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

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24 **Abstract**

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

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

47

48 Introduction

49 Sexual reproduction involves pre-ejaculatory and post-ejaculatory interactions between the sexes
 50 with sexual selection influencing male and female traits that mediate the fitness outcome of these
 51 interactions. While aspects of reproduction can be cooperative, the sexes also diverge over the
 52 optima of reproductive traits, such as courtship signals, fertilization and offspring production. The
 53 intensity of sexual selection is linked to the extent to which reproductive fitness optima differ
 54 between the sexes and can generate sexual antagonism, in which selection acts in opposing
 55 directions on the sexes (Rice, 1996; Holland and Rice, 1999). As the sexes share the majority of
 56 their genome, intra-locus sexual conflict can occur over phenotypes that are influenced by these
 57 shared genes. Resolving this conflict can occur by the evolution of sexual dimorphism, mediated
 58 by changes in the shared genome, such as sex-specific regulation of gene expression (Mank
 59 2017). Comparative genomic studies have found that genes with rapid divergence and that show
 60 stronger signatures of positive divergent selection are often sex-biased or sex-limited in expression
 61 (e.g. Cheng *et al.* 2016; Pröschel *et al.*, 2006; Ellegren & Parsch, 2007; Zhang *et al.*, 2007). This
 62 pattern is sometimes stronger in species that have traits associated with indicators of sexual
 63 selection, such as increased sexual dimorphism (Harrison *et al.* 2015; Wright *et al.* 2019).

64 In internally fertilizing species, the main arena for post-ejaculatory molecular interactions is the
 65 female reproductive tract which includes sites of sperm transfer, storage and subsequent
 66 fertilization of eggs transiting from the ovaries. Postmating female responses are extensive,
 67 influencing female behaviour, morphology and physiology. These responses are mediated by
 68 interactions between components of the male ejaculate, including sperm produced in the testes,
 69 rapidly evolving seminal fluid proteins (Sfps) produced by accessory glands, and female
 70 reproductive proteins in the female reproductive tract and ovaries (Sirot *et al.* 2015; Oku *et al.*
 71 2019; Wolfner 2009). Postmating changes in females include altering investment in oogenesis
 72 (Wolfner 2009), remating propensity (Chapman *et al.* 2003), and sperm storage and usage (Avila
 73 *et al.* 2010; Avila *et al.* 2015). Other aspects of physiology are also altered, for example, female
 74 hunger (Carvalho *et al.* 2006), female aggression (Bath *et al.* 2017), and homeostasis (Ribeiro and
 75 Dickson 2010; Cognigni *et al.* 2011). Genes associated with immunity and stress have altered
 76 gene expression upon mating in females, which may impact susceptibility or resistance to
 77 pathogens and/or parasites (Oku *et al.* 2019; Zhong *et al.* 2013).

78 An increasing number of studies have characterized transcriptomic postmating changes and used
 79 gene ontology to determine the associated gene functions, although many studies typically
 80 examine either whole bodies or abdomens of females (Lawniczak and Begun 2004; McGraw *et al.*
 81 2004, 2008, Innocenti and Morrow 2009; Innocenti *et al.* 2014; Hollis *et al.* 2014, 2016; Delbare *et al.*
 82 2017; Fowler *et al.* 2019) or examine only one component of the female reproductive tract
 83 (“lower” reproductive tract, defined by the female sperm storage organs, Mack *et al.* 2006,

84 Prokupek *et al.* 2008; or the “upper” reproductive tract, defined by the oviducts, Kapelnikov *et al.*
85 2008). No study has simultaneously assessed postmating effects on the sperm receiving
86 components of the female reproductive tract (the uterus and sperm storage organs) and the egg
87 production components (oviducts and ovaries). Receipt of the male ejaculate effects sperm
88 storage dynamics, oogenesis and oviposition (Sirot *et al.* 2015) so all are subject to sexual
89 selection (and sexual conflict). Likewise, for males, the main focus on the role of sexual selection
90 and sexual conflict has been on Sfp evolution given that they are among the most rapidly evolved
91 proteins known (Ahmed-Braimah *et al.* 2017; Ellegren and Parsch 2007), although sperm and the
92 cellular architecture of the testes also can be subject to rapid morphological evolution and sexual
93 selection (Lüpold *et al.* 2009).

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

107

108 Experimental sexual selection, in which populations are subjected to either polyandrous conditions
109 that facilitates sexual selection or enforced monandrous conditions that eliminate sexual selection,
110 has been used to examine the evolution of gene expression responses to sexual selection, and
111 link these to macroevolutionary patterns of sex-biased and sex-limited gene expression evolution
112 (Hollis *et al.* 2014, 2016; Immonen *et al.* 2014; Veltsos *et al.* 2017). Previous studies supported the
113 role of sexual selection in divergent sex-biased gene expression, but whether male- or female-
114 biased genes responded the most depended on species (Hollis *et al.* 2014; Immonen *et al.* 2014;
115 Veltsos *et al.* 2017; Parker *et al.* 2019; Hollis *et al.* 2019). Additionally, these studies were limited in
116 that postmating responses and/or sex-specific tissue responses were rarely examined.

117 Consequently, the understanding of how sexual selection impacts sex-limited and reproductive
118 tissue-specific gene expression, the consequences of this divergence on postmating responses,
119 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 ($\chi^2 = 3.81$, $df = 1$, $p = 0.051$) 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 ($\chi^2=1.8$, $p=0.18$), the proportion of DE genes was higher among all genes expressed in

156 accessory glands than all genes expressed in testes (0.031 vs 0.007; $\chi^2=179.32$, $df=1$, $p<0.001$).
 157 The 34 accessory gland genes with higher expression under polyandry were enriched for BP terms
 158 related to eggshell chorion assembly, development, and neuropeptide signaling (Supplementary
 159 File 1). The 46 genes upregulated in monandry males were enriched for the BP term “detection of
 160 chemical stimulus involved in sensory perception” (Supplementary File 1).

161 Sfps are some of the most rapidly evolving proteins, hypothesized to be in response to sexual
 162 selection. To examine whether Sfps were more likely to be upregulated in polyandrous males
 163 (Hollis *et al.* 2016), we compared expression levels between the sexual selection treatments of
 164 different sets of accessory gland proteins recently described for *D. pseudoobscura* (Karr *et al.*
 165 2019). Karr and colleagues identified 3281 proteins produced in the accessory gland (the
 166 accessory gland “proteome”), of which 528 had protein secretory signals (the “secretome”) and
 167 163 were orthologous to *D. melanogaster* seminal fluid proteins (putative Sfps; also referred to as
 168 the “exoproteome”). In contrast to the prediction, we found that monandry males had higher overall
 169 expression of secretome proteins compared to polyandry males ($W=21345000$, $p<0.001$; Figure
 170 2a) and there were no significant treatment effects on the proteome or Sfps. However, of the 80
 171 accessory gland genes that were differentially expressed between treatments, we found a non-
 172 significant trend towards higher expression in polyandry males (Figure 2b).

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

177 **Gene expression divergence in virgin female tissues**

178 Virgin female reproductive tracts had 120 differentially expressed genes, 66 (54) of which were
 179 upregulated in polyandry (monoandry) females (Figure 1b), a nonsignificant difference ($\chi^2 = 1.2$, df
 180 $= 1$, $p = 0.27$). The 66 genes upregulated under polyandry were enriched in GO terms associated
 181 with the immune system and egg production, such as egg activation whereas the 54 genes
 182 upregulated under monandry were enriched for multicellular reproduction biological processes
 183 (Supplementary File 1).

184 Virgin ovaries had 417 differentially expressed genes between the sexual selection treatments
 185 (Figure 1b), with significantly more genes upregulated in polyandry (299) than in monandry (118)
 186 ($\chi^2 = 78.6$, $df = 1$, $p < 0.001$). The proportion of DE genes in ovaries was significantly greater than
 187 that in FRT (0.040 vs 0.014; $\chi^2=120.1$, $df=1$, $p<0.001$). Upregulated polyandry genes were
 188 enriched in BP terms associated with eggshell chorion assembly whereas upregulated genes in
 189 monandry ovaries were enriched for negative regulation of BMP signaling pathway
 190 (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; $\chi^2=2.4425$, $p=0.12$) (Figure S1). However, polyandry females had significantly more postmating responsive genes showing downregulation ($n=246$) than upregulation ($n=154$) after mating ($\chi^2=21.16$, $p<0.001$), whereas monandry females showed the opposite pattern, with significantly more genes upregulated ($n=233$) than downregulated ($n=124$) after mating ($\chi^2=33.28$, $p<0.001$) (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 ($n=1$) ($\chi^2=19.174$, $p<0.001$). Of the 22 genes involved in postmating divergence of polyandry ovaries, 14 were upregulated after mating and 8 downregulated, not significantly different ($\chi^2=1.64$, $p=0.2$; 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).

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

275

276 ***Different female, same male***

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

289 In the female reproductive tract contrast between a coevolved MM and non-coevolved EM mating,
 290 106 of 196 genes that showed downregulation when a E female mated to a M male (Figure 3c,
 291 purple pluses) were not DE in coevolved postmating responses (Figure 3a). These downregulated
 292 genes were significantly enriched for various metabolic processes, proteolysis and antimicrobial
 293 humoral response. 213 of the 235 genes that showed relatively higher expression in E females
 294 after mating with M males (Figure 3c, orange crosses) did not change expression in coevolved
 295 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, vitellogenesis 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 ($\chi^2 = 44.73$, $df = 1$, $P < 0.001$) 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: $\chi^2=10.28$, $df=1$, $p<0.001$; E: $\chi^2=10.4$, $df=1$, $p=0.0012$).

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

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

341 An evolutionary history of polyandry previously has been suggested to select for higher expression
342 of genes affecting sperm competition and/or showing antagonistic effects on female reproductive
343 behavior. This hypothesis has been examined in male abdomens of *D. melanogaster* testing the
344 effects of experimental sexual selection on gene expression of 138 Sfps (Hollis *et al.* 2016). That
345 study found an overall lower gene expression across all Sfps in monandry-selected males, while
346 no individual Sfp was differentially expressed. Here we separate the effects of sexual selection on
347 evolutionary divergence in both accessory gland and testes. Testes are also subject to sexual
348 selection via increased sperm number for male-male competition and/or in testicular architecture to
349 support rapid sperm production (Lüpold *et al.* 2009; Pitnick *et al.* 2008). Experimental sexual
350 selection can alter relative testes size and accessory gland size, although this varies between
351 species, between studies of the same species, and has different fitness outcomes (Hosken *et al.*
352 2001; Hosken and Ward 2001; Pitnick *et al.* 2001; Wigby and Chapman 2004; Linklater *et al.* 2007;
353 Reuter *et al.* 2008; Crudgington *et al.* 2009; Hollis *et al.* 2019). Previously we found that *D.*
354 *pseudoobscura* polyandry males had evolved larger accessory glands but not testes, and
355 polyandry males had greater mating capacity (Crudgington *et al.* 2009). Although we found that
356 accessory glands had proportionally more DE genes than testes, contrary to predictions, virgin
357 monandry males had more upregulated genes both in the accessory glands and testes than
358 polyandry males. A similar result was observed when we limited our analysis to putative *D.*
359 *pseudoobscura* Sfps (either the secretome or Sfps with *D. melanogaster* orthologs; Karr *et al.*
360 2019).

361 The male genes that changed in expression in response to sexual selection treatment were
362 associated with different biological processes. In the accessory glands, polyandry males had
363 higher expression of neuropeptides, which are diverse neuronal signaling molecules that regulate
364 physiological processes and behaviour in animals (Elphick *et al.* 2018). Given that postmating
365 female responses frequently involve changes in female reproductive physiology and behavior
366 (Sirot *et al.* 2015), polyandry may generally increase investment by males in the expression of
367 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 (Crudginton *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 (Crudginton *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 (Crudginton *et al.* 2010). Virgin males and females were collected under light CO₂ 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

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

Sequencing and mapping

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

Statistical analyses

We analysed the count data using edgeR v3.18.1 (Robinson *et al.* 2010) running in R v.3.4.0 (R Development Core Team, 2007). Libraries were normalised with the default edgeR normalisation procedure (TMM) and only genes with at least 3 counts per million across all libraries used in each analysis were retained. Dispersion was measured with default parameters using a negative binomial model using only the directly contrasted libraries. We performed different contrasts, but within each we tested for mean expression differences between the significantly upregulated and downregulated genes using Mann-Whitney rank tests using R v3.4.0 (R Development Core Team 2007). We considered genes to be differentially expressed if they were below the 5% false discovery rate (FDR) threshold (Benjamini and Hochberg, 1995). We did not employ a log₂FC threshold because allometry is unlikely to influence results obtained from specific tissues (Montgomery and Mank 2016).

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

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

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

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

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616 Figures

617 **Figure 1.** Differential gene expression (absolute $\log_2$ FC changes, y axis) and number of
 618 significantly upregulated genes (numbers to the right of the ridge plot) when comparing monoandry
 619 (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).

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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842 **Supplementary Files**

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

851

Figure S1. Differential gene expression (absolute log₂FC 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) in monandry (M) crosses and in polyandry (E) crosses. The direction of gene expression is defined relative to mated females (e.g. Down means the mated female had significantly lower expression than in the virgin status; Up means the mated female had significantly higher expression than in the virgin status).

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).

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).