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.

120 In this study, we use replicate populations of *D. pseudoobscura* following over 150 generations of
121 experimental evolution in which either monandry is enforced (referred to as M), eliminating sexual
122 selection and conflict, or elevating the opportunity for polyandry (referred to as E). We take a
123 quantitative transcriptomics approach to investigate the impact of sexual selection on gene
124 expression divergence. We separate the effects between different reproductive tissues of males
125 (testes and accessory glands) and females (female reproductive tract and ovaries), and examine
126 the postmating response within the separate female reproductive tissues to determine whether
127 sexual selection acts on these similarly or differently. This approach also allows us to polarize the
128 direction of postmating gene expression changes arising from variation in sexual selection intensity
129 to then compare the female postmating response when mating occurs between individuals of the
130 different sexual selection treatments. Previous work on these populations has documented the
131 action of sexual selection and sexual conflict on phenotypic reproductive interactions between the
132 sexes (Crudgington *et al.* 2005; 2009; 2010; Debelle *et al.* 2014; Debelle *et al.* 2016; Snook *et al.*
133 2005), including those that could influence postmating responses. For example, changes in
134 accessory gland investment (Crudgington *et al.* 2009) of males and ovariole number and
135 subsequent offspring production in females (Crudgington *et al.* 2010; Immonen *et al.* 2014). Sex-
136 biased gene expression evolution has also occurred but previous studies of expression divergence
137 either examined whole bodies of only one sex (Immonen *et al.* 2014) or in heads and abdomens of
138 each sex, but not following mating (Veltsos *et al.* 2017).

139 **Results**

140 **Gene expression divergence in male tissues**

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

152 In male accessory glands, 80 genes were differentially expressed between sexual selection
153 treatments, with 34 genes upregulated in polyandry males (Figure 1a) and 46 upregulated in
154 monandrous males. While the number of DE genes did not differ between sexual selection
155 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).

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

195 **Sexual selection causes divergence in the female postmating response**

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

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

223 **Unique and shared postmating responses between sexual selection treatments**

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

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

243

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

246 ***Same female, different male***

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

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

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

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

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

329

330 **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

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

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

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

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

404 selection treatments. Differential expression of these genes could be either congruent (both sexual
405 selection treatments upregulated or downregulated these genes) or non-congruent, in which
406 monandry upregulated the postmating gene expression, whereas the same gene in polyandry
407 females was downregulated. Both upregulated and downregulated congruent genes were related
408 to fatty acid elongation and some upregulated congruent genes were related to stress/immune
409 responses. Fatty acids are critical to signaling pathways and have been associated with sex
410 pheromone synthesis and with maturation of egg follicles in *D. melanogaster* (Vrablik and Watts
411 2013). The shared responses between sexual selection treatments suggests a shared critical
412 postmating reproductive function. Non-congruent genes were enriched for immune response (see
413 later) and egg production.

414 In polyandrous species, interactions between the sexes that impact fertility are thought to be
415 particularly dynamic and coevolve, perhaps antagonistically. In turn, such coevolution may
416 generate postmating prezygotic (PMPZ) incompatibilities between populations, contributing to
417 reproductive isolation. Standard approaches in speciation research to identify reproductive
418 isolation use crosses between males and females within and between populations. Several studies
419 have compared postmating transcriptomic responses to test the hypothesis that misregulation
420 between components in the male ejaculate and female reproductive tissues could generate PMPZ
421 incompatibilities (Bono *et al.* 2011; Ahmed-Braimah *et al.* 2020). However, it is very difficult to infer
422 the historical role of different evolutionary processes from patterns of contemporary divergence
423 between species. Experimental evolution introduces fewer confounding variables but lacks the full
424 realism of natural conditions. Previous experimental evolution work has been criticized for crossing
425 different populations to infer the direction of sexual conflict (Pizzari and Snook, 2005; Rowe *et al.*
426 2005). Here we can use known coevolved population differentiation in gene expression to examine
427 the consequences on mismatches in gene expression when crossed with a non-coevolved
428 individual. The effect of mating with a non-coevolved individual depended on whether we were
429 contrasting the effect of either the male or female treatment, what female reproductive tissue was
430 being analyzed, and the sexual selection treatment itself. Regardless of these factors, most of the
431 postmating genes that were differentially expressed in non-coevolved crosses were distinct from
432 the postmating differentially expressed genes within a coevolved cross. This pattern indicates
433 much of the non-coevolved postmating response is unique, supporting substantial and rapid
434 coevolution between the male ejaculate and female reproductive tissues within each treatment that
435 differs between sexual selection treatments. Such novel mismatches may have negative fitness
436 consequences that could potentially contribute to the evolution of PMPZ reproductive isolation.

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

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

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

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

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

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

509

510 **Material and methods**

511 **Experimental evolution lines**

512 The origin, establishment, and maintenance of the selection lines are described in detail elsewhere
513 (Crudgington *et al.* 2005). Briefly, 50 wild-caught females of *D. pseudoobscura* from a population in
514 Tucson, AZ USA were brought into the laboratory and reared for three generations, then four
515 replicate lines of two different sexual selection treatments were established. We modified the
516 opportunity for sexual selection by manipulating the adult sex ratio in food vials (2.5 x 80 mm) by
517 either confining one female with a single male (enforced monogamy treatment; M) or one female
518 with six males ("elevated" polyandry treatment; E). This species is naturally polyandrous with wild-
519 caught females frequently being inseminated by at least two males at any given time (Anderson
520 1974). We successfully equalized effective population sizes between the treatments (Snook *et al.*
521 2009). At each generation, offspring were collected and pooled together within each replicate line
522 for each treatment, and a sample from this pool was used to start the next non-overlapping
523 generation in the appropriate sex ratios. Thus, this proportionally reflected the differential offspring
524 production across families within a replicate and treatment. Generation time was 28 days and all
525 populations were kept at 22°C on a 12L;12D cycle, with standard food media and added live yeast.
526 Note that 'monandry' versus 'polyandry' as used here refers to the evolutionary history under which
527 the individuals have evolved, not their current reproductive status.

528 **Sample preparation**

529 To generate experimental males and females, parents were collected from each replicate at
530 generation 157-158. We standardized for maternal and larval environments as previously
531 described (Crudgington *et al.* 2010). Briefly, parents were mated en masse in food bottles,
532 transferred to containers with oviposition plates, allowed to oviposit for 24 h, and then 48 hr later,
533 100 first instar larvae were seeded in standard food vials (Crudgington *et al.* 2010). Virgin males
534 and females were collected under light CO_2 anesthesia on the day of eclosion and kept in vials of
535 10 individuals for 5 days to ensure reproductive maturity (Snook and Markow 2001). On Day 5,
536 within a 2 h window after lights turned on, one virgin female was placed in a food vial with one
537 virgin male that was from either the same experimental replicate ("coevolved"; MM, EE where the
538 first letter is the female) or the other treatment ("non-coevolved"; ME, EM). We dissected age- and
539 circadian rhythm-matched virgin males and females from the same collections. Each treatment
540 used 100 individuals, the tissues of which were equally split into 4 separate tubes, for easy
541 pooling. For the mating treatments, males were put first in individual vials with fly food and allowed
542 to settle. Females were then added, and were dissected 6 h after the first couple mated, in the
543 order of mating, within a 2 h block. Dissections were performed under ether anaesthesia in RNA
544 later (Ambion) on ice blocks. We separated the ovaries and the remainder of the female
545 reproductive tract, including the sperm storage organs (seminal receptacle and spermathecae).
546 We refer to these different female tissue sets as ovaries and the female reproductive tract. The
547 male accessory glands and testes were dissected separately (ejaculatory bulbs were not included).
548 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 ($\log_2$FC) 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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613 analysis are available at https://osf.io/z7fm9/?view_only=054171ba3f534f839a0814fa1b8f9f61. We
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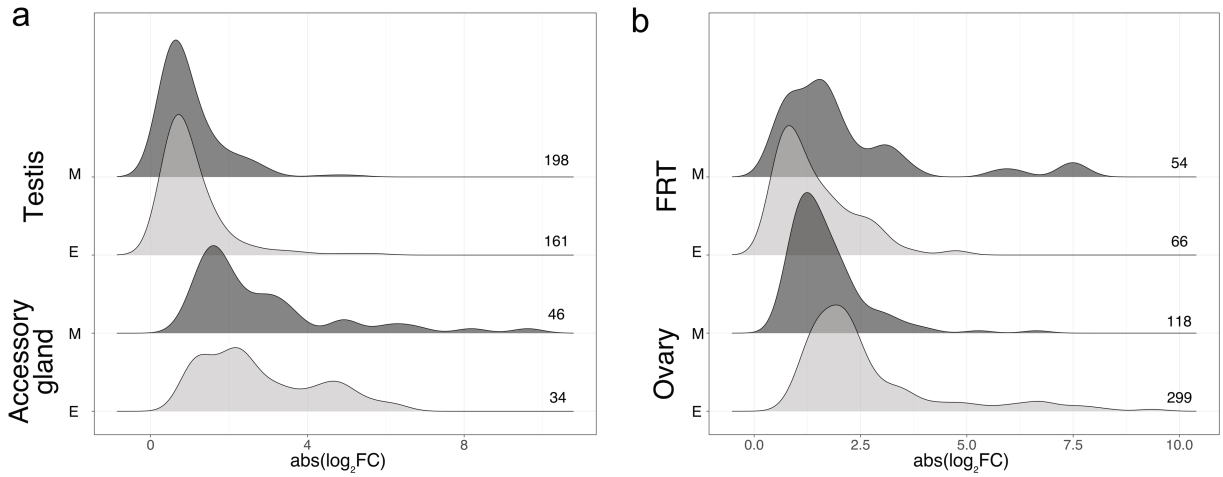
615 experimental sexual selection lines in RRS's lab and R Axel W Wiberg for help with dissections.

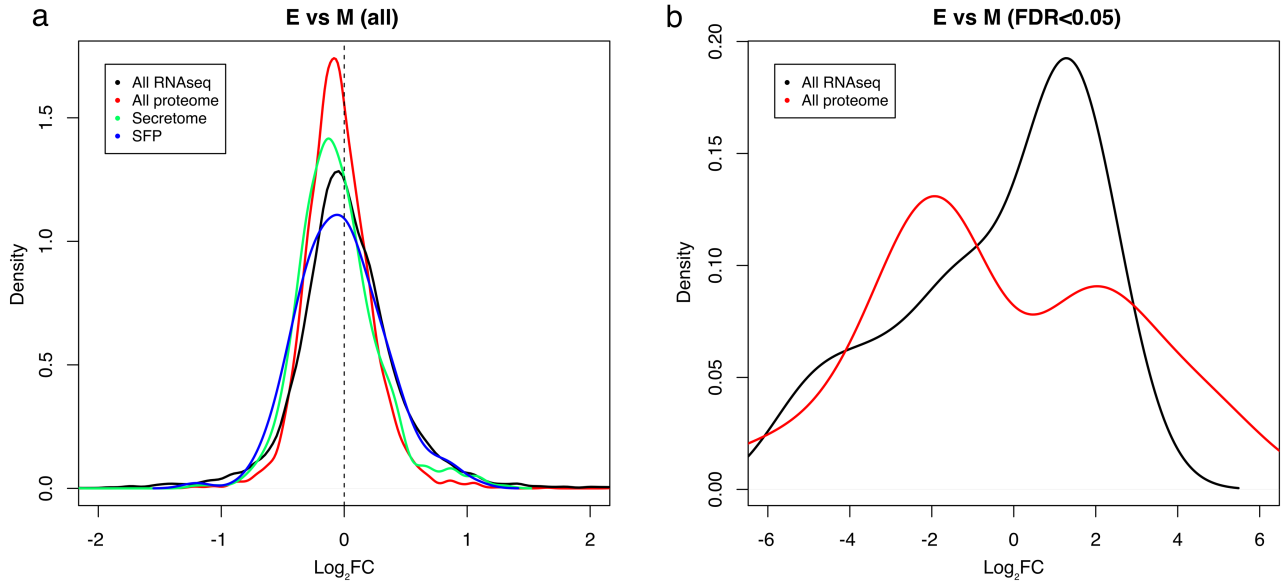
616 **Figures**

617 **Figure 1**. Differential gene expression (absolute log_2FC 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.



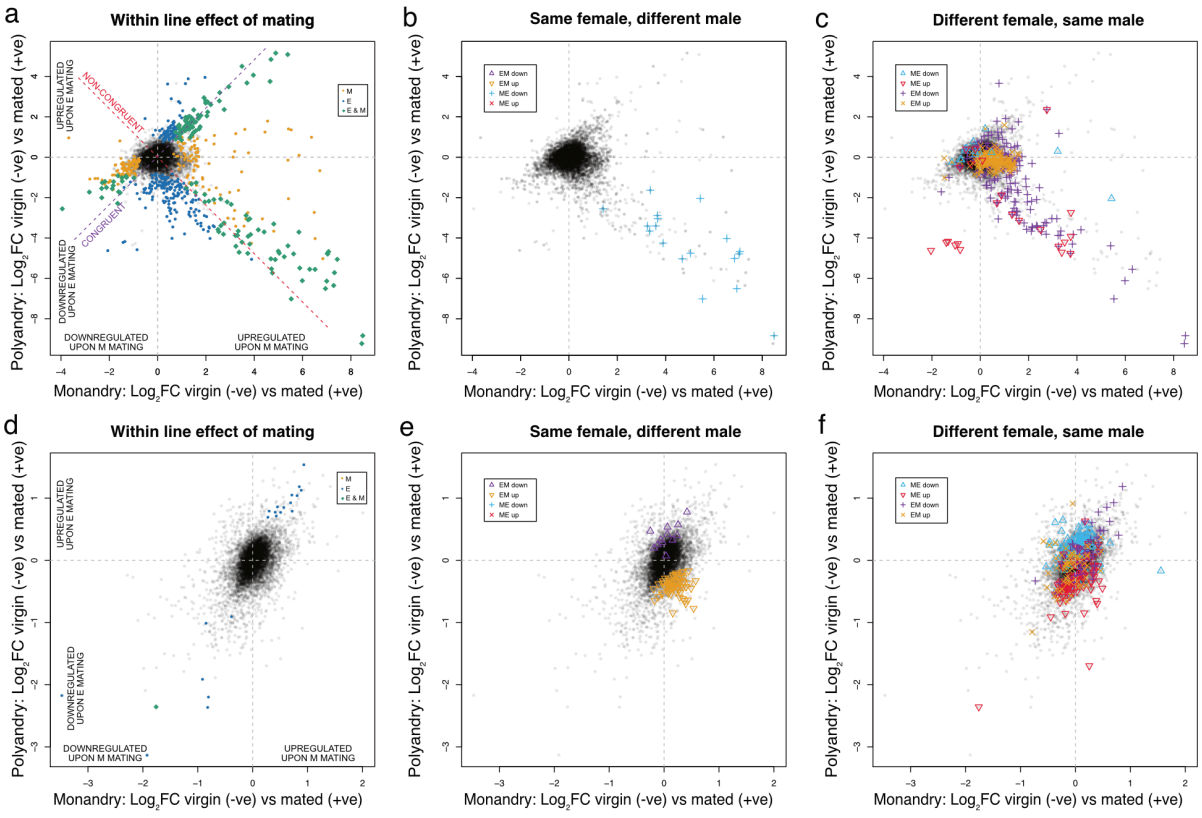
620

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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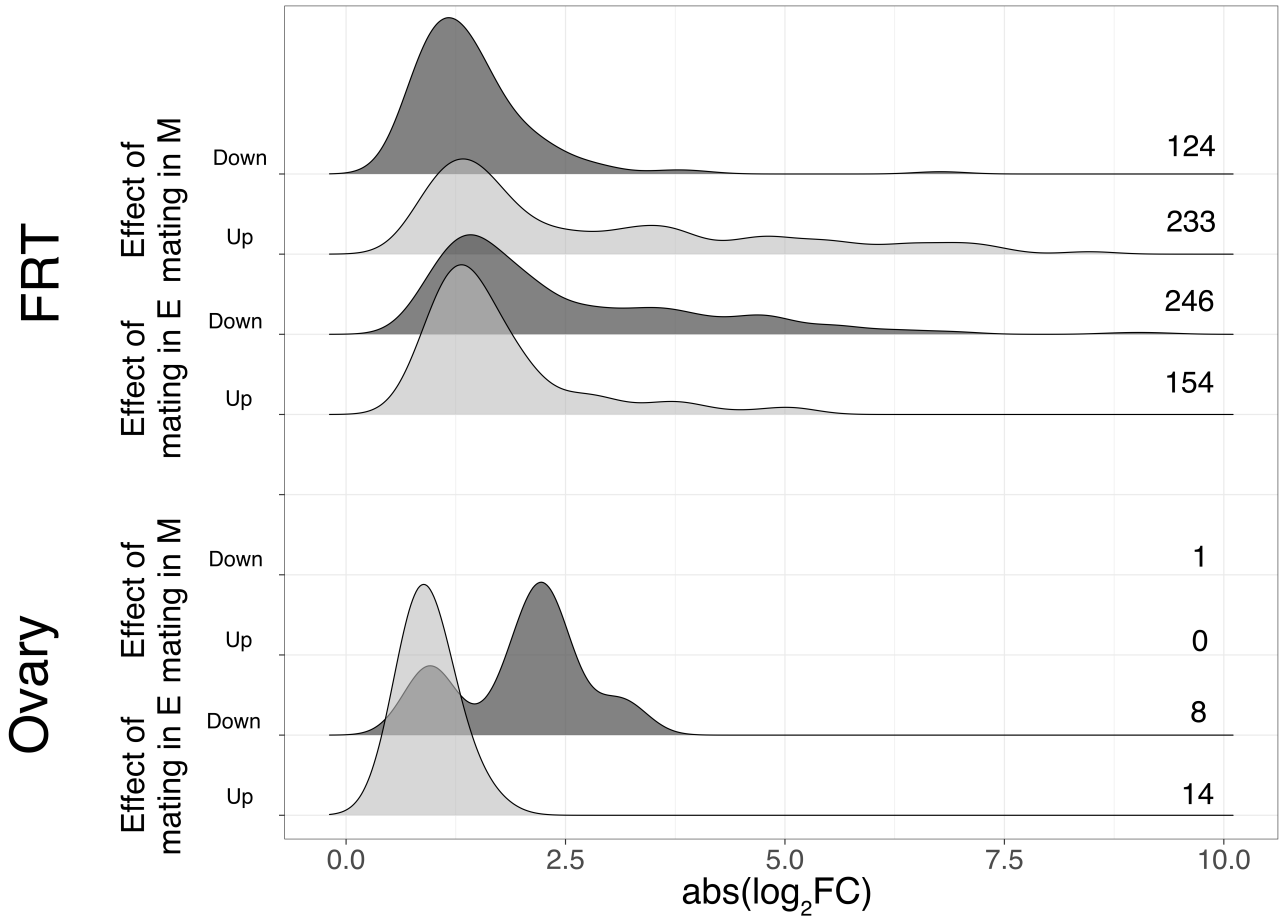
842 **Supplementary Files**

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

852 **Figure S1**. Differential gene expression (absolute $\log_2$FC 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

Figure S2. 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 female effect (i.e., when a monandry (M) female is mated to a polyandry (E) male (Effect of male to M female), or when a polyandry female (E) is mated to a monandry (M) male (Effect of male to the E female)). The direction of gene expression is defined relative to the relevant coevolved cross (e.g. Down means the female mated to the non-coevolved male (i.e., either ME or EM) 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).

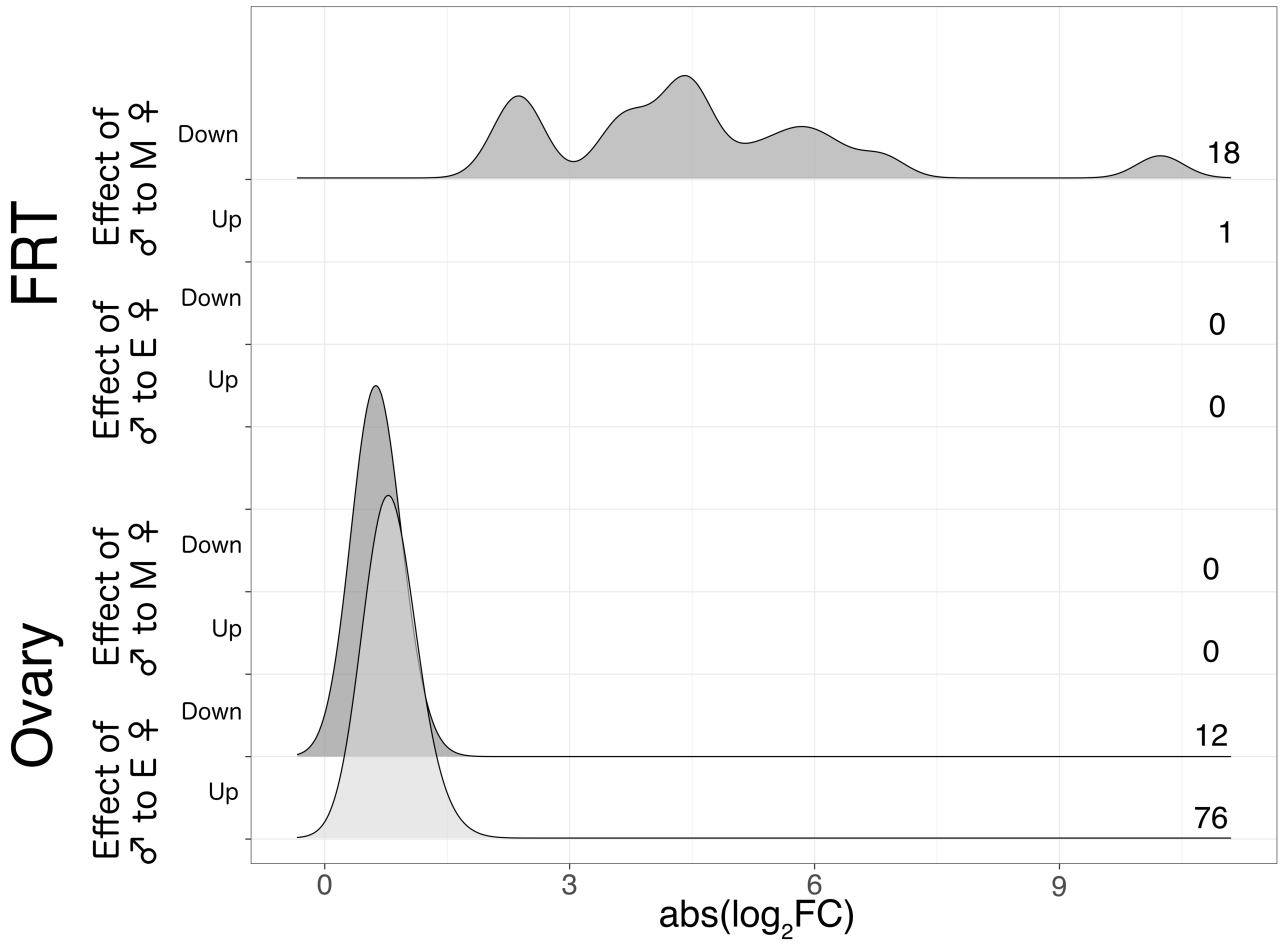
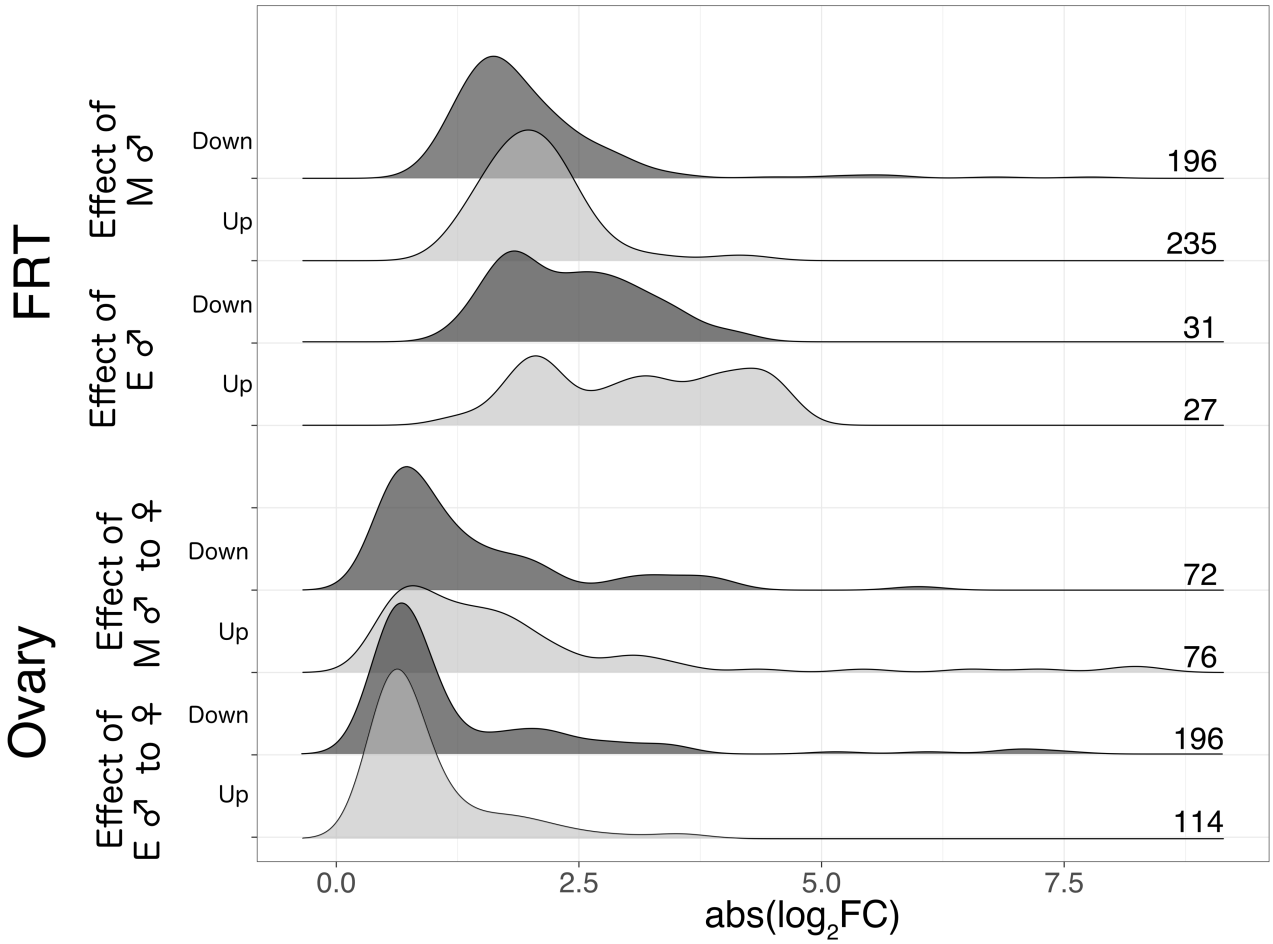



Figure S3. Differential gene expression (absolute $\log_2$FC changes, y axis) and number of significantly upregulated (Up) or downregulated (Down) genes (numbers to the right of the ridge plot) when comparing the postmating response in the female reproductive tract (FRT) or ovaries (Ovary) for the male 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).