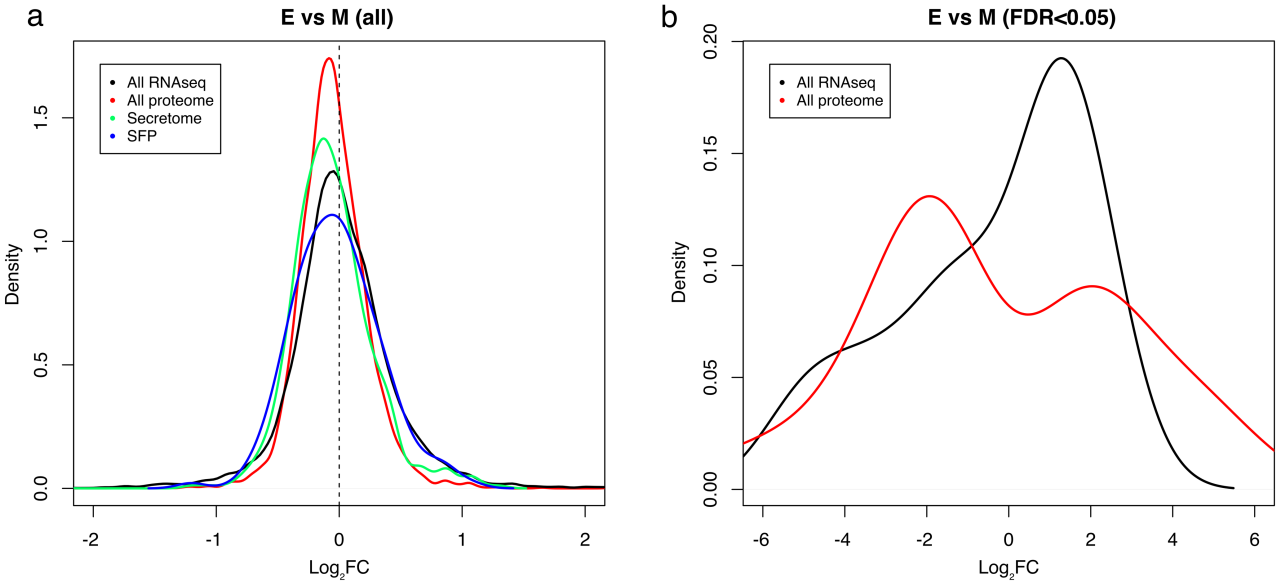
Figures

Figure 1. Differential gene expression (absolute log_2FC changes, y axis) and number of significantly upregulated genes (numbers to the right of the ridge plot) when comparing monoandry (M) and polyandry (E) tissues of a) males and b) females.

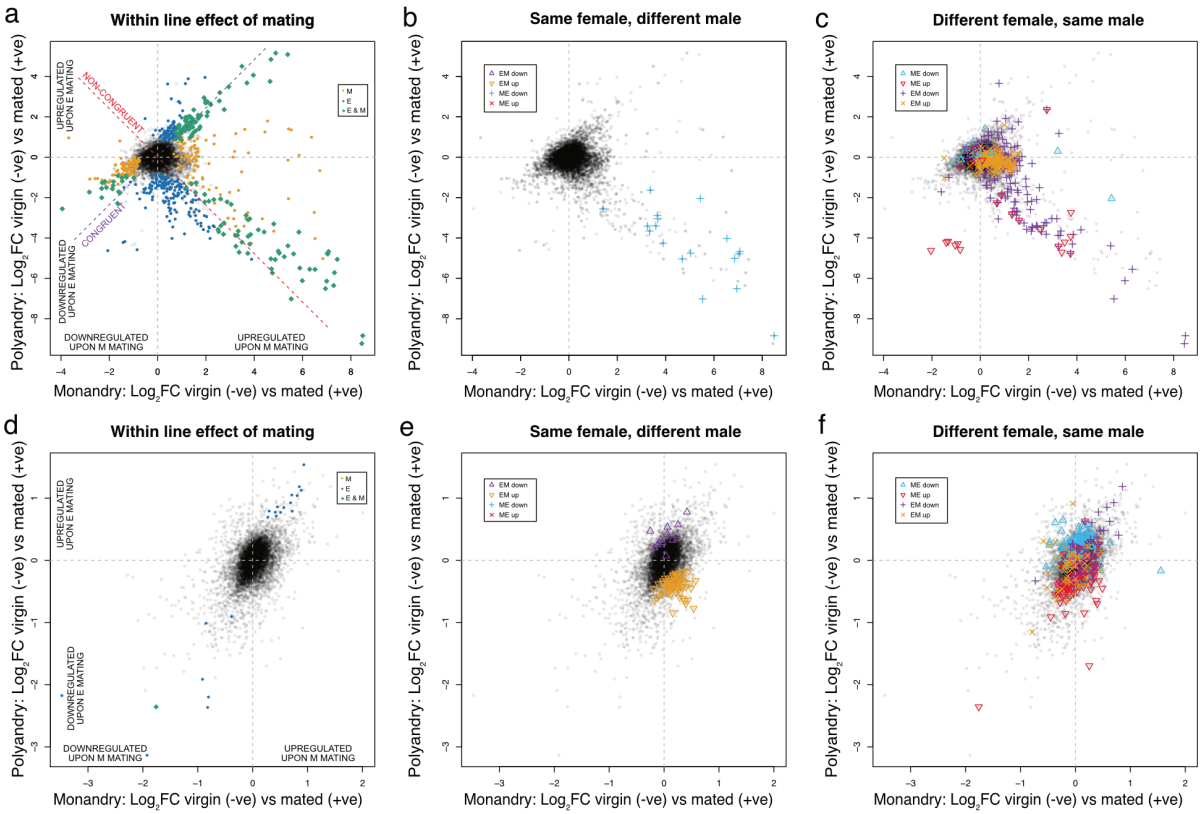


621 **Figure 2**. Effect of sexual selection on expression of genes coding for a) all and b) differentially
622 expressed male accessory gland genes between polyandry (positive x axis values) and monandry
623 (negative x-axis values) treatments. The line colours differentiate between independently
624 categorized parts of the transcriptome as proteome, secretome or seminal fluid proteins (Karr *et al.*
625 2019).



626

Figure 3. Postmating responses of differentially expressed genes (log_2FC) in the female reproductive tracts (top; a-c) and ovaries (bottom; d-f). a,c) Within line mating contrasts showing either upregulation (positive values) or downregulation (negative values) of genes following mating within lines, either in monandry (x axis) or polyandry (y axis) females. Gene expression response direction is defined relative to virgins. In panel a, a similar response (congruent) in both selection lines is shown with a purple diagonal line, and a non-congruent response is shown with a red diagonal line. Panel a and c legends indicate genes uniquely differentially expressed in the monandry line (M) in yellow circles, those in the polyandry line (E) in blue circles and genes differentially expressed in both selection lines (M+E) in green diamonds. Grey circles are genes that are not differentially expressed. b,d) Same female, different male contrast showing either upregulation (yellow triangle, red cross) or downregulation (purple triangle, blue plus) of genes following mating with a non-coevolved male, focusing on the response within each female line. Panel b and d legends show the female line in the first letter and the male line the second letter (i.e., ME = monandry (M) female mated to polyandry (E) male; EM = polyandry female (E) mated to monandry (M) male). Contrast made relative to relevant coevolved cross (i.e., for ME, contrast with MM; for EM, contrast with EE). c,f) Different male, same female contrast showing either upregulation (red triangle, yellow cross) or downregulation (blue triangle, purple plus) of genes following mating with a non-coevolved male, focusing on the response of each male line. Panel c and e legends show the female line in the first letter and the male line the second letter. Contrast made relative to relevant coevolved cross (i.e., for ME, contrast with EE; for EM, contrast with MM).



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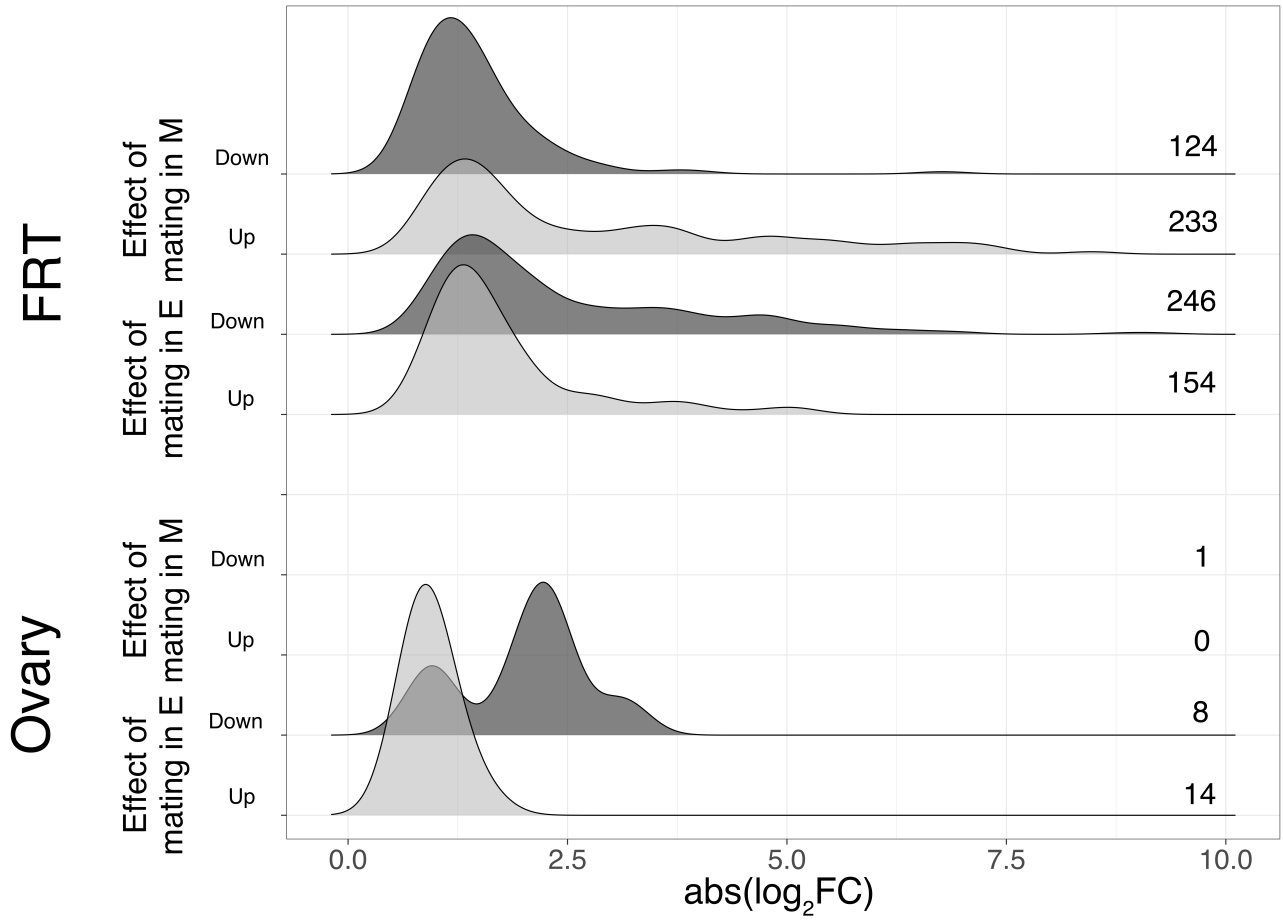
Supplementary Files

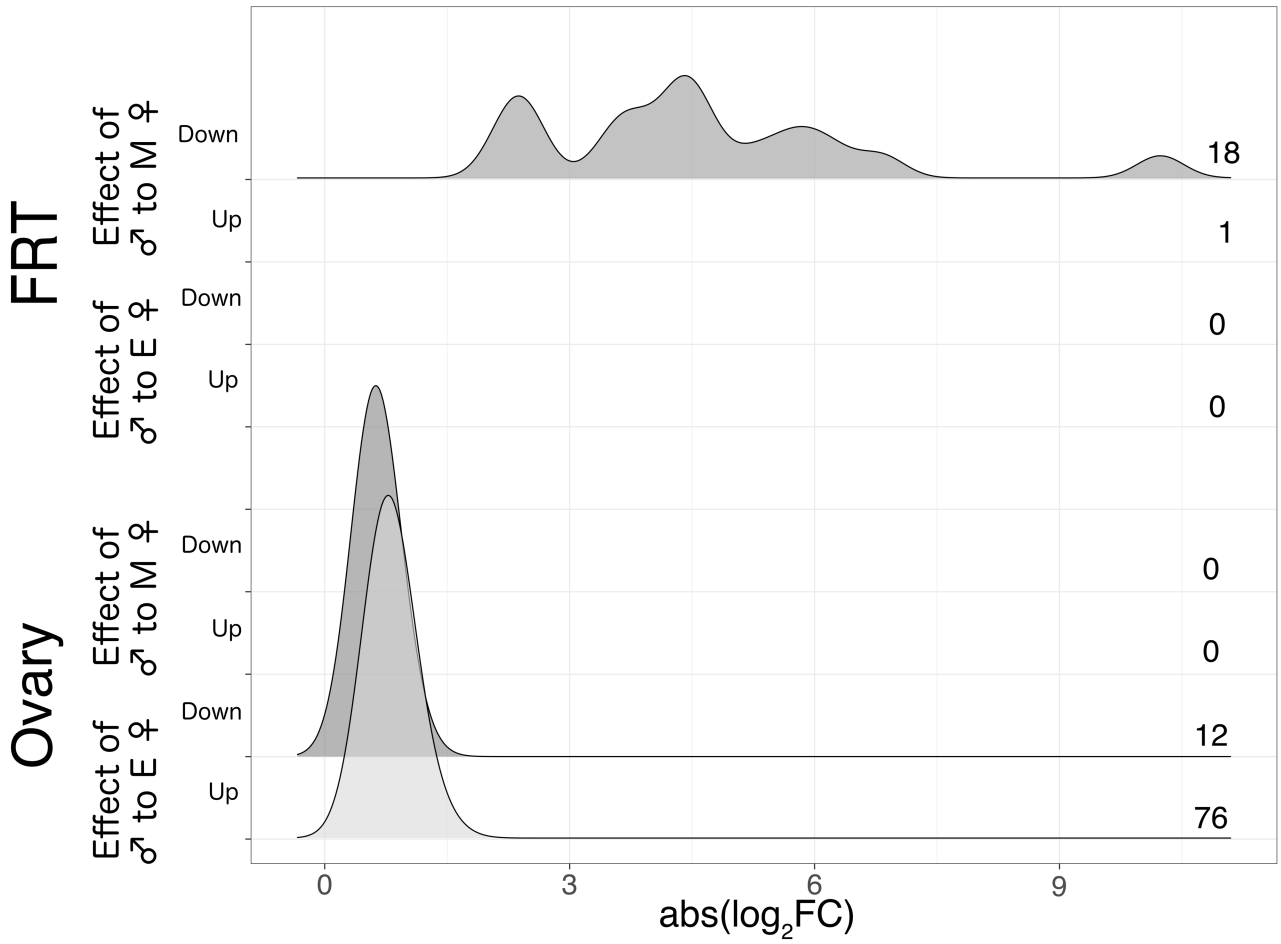
Supplementary File 1. Summary of gene ontology enrichment analysis for genes and all contrasts, shown in Figures 1 and 3 (and Figures S1-3). The order of the tabs in the file corresponds to the order of appearance of the genes in the Figures. Each tab shows all DE genes, and separately upregulated and downregulated genes. Tab names refer to Figure, tissue and contrast. Contrast names refer to the polyandry (E) and monogamous (M) lines. The virgin treatment is represented by the letter indicating the selection line, while the mated treatment is represented by two letters, starting with the sexual selection line of the female and followed by the male partner (i.e., “EM” indicates the E females mated with M males).

852 **Figure S1**. Differential gene expression (absolute log_2FC changes, y axis) and number of
853 significantly upregulated (Up) or downregulated (Down) genes (numbers to the right of the ridge
854 plot) when comparing the postmating response in the female reproductive tract (FRT) or ovaries
855 (Ovary) in monandry (M) crosses and in polyandry (E) crosses. The direction of gene expression is
856 defined relative to mated females (e.g. Down means the mated female had significantly lower
857 expression than in the virgin status; Up means the mated female had significantly higher
858 expression than in the virgin status).



859

860 **Figure S2**. Differential gene expression (absolute log_2FC changes, y axis) and number of
861 significantly upregulated (Up) or downregulated (Down) genes (numbers to the right of the ridge
862 plot) when comparing the postmating response in the female reproductive tract (FRT) or ovaries
863 (Ovary) for the female effect (i.e., when a monandry (M) female is mated to a polyandry (E) male
864 (Effect of male to M female), or when a polyandry female (E) is mated to a monandry (M) male
865 (Effect of male to the E female)). The direction of gene expression is defined relative to the
866 relevant coevolved cross (e.g. Down means the female mated to the non-coevolved male (i.e.,
867 either ME or EM) had significantly lower expression than when mated to a coevolved male (i.e.,
868 MM or EE, respectively); Up means the female mated to non-coevolved male had significantly
869 higher expression than when mated to a coevolved male).



870

Figure S3. Differential gene expression (absolute log_2FC changes, y axis) and number of significantly upregulated (Up) or downregulated (Down) genes (numbers to the right of the ridge plot) when comparing the postmating response in the female reproductive tract (FRT) or ovaries (Ovary) for the male effect (i.e., when a polyandry (E) female is mated to a monandry (M) male (Effect of M male), or when a monandry female (M) is mated to a polyandry (E) male (Effect of E male)). The direction of gene expression is defined relative to the relevant coevolved cross (e.g. Down means a female mated to the non-coevolved male (i.e., either EM or ME) had significantly lower expression than when mated to a coevolved male (i.e., MM or EE, respectively); Up means the female mated to non-coevolved male had significantly higher expression than when mated to a coevolved male).