The evolution of sexual dimorphism in gene expression in response to a manipulation of mate competition

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Running head: Mate competition and gene expression evolution

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Abstract

Many genes are differentially expressed between males and females and patterns of sex-biased gene expression (SBGE) vary among species. Some of this variation is thought to have evolved in response to differences in mate competition among species that causes varying patterns of sex-specific selection. We used experimental evolution to test this by quantifying SBGE and sex-specific splicing in 15 *Drosophila melanogaster* populations that evolved for 104 generations in mating treatments that removed mate competition via enforced monogamy, or allowed mate competition in either small, simple or larger, structurally more complex mating environments. Consistent with sex-specific selection affecting SBGE, initially sex-biased genes diverged in expression more among treatments than unbiased genes, and there was greater expression divergence for male- than female-biased genes. It has been suggested the transcriptome should be 'feminized' under monogamy because of the removal of sexual selection on males; we did not observe this, likely because selection differs in additional ways between monogamy vs. polygamy. Significant divergence in average expression dimorphism between treatments was observed and, in some treatment comparisons, the direction of the divergence differed across different sex-bias categories. There was not a generalized reduction in expression dimorphism under enforced monogamy.

Keywords: sex-biased gene expression, sex-specific splicing, sexual selection, sexual conflict, *Drosophila*,

43 **Introduction**

44 In many animals, substantial differences between the sexes exist in a myriad of phenotypes involving
45 morphology, behaviour, physiology, and life history. Dimorphism in these phenotypes arises, at least in
46 part, from sex differences in expression of the underlying genes, which themselves are another set of
47 phenotypes for which sexual dimorphism can be considered. Studies across many taxa report the
48 existence of sex-biased expression in a large fraction of genes for any given tissue and/or developmental
49 stage (Grath & Parsch, 2016). However, the extent of transcriptional dimorphism can vary considerably
50 between species (Ingleby et al., 2015), echoing patterns of variation in phenotypic dimorphism.

51 Contrasting selection between the sexes is widely believed to be the major reason for sexual
52 dimorphism. Sex-specific optima exist for many traits, causing intralocus sexual conflict over optimal
53 expression of underlying genes that are shared between males and females (Bonduriansky & Chenoweth
54 2009). Such conflict can be resolved via the evolution of sex-biased gene expression (Parsch & Ellegren,
55 2013). The different reproductive strategies of males and females are thought to be a major source of
56 such sex-specific selection. In particular, selection arising from mate competition, including pre- and
57 post-copulatory sexual selection, often differs between the sexes, shaping the traits that mediate intra-
58 and intersexual interactions. We use "mating system" to refer to the set of inter- and intrasexual
59 interactions related to mating and reproduction.

60 Mating systems vary widely among species and, hence, this variation is presumed to be a major source
61 of variation in sexual dimorphism, though this has received limited direct attention (Fernandes Martins
62 et al., 2017). At the expression level, Harrison et al. (2015) used a comparative approach to examine the
63 effect of mating system on variation in dimorphism among six bird species. The proportion of genes that
64 were male-biased was positively correlated with presumed indices of both pre- and post-copulatory
65 sexual selection (e.g., sexual ornamentation, sperm number, residual testis mass). Further, the rate of
66 turnover of male-biased genes was positively associated with male sexual ornamentation. This suggests
67 that sexual selection drives the evolution of expression dimorphism. Further evidence consistent with
68 the importance of mating system comes from studies showing that sex-biased gene expression differs
69 between alternative reproductive morphs, for example between 'dominant' vs. 'auxiliary' males (Dean
70 et al., 2017; Pointer et al., 2013; Stuglik et al., 2014).

71 Experimental evolution offers a powerful means to directly test whether a change in mating system
72 drives evolutionary changes in the transcriptome. In one such study, Hollis et al. (2014) subjected

replicate populations of the naturally polygamous *D. melanogaster* to experimental evolution under two mating systems: polygamy and enforced monogamy (i.e., randomly assigned male-female pairings). The motivation for imposing monogamy was to eliminate sexual selection on males, which is thought to be the primary reason why selection differs between the sexes. Hollis et al. predicted that in the absence of sexual selection on males, a population would no longer be constrained by conflicting selection between the sexes, and hence phenotypes in males and females would evolve towards female optima. Assuming existing patterns of sex-biased gene expression represent only a partial resolution of intralocus sexual conflict, they predicted that evolution under monogamy would result in 'feminisation' of the transcriptome (i.e., upregulation of female-biased genes and down-regulation of male-biased genes) in both sexes. These predictions were borne out, with expression being feminised in males and females under enforced monogamy compared to polygamy. However, a similar evolution experiment using *D. pseuodoobscura* found results that were very different from these predictions, with expression being largely masculinised in populations evolving under monogamy compared to polygamy (Veltsos et al., 2017).

Though Hollis et al. (2014) used enforced monogamy with the goal of eliminating sexual selection on males, such manipulations of mating systems are likely to have additional consequences on selection more broadly in both sexes (Rowe & Rundle, 2021). Males are known to inflict harm on females, for example through persistent courtship and toxic seminal fluid proteins (Fowler and Partridge 1989, Partridge and Fowler 1990; Chapman et al. 1995, Arnqvist and Rowe 2005). Under enforced monogamy, there should be strong selection on males to be less harmful to females, and experimental evidence supports this (Holland and Rice 1999; Yun et al. 2021). Selection on females also likely differs between these mating systems. Reduced harm by males may subsequently yield selection against costly female traits involved in avoiding or reducing male harm (Wigby & Chapman, 2004). In addition, under some polygamous but not monogamous conditions, high-quality females may suffer a 'cost of attractiveness' as a result of being the targets of preferential male harassment (Long et al., 2009; Yun et al. 2017, MacPherson et al., 2018), ultimately weakening natural selection through females. A change from polygamy to enforced monogamy is thus likely to alter selection in both sexes in a variety of ways, such that contrasts between these mating systems are more nuanced than simply the presence vs. absence of sexual selection. Expression changes may therefore be more difficult to predict than Hollis et al. (2014) suggested. Further complicating this contrast is that 'polygamy' can take many forms, characterized by differences in inter- and intrasexual interactions that occur under different contexts (e.g., when reproductive interactions and mating happen in different environments).

105 Here, we analyze sex-biased gene expression in replicate populations of *D. melanogaster* evolved in
106 three treatments that varied with respect to mating system. One treatment involved the absence of
107 mate competition (MCabs) via the application of enforced monogamy. The remaining two treatments
108 allowed for mate competition, but in distinct 'mating environments'. In the first of these treatments
109 (MCsim), mate competition occurred in a relatively simple environment (e.g., *Drosophila* vials), similar to
110 the 'polygamy' treatment of earlier studies (i.e., Hollis et al. 2014; Veltsos et al. 2017). In the third
111 treatment (MCcom) mate competition occurred in larger, less dense containers with multiple food
112 sources and greater spatial complexity, presumably allowing females to more readily evade males.
113 Consistent with this, in the 'complex' relative to the 'simple' mating environment, intersexual
114 interactions and mating are less frequent, males are less harmful to females, and females no longer
115 suffer a 'cost of attractiveness' that weakens viability selection on them in the simple environment (Yun
116 et al. 2017, 2019; MacPherson et al. 2018). Populations maintained in the complex mating environment
117 also evolved males that are less harmful to females compared to their counterparts from the simple
118 mating environment (Yun et al. 2021), yet these males are highly successful in siring offspring in
119 competition with other males (Yun et al 2019).

120 Here we analyze gene expression divergence among these three mating treatments from several
121 perspectives. We begin by asking whether divergence among mating treatments is random across the
122 transcriptome with respect to pre-existing sex-bias in expression. If sex-biased gene expression (SBGE)
123 evolves because of differential selection arising from mating interactions, then one would expect that
124 sex-biased genes would be more likely to diverge among mating treatments than would unbiased genes.
125 Second, we contrast female- vs. male-biased genes. Past studies have documented that male-biased
126 genes diverge in expression more rapidly among populations or species than female-biased genes (e.g.,
127 Meiklejohn et al. 2003, Zhang et al. 2007, Allen et al. 2018), but this is yet to be considered in relation to
128 variation in mating system. We ask whether male-biased genes are more likely to diverge than female-
129 biased genes in response to changes in mating system.

130 Third, we examine the directionality of expression changes from the perspective of Hollis et al. (2014),
131 testing their prediction that enforced monogamy should lead to a feminization of the transcriptome
132 relative to polygamous mating systems in which there is a much greater opportunity for sexual selection
133 on males. We compare our enforced monogamy treatment with each of two different polygamous
134 mating treatments in which mate competition occurs in different environments. We also compare the

135 latter two mate competition treatments with one another, asking whether they differ with respect to
136 transcriptome feminization or masculinization.

137 Changes in dimorphism can occur because of changes in just one sex or both. Moreover, examination of
138 dimorphic traits in each sex separately can provide clues as to whether dimorphism is hindered by
139 intersexual genetic covariances (Lande 1980; Prasad et al. 2007). For such reasons, we examine gene
140 expression in each sex separately, as have past studies (Hollis et al. 2014; Veltsos et al. 2017). For
141 example, Hollis et al. (2014) reported feminization in the transcriptomes of females, and also of males,
142 under enforced monogamy. However, reporting results in this way alone does not provide a clear view
143 of how dimorphism itself changed (this was not a goal of past work). For example, feminization in both
144 sexes under enforced monogamy could mean that dimorphism had increased, decreased, or remained
145 constant, depending on the relative magnitudes of the changes within each sex. Though we are
146 interested in the evolutionary divergence of dimorphic traits within each sex, like past studies (Hollis et
147 al. 2014; Veltsos et al. 2017), we are also interested in whether mating system affects dimorphism per
148 se, so we also explicitly compare dimorphism across mating treatments.

149 We examine the above questions with respect to expression dimorphism in whole bodies as well as
150 heads. In being less sexually dimorphic, heads offer a point of contrast. Finally, in addition to examining
151 SBGE, we also examine some of these questions with respect to another form of expression
152 dimorphism, sex-specific splicing (SSS), i.e., quantitative differences between the sexes in the relative
153 usage of different isoforms.

154

155 **Methods**

156 *Samples for RNAseq*

157 We used 15 populations from a previously described evolution experiment (Yun et al. 2018, 2019, 2021)
158 that were created in September 2014 by sampling from a single laboratory stock population of *D.*
159 *melanogaster*. During experimental evolution, all populations used here were raised under the same
160 larval conditions consisting of standard cornmeal medium supplemented with 5% NaCl (6% after
161 generation 6) and a constant exposure to 28 C (rather than the standard 25 C of the ancestor) during
162 larval development. The 15 populations were divided equally among three treatments that manipulated
163 the mating system that adults experienced, including the opportunity for mate competition and the
164 environment in which this occurred. The treatments were: mate competition absent (MCabs) via

165 randomly assigned single-pair monogamy, and two polygamy treatments in which mate competition
166 was permitted either in small, structurally simple *Drosophila* vials (MCsim) or in larger, 1.65-L cylindrical
167 containers with added structural complexity (MCcom).

168 Populations were maintained via 3-week non-overlapping generations. Within a given population, 140
169 male and 140 female adults spent 6 days each generation in their respective mating treatment. In
170 MCabs, this involved 140 male-female pairs separately allocated to individual wide-mouth straws, while
171 in the polygamy treatments this involved four groups of 35 males and 35 females, each group being held
172 in either separate vials (MCsim) or separate containers (MCcom). Within each of the MCcom containers
173 were five small cups of food (with plastic barriers inserted into the food that further subdivided the
174 surface) and two coiled pipe cleaners, anchored in the lid, that extended into the interior space. After 6
175 d in these mating treatments, males were discarded and 105 of the surviving females were randomly
176 allocated among seven vials to lay eggs for ~24 h. Females were subsequently discarded and the
177 resulting offspring developed under the same larval conditions in all populations (i.e., cornmeal food
178 with added salt at 28 C). Adults that eclosed were used to create the next generation's mating
179 treatments.

180 After 104 generations of selection, samples for RNAseq were obtained as follows. Flies were reared
181 under a controlled density of 40 larvae per vial on a benign yeast-agar food (no salt). Adults emerged
182 eight days after hatching and were collected under light CO_2 (< 20 seconds) as virgins (within 8 hours of
183 emergence) and then held in single-sex vials at low density (10 flies per vial). Two days later, flies were
184 processed. For whole body samples, flies were transferred under light CO_2 (< 20 sec) to microcentrifuge
185 tubes (10 flies per tube). A few minutes later (and well after awakening from CO_2) tubes were flash
186 frozen in liquid nitrogen and then stored at -80 C. For head samples, the same procedure was used
187 except after flash freezing, tubes were vortex shaken for ca. 10 s to separate heads from bodies. Heads
188 were transferred to a new microcentrifuge tube (8-10 heads per tube) and then stored at -80 C. RNA
189 extraction was performed using ThermoFisher PicoPure RNA Isolation Kit. Paired-end (100 bp)
190 sequencing was performed using Illumina NovaSeq S4. For each of the 15 populations, there was a
191 single replicate of each of the four sample types (i.e., body and head samples of each sex).

192 One of the female replicates from MCsim treatment was found to have elevated expression of Y-linked
193 genes, indicating possible contamination with male tissue. Consequently, we excluded this replicate
194 from all further analyses.

Differential Expression between Mating Treatments

Differential expression was analysed between each pair of mating treatments, separately for each sex and tissue. Each RNAseq file was aligned to the *D. melanogaster* reference genome (dos Santos et al., 2015) using STAR v2.7 (Dobin et al., 2013) with default parameters. The resulting alignment files were processed with *htseq-count* to obtain gene-level read counts for each sample (Anders et al., 2015). The R package *DESeq2* (Love et al., 2014) was used to estimate differential expression between pairs of mating treatments. For all genes averaging >50 reads across all replicates, we estimated the $\log_2$ fold change in expression between treatments (henceforth, 'treatment effect'). For any genes thus tested, *DESeq2* yields an adjusted p-value by applying Benjamini and Hochberg corrections for multiple testing. A gene was designated to have significant differential expression between treatments if the treatment effect was accompanied by an adjusted p-value < 0.1. Such genes are called 'Treatment Differentially Expressed genes' or 'TDE genes'.

Sex-Biased Gene Expression

In analyses where SBGE is treated as an independent variable, we used an external dataset (Osada et al., 2017) to characterise genes with respect to sex-biased gene expression for our analyses to avoid circularity that could generate spurious associations if the same data contribute to both dependent and independent variables. This choice for an external dataset is not critical for our purposes; though expression in this external dataset undoubtedly differs somewhat from our own, variation among genes in SBGE tends to be much larger than variation in SBGE for a gene across studies (e.g., genes that are male- or female-biased in one study are generally male- or female-biased in other studies, though they may vary quantitively the magnitude of bias). Even across species separated by millions of years, among-gene SBGE is strongly correlated (Zhang et al. 2007).

The Osada et al. RNAseq dataset consisted of data for whole bodies and heads for a male and female sample from each of 18 lines. Gene-level read counts were obtained as described above. Sex-biased gene expression was estimated using the R package *DESeq2* (Love et al., 2014). In estimating differential expression between sexes, we excluded all genes which averaged <50 reads across all male and female replicates in the given tissue. *DESeq2* yields $\log_2$ estimates of fold change in male to female expression ("$\log_2$ FC male/female"). For some analyses, we assigned genes into one of three sex bias categories: female-biased ($-\infty < \text{log2FC} \leq -0.5$), unbiased ($-0.5 < \text{log2FC} \leq 0.5$), and male-biased ($0.5 < \text{log2FC} < \infty$).

225

226 *Differential Isoform Usage Analysis*

227 In addition to analysing changes to total gene expression, we also considered changes in patterns of
228 alternative splicing using the R package *JunctionSeq* (Hartley and Mullikin 2016). *JunctionSeq* utilises
229 read count data from *QoRTs* (Hartley & Mullikin, 2015), which partitions genes into exons and splice
230 junctions. It then counts the number of reads that overlap an annotated feature (i.e., exon or splice
231 junction). Then, through *JunctionSeq*, generalised linear models (GLM) are used to test for differential
232 splicing. At the gene level, corrections for multiple testing are applied through the Benjamini and
233 Hochberg method, yielding an adjusted p-value for each gene.

234 We used *QoRTs* to gather read count data from alignment files, previously obtained using *STAR* aligner
235 on the RNA-seq data. Following this, we utilised *JunctionSeq* to analyse differential isoform usage
236 between the sexes, as well as between treatments. In both, we restricted our analyses to features which
237 averaged >50 reads across all replicates. A gene was considered to have significant differential isoform
238 usage between sexes if the gene-wise p-adjusted was below 0.1. For comparisons between treatments,
239 a gene was considered to have significant differential isoform usage if p-adjusted < 0.1. The latter set of
240 genes are referred to as 'TDS genes' (for 'Treatment Differentially Spliced genes').

241

242 *Identification of Gonad-Specific Genes*

243 Patterns of differential expression or isoform usage in whole bodies might be driven in part by the
244 gonads. To examine this possibility, we repeated our analyses for the whole bodies without genes that
245 are expressed specifically in the gonads ('gonad-specific genes', GSGs). We identified GSGs using tissue-
246 specific estimates of gene expression from the supplementary data of Witt et al. (2017), which in turn is
247 based on RNAseq data from FlyAtlas2 (Leader et al., 2018). Each gene's expression in a tissue was
248 expressed in terms of FPKM. We estimated a 'gonad specificity index' (GSI) separately for males and
249 females, as per the following expression:

250 $$\mathrm{GSI_j} = \frac{\mathrm{FPKM_{gonads,j}}}{\Sigma_i \mathrm{FPKM_{i,j}}},$$

251 where *j* = sex and *i* = tissue. For the denominator, we only included FPKMs from non-overlapping tissues.
252 Any gene that yielded a GSI > 0.95 in either sex was designated as gonad-specific. We then proceeded to
253 repeat our analyses excluding this set of genes.

254

255 **Results**

256 *Divergence in expression is non-random with respect to SBGE*

257 We first examined the frequency of SBGE within each mating treatment. The frequency of genes with
258 significant sex bias in expression was similar across treatments in whole bodies and in heads (Table 1).
259 Though we found statistically significant variation in the frequency of SBGE among treatments in the
260 whole body, these differences are not drastic. This is not surprising because, on a short
261 microevolutionary time, one would not expect large scale expression divergence that would cause genes
262 to change categories of SBGE (especially given that SBGE is fairly consistent across species; Zhang et al.
263 2007). However, this does not preclude the possibility of many subtle, quantitative changes in
264 expression.

265

266

Table 1: Frequency of genes with significant sex-biased gene expression (p-adj < 0.05) and significant sex-specific splicing (SSS; p-adj < 0.1) for whole bodies and heads. P-values are from Fisher's exact tests for non-random association between the percentage of genes with SBGE/SSS and mating treatments. For sex-biased gene expression, the numerator includes only genes that are sex-biased (i.e., $|\log_2 FC| > 0.5$) with p-adj < 0.05, while the denominator includes all genes.

	Sex-biased gene expression (SBGE)					**Sex-specific splicing (SSS)**				
		% with SBGE					**% with SSS**			
Tissue	**# of genes**	**MCabs**	**MCsim**	**MCcom**	**p-value**	**# of genes**	**MCabs**	**MCsim**	**MCcom**	**p-value**
Body	11,717	88.4%	87.6%	87.2%	**3.4×10^{-5}**	7,250	49.8%	48.1%	49.0%	0.097
Head	9,356	9.8%	10.4%	10.5%	0.56	7,547	4.2%	13.6%	10.4%	**$<2.2 \times 10^{-16}$**

To that end, we performed pairwise contrasts of mating treatments within each sex to identify genes that were differentially expressed between treatments (hereafter 'Treatment Differentially Expressed', TDE, genes). Though a relatively small number of genes qualified as TDE (Table S1), we considered whether these TDE genes are disproportionally represented among different categories of sex-biased expression. If genes that are sex-biased had previously evolved to be so because of a history sex-differential selection arising from mate competition, one might expect that sex-biased genes would be disproportionately likely to diverge among populations evolving in varying mating treatments. However, we may not expect male- vs. female-biased genes to be equally likely to diverge in expression among mating treatments, given results of past studies: relative to the female-biased and unbiased genes, male-biased genes have been reported to have higher rates of evolution in sequence (Zhang et al., 2004) and expression (Meiklejohn et al., 2003).

In female bodies, TDE genes are significantly more common among sex-biased than unbiased genes (Table 2), with the exception of the MCabs-MCsim comparison for which too few TDE genes exist to draw any conclusions. No significant difference is observed in the frequency of TDE genes among unbiased vs. sex-biased genes in comparisons for male bodies, however. Comparing male- vs. female-biased genes, TDE genes are significantly more frequent among male-biased genes in both male and

292 female body samples (again ignoring the MCabs - MCsim comparison due to the low total number of
293 TDE genes). Both of these patterns hold to some degree in heads too: in those comparisons with
294 statistically significant differences, TDE genes are more common among sex-biased than unbiased genes
295 and TDE genes are more common among male- than female-biased genes.
296
297 A possible proximate mechanism for the body results is a change in the relative sizes of gonads (Mank,
298 2017). We repeated the analysis presented in Table 2 after excluding genes with highly gonad-specific
299 expression. The results remain similar (Table S2). While this does not rule out the possibility that
300 changes in gonads play an important role in expression divergence among treatments, the fact that the
301 patterns are not weakened after removal of gonad-specific genes—along with the existence of the
302 patterns in heads—suggests that there is more to the underlying mechanisms behind expression
303 divergence than solely a change in gonad size.
304
305 We analysed the TDE genes for significant enriched biological processes or functions using a gene
306 ontology enrichment test (Eden et al., 2009), but failed to find any significant associations. However,
307 among the 574 TDE genes in total, we found 16 genes that putatively code for seminal fluid proteins
308 (SFPs), identified in a recent study of the seminal fluid proteome (Wigby et al., 2020). One of these 16
309 putative SFP genes (CG42807) was divergently expressed in two pairwise comparisons (MCabs vs
310 MCcom, MCsim vs MCcom).
311
312
313

Table 2: Results of Fisher's exact tests for the proportion of treatment differentially expressed (TDE) genes in each sex bias category. Values in parentheses represent the fraction of TDE genes out of all genes in the sex bias category. Genes were assigned to sex-bias categories based on external data source.

Comparison	Percent TDE genes		p-value (UB vs SB)	Percent TDE genes		p-value (MB vs FB)
	UB	SB		FB	MB	
A) Female body						
MCabs vs MCsim	0% (0/1693)	0.03% (2/7025)	1	0.03% (1/3721)	0.03% (1/3304)	1
MCabs vs MCcom	0.5% (9/1693)	2.0% (137/7025)	**7.0×10^{-6}**	0.7% (26/3721)	3.4% (111/3304)	**2.3×10^{-16}**
MCsim vs McCom	1.2% (21/1693)	2.5% (177/7025)	**1.0×10^{-3}**	0.8% (29/3721)	4.5% (148/3304)	**$<2.2 \times 10^{-16}$**
B) Male body						
MCabs vs MCsim	0.5% (9/1699)	0.25% (23/9101)	0.082	0.1% (5/3546)	0.3% (18/5555)	0.13
MCabs vs MCcom	1.6% (27/1699)	1.5% (139/9101)	0.83	0.6% (21/3546)	2.1% (118/5555)	**1.1×10^{-9}**
MCsim vs MCcom	0.7% (12/1699)	0.8% (75/9101)	0.77	0.3% (9/3546)	1.2% (66/5555)	**3.0×10^{-7}**
C) Female head						
MCabs vs MCsim	0.7% (54/7767)	3.7% (32/872)	**1.5×10^{-11}**	3.4% (24/698)	4.6% (8/174)	0.50
MCabs vs MCcom	0.3% (27/7767)	0.8% (7/872)	0.076	0.4% (3/698)	2.3% (4/174)	**0.032**
MCsim vs MCcom	0.3% (26/7767)	1.0% (9/872)	**6.6×10^{-3}**	0.9% (6/698)	1.7% (3/174)	0.39
D) Male head						
MCabs vs MCsim	0.5% (35/7774)	1.1% (10/874)	**0.021**	1.2% (8/688)	1.1% (2/186)	1
MCabs vs MCcom	1.4% (108/7774)	3.0% (26/874)	**1.2×10^{-3}**	2.2% (15/688)	5.9% (11/186)	**0.013**
MCsim vs MCcom	0.7% (56/7774)	1.9% (17/874)	**1.2×10^{-3}**	1.7% (12/688)	2.7% (5/186)	0.38

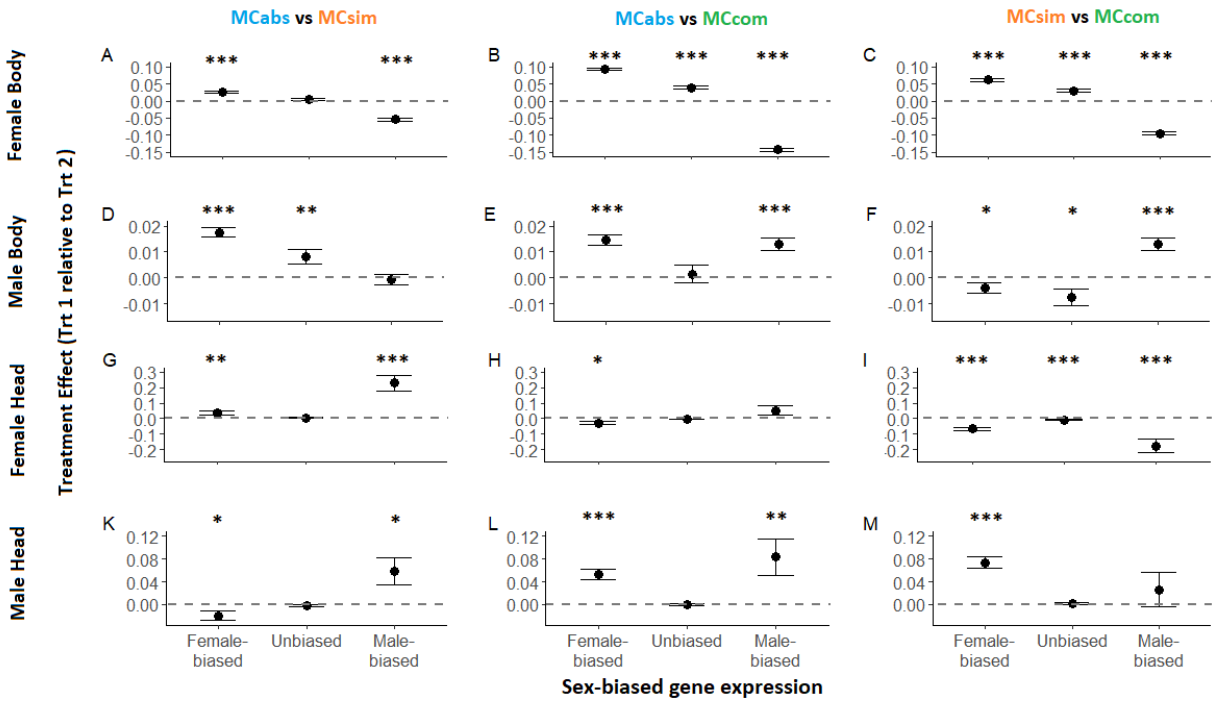
320

321 *Divergence cannot be summarized as feminization/masculinization of the transcriptome*

322 In the previous section we tested whether different types of genes (with respect to sex-bias) are more
323 likely to diverge, but we did not test for patterns in the direction and magnitude of divergence. In their
324 study, Hollis et al. (2014) predicted that, relative to polygamy, expression would be feminized in
325 populations evolving in monogamy, i.e., female-biased genes evolve to be upregulated in monogamy,
326 while male-biased genes become downregulated. Similar patterns are predicted in both female and
327 male tissues due to the presumed shared genetic architecture that constrains dimorphism under any
328 given mate competition regime (Prasad et al. 2007; Hollis et al. 2014).
329
330 We evaluated expression divergence in our populations from this Hollis et al. perspective by examining
331 divergence separately for female- and male-biased genes, focusing on the contrasts of our monogamy
332 treatment (MCabs) with each the two polygamy treatments (MCsim, MCcom). To visualise and test for
333 patterns of transcriptomic feminization/masculinization, we used the 'treatment effect' for each gene as
334 a measure of expression change, defined as the logarithmic ratio of expression in treatment 1 relative to
335 treatment 2. A positive treatment effect in a male-biased gene, for instance, would imply that its
336 expression is relatively masculinized in treatment 1; for female-biased genes, a positive treatment effect
337 would similarly imply relatively feminized expression in treatment 1. (We included unbiased genes for
338 completeness but these are not pertinent to the Hollis et al. prediction.) Results (Fig. 1) suggest that the
339 relationship between treatment effect and sex bias is inconsistent across treatment pairs, sexes and
340 sample types (head/body). Matching the Hollis et al. (2014) prediction, there is an overall feminization
341 of the female transcriptome in MCabs relative to the other two treatments, with a significant increase in
342 expression of female-biased genes and a significant decrease in expression of male-biased genes in
343 MCabs (Fig. 1A-B). In male bodies (Fig. 1D-E), the net changes are smaller, but more importantly, the
344 pattern is different. Though female-biased genes have increased expression in MCabs relative to the
345 other two treatments (similar to the female body result), male-biased genes are not significantly
346 reduced in expression in MCabs relative to MCsim and, in fact, have significantly increased expression in
347 MCabs relative to MCcom (i.e., opposite the prediction). A further layer of complication is added when
348 examining the heads, where the results differ between female and male samples and in neither sex is
349 there a consistent pattern of the predicted feminization of MCabs (Fig 1G-H, K-L).
350

Fig. 1 depicts mean treatment effects in discrete, and somewhat arbitrarily-bounded categories of sex bias. We also performed LOESS regressions of treatment effect against continuously varying sex bias to discern any patterns missed by treating variation in sex-biased gene expression as discrete. These regressions (Fig. S2) show that the relationship between treatment effect and sex bias can vary within a sex bias category, a nuance not captured in the discrete version of the plots. However, the overall sign of mean treatment effect for a sex bias category is largely consistent in the two analyses.

As another approach to evaluating the Hollis et al. prediction, we looked for evidence of net changes in feminization/masculinization via a different method using only the TDE genes. In female bodies, there is a significant net feminization of TDE genes in MCabs relative to MCcom (Fig. S3), similar to the results depicted in Fig. 1 and matching the Hollis et al. prediction. In male bodies, there is a significant net masculinization of TDE genes in MCabs relative to MCcom (Fig. S3), opposite to the Hollis et al. prediction. In most other contrasts, there is no significant net feminization or masculinization.



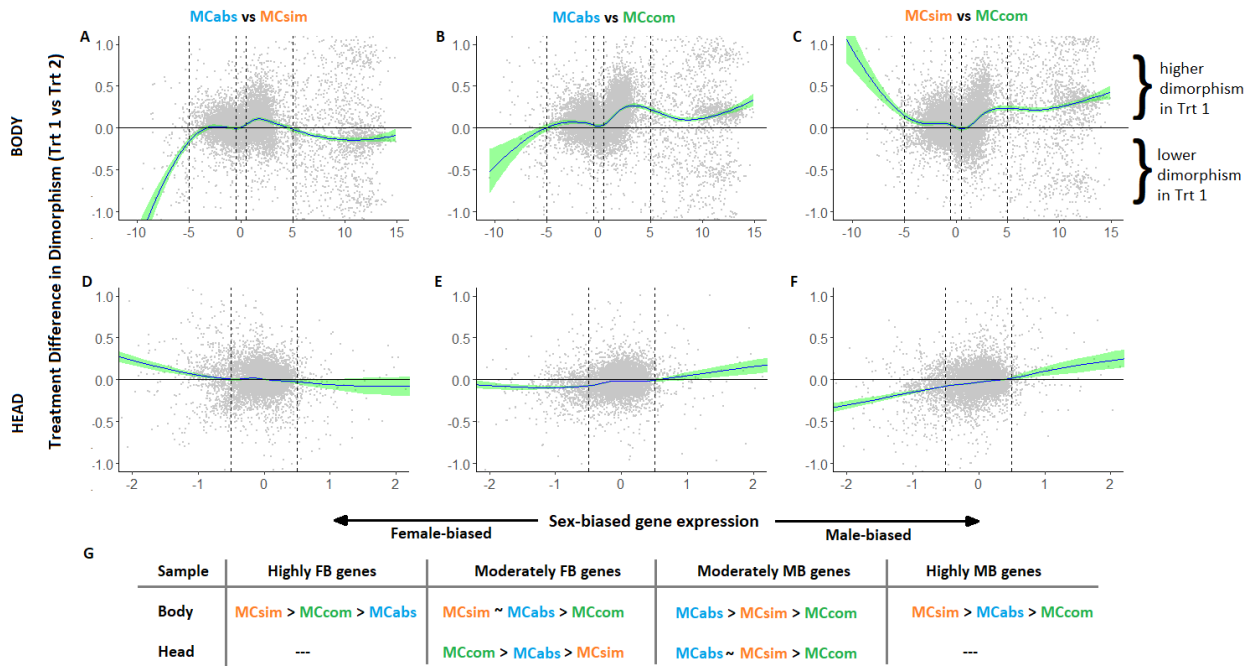
N

Sample	Female-biased genes	Male-biased genes
Female Body	MCabs > MCsim > MCcom	MCcom > MCsim > MCabs
Male Body	MCabs > MCcom > MCsim	MCsim ~ MCabs > MCcom
Female Head	MCcom > MCabs > MCsim	MCcom ~ MCabs > MCsim
Male Head	MCsim > MCabs > MCcom	MCabs > MCsim ~ MCcom

Figure 1: Average treatment effect (± 1 SE) for three sex bias categories for female body (A-C), male body (D-F), female head (G-I), male head (K-M), in pairwise comparisons between mating treatments. Asterisks denote the mean treatment effect is significantly different from zero, determined using one-sample permutation tests (i.e., by changing the sign of the treatment effect of a randomly chosen 50% of the genes and re-calculating the mean in each permutation). Significance is denoted as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Panel N summarises the order of expression levels for female- and male-biased genes in panels A-M; tilda (~) denotes no significant difference. An analogous figure from an analysis excluding gonad-specific genes is shown in Fig. S1; most of the patterns are very similar. Genes were assigned to sex-bias categories based on external data source.

Changes in degree of sexual dimorphism

383 In the previous section we examined expression changes for each sex separately, but these within-sex
384 changes are not directly informative of changes in sexual dimorphism. Here we investigate differences
385 between treatments in the degree of expression dimorphism across genes with different levels of sex-
386 bias as determined from an external dataset (see Methods). We used LOESS regressions to evaluate how
387 treatment differences in 'local average dimorphism' varied with sex-bias (where local average refers to
388 the difference in dimorphism averaged across a local range of sex-bias). Patterns were complex (Fig. 2)
389 though treatment differences in local average dimorphism did tend to be larger for sex-biased than
390 unbiased genes. Among the sex-biased genes, the magnitude and even direction of treatment
391 differences in local average dimorphism varied between, but also within, sex-bias categories. In bodies,
392 local average dimorphism is highest in MCsim for both highly female- and male-biased genes, but
393 patterns differ for moderately-biased genes (summarized in Fig. 2G). In heads, where phenotypic sexual
394 dimorphism is not as marked, changes in local average dimorphism are detectable despite the relatively
395 low number of sex-biased genes. Female-biased genes are, on average, most dimorphic in MCcom, but
396 male-biased genes are least dimorphic in MCcom. Analyses excluding gonad-specific genes yielded
397 nearly identical results.
398
399



Sample	Highly FB genes	Moderately FB genes	Moderately MB genes	Highly MB genes
Body	MCsim > MCcom > MCabs	MCsim ~ MCabs > MCcom	MCabs > MCsim > MCcom	MCsim > MCabs > MCcom
Head	---	MCcom > MCabs > MCsim	MCabs ~ MCsim > MCcom	---

Figure 2: Treatment difference in dimorphism as a function of sex-biased gene expression in A-C) body and D-F) head. The vertical axis is (sign of sex bias)*(difference in dimorphism between treatments) so that positive (negative) values indicate increased (reduced) dimorphism in treatment 1 relative to treatment 2. In panels A-F, the fitted line is obtained from LOESS regressions (fit using *geom_smooth* in the R package *ggplot2* with the width of the sliding-window set at 0.5); 95% confidence intervals are indicated in green. The plots show a subset of the genes within the vertical axis range [-1,1] to better display the pattern for the majority of genes. Dashed vertical lines demarcate the sex bias categories listed in the panel G. Panel G summarises the relative strength of dimorphism among treatments for each SBGE category. Sex-bias values were determined from an external data source. The categories used in panel G are designated as follows: highly FB genes = (-∞,-5], moderately FB genes = (-5, -0.5], moderately MB genes = [0.5, 5), [5, ∞). No highly male- or female-biased genes are depicted for heads due to the low numbers of such genes.

417 *Differential splicing amongst mating treatments*

418 We also examined sex-specific splicing (SSS), testing whether alternative splicing differs between mating
419 treatments. Congruent to our analysis for TDE genes, we tested for genes with significant differential
420 splicing between treatments, i.e., 'treatment differentially spliced' (TDS) genes. Frequencies of TDS
421 genes are in the range of 0.2-2.3% of all genes analysed (Table S3). We performed Fisher's exact tests to
422 ascertain if TDS genes are disproportionately represented among sex-biased genes. In contrast to the
423 analogous test for TDE genes (Table 2, S2), there was no over-representation of TDS genes among sex-
424 biased genes, though there was some evidence of TDS genes being more common among male- than
425 female-biased genes (Tables S4, S5). Considering TDE and TDS together, there was significant overlap
426 between these in most sample types (Table S6).

427 We also tested for sex-specific splicing (SSS) within each treatment. The proportion of SSS genes was
428 similar among treatments, with one exception: the proportion of such genes is noticeably lower in the
429 heads in MCabs than the other two treatments (Table 1). The lower frequency of SSS in MCabs heads is
430 unlikely to be due to reduced power to detect SSS: MCabs heads had the highest mean coverage (Table
431 S7) and there was no comparable in reduction in the frequency of sex-biased expression (Table 1). This
432 difference in treatment effects on the prevalence SBGE and SSS suggests that these two forms of
433 expression dimorphism evolve somewhat independently.

434

435 **Discussion**

436 Differential selection on the sexes is thought to drive the evolution of sexual dimorphism in gene
437 expression (Parsch & Ellegren, 2013). Selection arising from sexual interactions and mate competition is
438 hypothesized to be a major cause of this differential selection. Thus, variation in mate competition
439 among populations or species should be a major source of variation in SBGE across such taxonomic
440 groups. Here, we tested this idea by measuring shifts in gene expression in response to evolutionary
441 manipulations of mate competition in *D. melanogaster*. The MCabs treatment eliminates mate
442 competition through enforced monogamy, while the two other treatments (MCsim, MCcom)
443 incorporate mate competition in differing mating environments that we know alter intersexual
444 interactions and the selection these generate (MacPherson et al., 2018; Yun et al., 2017, 2019).

445 An implicit assumption in evaluating the predictions raised in the Introduction is that the observed
446 differences in expression are, at least to some extent, adaptive. This warrants consideration. The *DEseq2*

447 analysis identifies changes that are consistent across populations (i.e., evolutionarily independent
448 replicates) within selective treatments; that is, these are expression differences that can be attributed to
449 the effect of the selective treatments. Previous studies of fitness-related traits (Yun et al. 2019; 2021)
450 show that these populations have adapted to their mating treatments. However, as with other "evolve
451 and resequence" (E&R) expression studies (e.g., Huang et al. 2014, Hollis et al. 2014, Veltsos et al. 2017,
452 Hsu et al. 2020), we do not have direct evidence that the observed expression differences contribute to
453 the observed fitness changes. Though we suspect the expression changes will tend to be adaptive, some
454 of the expression changes are likely incidental outcomes of linked selection, which can cause parallel,
455 non-adaptive changes in E&R studies.

456 Differences in the mating regimes could also result in differences in the strength of drift among
457 treatments. However, drift is not expected to create consistent changes in allele frequencies across
458 evolutionarily independent replicates, and thus would not typically contribute to a statistically-identified
459 "selective treatment effect". There is one potentially important exception. A treatment with stronger
460 drift would have populations carrying a heavier burden of deleterious alleles and thereby lower average
461 fitness within each replicate, though the causative alleles would differ among replicate populations. If
462 the effects of deleterious alleles at different sites are somewhat interchangeable in that they contribute
463 to a common latent variable such as "condition" (*sensu* Rowe and Houle 1994), which itself affects
464 expression, then differences in drift would contribute to a "treatment effect". Indeed, Wyman et al.
465 (2010) showed that rearing flies on a poor quality diet, which could be viewed as a means to reduce
466 "condition", altered sex-biased gene expression. However, this is a specific manipulation of the
467 environment and not a demonstration that different sets of deleterious alleles would cause a consistent
468 expression effect. There is some evidence that lowering diet quality is not representative of the effects
469 of deleterious alleles (Bonduriansky et al. 2015).

470 Perhaps most importantly, Singh and Agrawal (2022) estimated N_e/N in *D. melanogaster* from direct
471 measures of male and female variance in fitness under three mating treatments that closely parallel
472 those used here, and found similar values for all three (see also Pischedda et al. 2015.). The common
473 intuition that mate competition will necessarily and dramatically alter N_e can be misleading for both
474 theoretical reasons (see Crow and Morton 1955; Singh and Agrawal 2021) and an empirical one: the
475 variance in female fitness increases under enforced monogamy (Long et al. 2009; Singh and Agrawal
476 2021). In summary, we have reasons to believe that N_e is similar among our treatments; it is
477 nevertheless possible that there are subtle differences in the strength of drift and these could

478 contribute to the treatment-level expression differences reported here, but only under the specific
479 circumstances outlined above. Given we know that these populations have evolved adaptively with
480 respect to their mating treatments (Yun et al. 2019; 2021), we suspect adaptive evolution is an
481 important process behind the observed expression changes. Nonetheless, as we interpret our results
482 below, it is important to bear in mind that some of the observed expression changes are likely non-
483 adaptive, resulting from linked selection or drift. (Similar issues would apply to related studies, e.g.,
484 Hollis et al (2014), Veltsos et al. (2017).)

485 If a history of sex-differential selection arising from mate competition is responsible for pre-existing
486 SBGE, then we expect that selection on genes that are initially sex-biased is more likely to be sensitive to
487 a change in mate competition than selection on unbiased genes. Thus, we predicted that sex-biased
488 genes would be more likely to diverge among mate competition treatments than unbiased genes. The
489 results largely supported this prediction (Table 2); all seven of the comparisons in which there was a
490 significant difference were in the predicted direction (though not all comparisons yielded a significant
491 difference).

492 Though the patterns are consistent with the prediction, other explanations likely also contribute to this
493 pattern. Core house-keeping genes will tend to be unbiased and their expression may be under strong
494 stabilizing selection that changes little across any of a large range of environments. For this reason, we
495 might predict that unbiased genes are less likely to diverge in expression than sex-biased genes across
496 any environmental change, not just changes to mate competition. A better test, which may be possible
497 via meta-analysis in future work, would be to ask whether the preferential divergence of sex-biased
498 relative to unbiased genes is more pronounced in response to mate competition treatments than it is
499 with other types of environmental changes.

500 We also compared the relative frequency of DE genes in male- versus female-biased genes. Past studies
501 have shown that male-biased genes evolve more rapidly than female-biased genes, both in DNA
502 sequence (Meisel, 2011; Zhang et al., 2004) and gene expression (Allen et al., 2018; Meiklejohn et al.,
503 2003), though some studies describe more mixed results (Whittle & Johannesson, 2013; Yang et al.,
504 2016). Adaptive evolution in response to sexual selection has been invoked as an explanation (Ellegren
505 & Parsch, 2007), with some studies suggesting that male-biased genes are targeted more often by
506 strong positive selection (Pröschel et al., 2006; Zhang et al., 2004; but see Singh and Agrawal 2023)
507 presumably resulting from reproductive interactions. Our manipulation of mate competition resulted in

508 more divergence in expression of male- than female-biased genes (Table 2): in the six comparisons
509 where there was a significant difference, male-biased genes had a higher propensity for expression
510 divergence than female-biased genes. In some sense, this matches with fitness data showing stronger
511 signatures of local adaptation to the mate competition environment in males than females (Yun et al.
512 2019), suggesting greater phenotypic divergence in males than females. However, Table 2 contrasts
513 male- and female-biased genes within each sex, rather than contrasting divergence of males versus that
514 of females. There are not obvious differences in the proportion of significantly diverged genes between
515 male and female samples. This apparent discrepancy could be reconciled if the expression divergence of
516 male-biased genes has greater phenotypic and fitness consequences in males than females.

517 Hollis et al. (2014) predicted that enforced monogamy would result in a "feminization" of the
518 transcriptome (i.e., increased expression of female-biased genes and decreased expression of male-
519 biased genes) in both sexes. This prediction has now been tested in three experiments with highly
520 heterogeneous results. The data of Hollis et al. (2014) matched their prediction, while the results of
521 Veltsos et al. (2017) were mixed but largely in the opposite direction. Our results are mixed with respect
522 to the predicted changes.

523 The basis of the Hollis et al. prediction is that the primary consequence of enforced monogamy is the
524 removal of sexual selection on males. However, enforced monogamy not only removes sexual selection,
525 but it imposes (potentially strong) selection on males to inflict less harm on their mates (Holland and
526 Rice 1999; Martin and Hosken 2003; Crudginton et al. 2005, 2010; Yun et al. 2021). This can lead to
527 changes in selection on female resistance to harm (Holland & Rice 1999, Martin and Hosken 2003). Even
528 in the absence of evolved changes in male harm, there are strong reasons to suspect that enforced
529 monogamy will change selection on females because males will be unable to bias their attention
530 towards particular phenotypes (Long et al., 2009; Arbuthnott and Rundle 2012; Chenoweth et al. 2015;
531 Yun et al. 2017). Considering this multitude of possible changes in selection, it is difficult to predict how
532 the transcriptome will respond, and it seems unlikely that net change will be consistent or easily
533 classifiable as 'feminization' or 'masculinization.'

534 Because selection in enforced monogamy can differ from selection under mate competition in a variety
535 of ways, other details could become important to how divergence occurs. These three studies differ in
536 various ways which could, in principle, contribute to the among-study heterogeneity in results (e.g.,
537 species studied, nature of starting variation (e.g., Hollis et al. (2014) add new mutations via chemical

538 mutagenisis to standing variation), food, temperature, density and population size, and maintenance
539 procedures (Li Richter & Hollis 2021)). One potentially important aspect is the precise nature of mate
540 competition, which can take many forms (e.g., there is no one true form of "polygamy" for a
541 "monogamy vs. polygamy" contrast). Our study directly shows that differences in how mate competition
542 occurs has major consequences for expression evolution. Within the context of our experiment, where
543 most other factors are not varied among treatments, the transcriptional differences between our two
544 different mate competition treatments (i.e., MCsim vs. MCcom) are generally as large as those between
545 either one of these with the enforced monogamy treatment (MCabs).

546 The hypothesis that the removal of sexual selection would 'feminize' the transcriptome was tested in a
547 very different way by Parker et al. (2019). They examined expression changes in five asexual species of
548 stick insects relative to their sexual sister species, under the idea that asexual females do not share a
549 gene pool with sexually-selected males. Contrary to expectation, Parker et al. (2019) found evidence
550 that asexual females had evolved 'masculinized' expression. They suggested that the most plausible
551 explanation was that much of feminized expression patterns in sexual females arises via traits involved
552 with female-male interactions and, because asexual females do not mate or interact with males,
553 expression evolves to be less 'feminized'. By this explanation, asexual species are not an ideal
554 counterfactual for inferring how sharing a gene pool with sexually-selected males constrains the
555 evolution of expression in sexual females because asexual females also experience substantially
556 different selection than sexual females.

557 Though selection arising from mate competition in sexual species is almost certainly an important driver
558 of SBGE (Parsch & Ellegren, 2013), there is little direct evidence (Harrison et al. 2014). It seems intuitive
559 that, in the absence of mate competition, expression dimorphism would become reduced. For example,
560 monogamous bird species are often less dimorphic with respect to plumage (and other traits) than non-
561 monogamous species, and presumably this reflects a reduction in expression dimorphism for genes
562 affecting such traits. Yet, it is unclear what the prediction for genome-wide expression dimorphism
563 should be as intralocus conflict on expression can easily exist under monogamy given that males and
564 females play different roles in reproduction and offspring rearing, even if overt traits such as plumage
565 do not experience divergent selection.

566 While the underlying motivation for the studies of Hollis et al. (2014) and Veltsos et al. (2017) was based
567 around sex-biased gene expression, neither attempted to examine how enforced monogamy changed
568 expression dimorphism per se. We examined how the average treatment effect on expression

569 dimorphism varied across local ranges of sex bias as determined in LOESS regressions. We found
570 differences in 'local average dimorphism' among mating regimes. These changes in local average
571 dimorphism tend to occur to a greater extent among sex-biased than unbiased genes, and more so in
572 bodies, which have much more sex-biased gene expression than heads (Fig. 2). However, we did not find
573 a consistent reduction in local average dimorphism between the enforced monogamy treatment and the
574 two treatments with mate competition; while some categories of sex-biased genes became less
575 dimorphic under monogamy, others became more so (Fig. 2). For example, relative to MCsim, MCabs
576 evolved reduced local average dimorphism of strongly sex-biased genes but increased dimorphism of
577 moderately male-biased genes in body samples. However, one aspect of expression was consistent with
578 the intuition of reduced dimorphism under monogamy: there was a notable reduction in the frequency
579 of genes with sex-specific splicing in heads of MCabs relative to either of the other two treatments,
580 though not in bodies (Table 1). While our experiment is the result of more than 100 generations of
581 selection, this is a relatively short period relative to other evolutionary time scales (e.g., sister species);
582 perhaps a more consistent reduction in expression dimorphism would be evident after enough time.
583 Nonetheless, this study shows that, though expression dimorphism readily evolves among mate
584 competition environments, enforced monogamy does not necessarily cause a rapid and consistent
585 reduction in dimorphism.

586 The most striking change in local average dimorphism occurred between the two treatments involving
587 mate competition, with MCsim showing greater dimorphism in bodies than MCcom across all sex bias
588 categories (Fig. 2C). Relative to MCcom, the MCsim environment is one in which intersexual
589 interactions, including mating, occur more frequently (Yun et al. 2017, 2019); exposure to males is more
590 harmful to females in the simple than complex environment and MCsim males have evolved to be more
591 harmful (Yun et al. 2017, 2021). Thus, it is reasonable to infer that interlocus sexual conflict is relatively
592 more important in MCsim than MCcom. However, this does not provide an obvious explanation for the
593 observed difference in expression dimorphism, as dimorphism is thought to evolve in response to intra-,
594 rather than interlocus, conflict.

595 In this study, we have treated each gene's expression as a trait (i.e., a measurable property) and
596 examined patterns in how these 'traits' change in response to an evolutionary manipulation of mate
597 competition. Just as with traditional traits (i.e., morphological, behavioral, or physiological phenotypes),
598 multiple possible genetic mechanisms could underly each change and a single genetic change could
599 affect multiple traits. At one (unlikely) extreme, every gene expression change could be due to a change

in that gene's *cis*-regulatory region. At the other extreme, a single genetic change could affect expression levels of many genes, possibly by changing the size of different organs or relative abundance of cell types within organs (Stewart et al. 2010). The truth likely lies somewhere between these two extremes. In an evolutionary manipulation of mating system in *Drosophila pseudoobscura*, Wiberg et al. (2021) found that genomically diverged sites were enriched near sites with expression divergence. That result would not be expected if a very small number of genetic changes underlay most expression differences, but it also does not imply that most expression changes are due to individual *cis*-regulatory changes.

It is not our objective here to resolve this issue for our own experiment (and we have little ability to do so). In the spirit of many past transcriptomic studies, we test for patterns among different 'types' of traits (e.g., genes with male-biased, female-biased, or unbiased expression). From this perspective, each gene represents a separate instance of that trait 'type' but it is important to remember that variation in each of those individual 'traits' need not be governed by independent proximate mechanisms. This unknown level of independence should temper inferences about genetic changes and the true nature and number of targets of selection (e.g., could some patterns result from changes in the relative size of gonads and, if so, why has selection caused such changes?). Our study clearly reveals patterns of expression evolution across SBGE categories in response to changes in mate competition, though we are limited in interpreting the reasons for these patterns. The type of work presented here represents only one step towards understanding the relationship between mate competition and SBGE, which can help evaluate hypotheses in the literature and provide fodder for new ones.

623 **Literature Cited**

624 Allen, S. L., R. Bonduriansky, and S. F. Chenoweth. 2018. Genetic constraints on microevolutionary
625 divergence of sex-biased gene expression. Philos. Trans. R. Soc. Lond. B Biol. Sci. 373:20170427.

626 Anders, S., P. T. Pyl, and W. Huber. 2015. HTSeq--a Python framework to work with high-throughput
627 sequencing data. Bioinformatics 31: 166-169.

628 Arbuthnott, D., and H. D. Rundle. 2012. Sexual selection is ineffectual or inhibits the purging of
629 deleterious mutations in *Drosophila melanogaster*. Evolution 66:2127–2137.

630 Arnqvist, G., and L. Rowe. 2005. Sexual conflict. Princeton University Press, Princeton, NJ.

631 Bonduriansky, R., and S. F. Chenoweth. 2009. Intralocus sexual conflict. Trends Ecol. Evol. 24:280–288.

632 Bonduriansky, R., M. A. Mallet, D. Arbuthnott, V. Pawlowsky-Glahn, J. J. Egozcue, and H. D. Rundle.
633 2015. Differential effects of genetic vs. environmental quality in Drosophila melanogaster suggest
634 multiple forms of condition dependence. Ecol. Lett. 18:317-26.

635 Chapman, T., L. F. Liddle, J. M. Kalb, M. F. Wolfner, and L. Partridge. 1995. Cost of mating in *Drosophila*
636 *melanogaster* females is mediated by male accessory gland products. Nature 373:241–244.
637 nature.com.

638 Chenoweth, S. F., N. C. Appleton, S. L. Allen, and H. D. Rundle. 2015. Genomic evidence that sexual
639 selection impedes adaptation to a novel environment. Curr. Biol. 25:1860–1866.

640 Crow J. F., and N. E. Morton NE. 1955. Measurement of gene frequency drift in small populations. Evol.
641 9:202-14.

642 Crudgington, H. S., A. P. Beckerman, L. Brüstle, K. Green, and R. R. Snook. 2005. Experimental removal
643 and elevation of sexual selection: does sexual selection generate manipulative males and resistant
644 females? Am. Nat. 165: S72–87.

645 Crudgington, H. S., S. Fellows, and R. R. Snook. 2010. Increased opportunity for sexual conflict promotes
646 harmful males with elevated courtship frequencies. J. Evol. Biol. 23:440–446.

647 Dean, R., A. E. Wright, S. E. Marsh-Rollo, B. M. Nugent, S. H. Alonzo, and J. E. Mank. 2017. Sperm
648 competition shapes gene expression and sequence evolution in the ocellated wrasse. Mol. Ecol.
649 26:505–518.

650 Dobin, A., C. A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, P. Batut, M. Chaisson, and T. R.
651 Gingeras. 2013. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29:15–21.

652 dos Santos, G., A. J. Schroeder, J. L. Goodman, V. B. Strelets, M. A. Crosby, J. Thurmond, D. B. Emmert,
653 W. M. Gelbart, and FlyBase Consortium. 2015. FlyBase: introduction of the *Drosophila*
654 *melanogaster* Release 6 reference genome assembly and large-scale migration of genome
655 annotations. Nucleic Acids Res. 43:D690–7.

656 Eden, E., R. Navon, I. Steinfeld, D. Lipson, and Z. Yakhini. 2009. GOrilla: a tool for discovery and
657 visualization of enriched GO terms in ranked gene lists. BMC Bioinformatics 10:48.

658 Ellegren, H., and J. Parsch. 2007. The evolution of sex-biased genes and sex-biased gene expression. Nat.
659 Rev. Genet. 8:689–698.

660 Fernandes Martins, M. J., G. Hunt, R. Lockwood, J. P. Swaddle, and D. J. Horne. 2017. Correlation
661 between investment in sexual traits and valve sexual dimorphism in Cyprideis species (Ostracoda).
662 PLoS One 12:e0177791.

663 Fowler, K., and L. Partridge. 1989. A cost of mating in female fruitflies. Nature 338:760–761.

664 Grath, S., and J. Parsch. 2016. Sex-biased gene expression. Annu. Rev. Genet. 50:29–44.

665 Harrison, P. W., A. E. Wright, F. Zimmer, R. Dean, S. H. Montgomery, M. A. Pointer, and J. E. Mank. 2015.
666 Sexual selection drives evolution and rapid turnover of male gene expression. Proc. Natl. Acad.
667 Sci. U. S. A. 112:4393–4398.

668 Hartley, S. W., and J. C. Mullikin. 2016. Detection and visualization of differential splicing in RNA-Seq
669 data with JunctionSeq. Nucleic Acids Res. 44:e127.

670 Holland, B., and W. R. Rice. 1999. Experimental removal of sexual selection reverses intersexual
671 antagonistic coevolution and removes a reproductive load. Proc. Natl. Acad. Sci. U. S. A. 96:5083–
672 5088.

673 Hollis, B., D. Houle, Z. Yan, T. J. Kawecki, and L. Keller. 2014. Evolution under monogamy feminizes gene
674 expression in *Drosophila melanogaster*. Nat. Commun. 5:3482.

675 Ingleby, F. C., I. Flis, and E. H. Morrow. 2014. Sex-biased gene expression and sexual conflict throughout
676 development. Cold Spring Harb. Perspect. Biol. 7:a017632.

677 Lande, R. 1980. Sexual dimorphism, sexual selection, and adaptation in polygenic characters. Evolution
678 34:292–305. JSTOR.

679 Leader, D. P., S. A. Krause, A. Pandit, S. A. Davies, and J. A. T. Dow. 2018. FlyAtlas 2: a new version of the
680 *Drosophila melanogaster* expression atlas with RNA-Seq, miRNA-Seq and sex-specific data. Nucleic
681 Acids Res. 46:D809–D815.

682 Li Richter, X.-Y., and B. Hollis. 2021. Softness of selection and mating system interact to shape trait
683 evolution under sexual conflict. Evolution 75:2335–2347.

684 Long, T. A. F., A. Pischedda, A. D. Stewart, and W. R. Rice. 2009. A cost of sexual attractiveness to high-
685 fitness females. PLoS Biol. 7:e1000254.

686 Love, M. I., W. Huber, and S. Anders. 2014. Moderated estimation of fold change and dispersion for
687 RNA-seq data with DESeq2. Genome Biol. 15:550.

688 MacPherson, A., L. Yun, T. S. Barrera, A. F. Agrawal, and H. D. Rundle. 2018. The effects of male harm
689 vary with female quality and environmental complexity in *Drosophila melanogaster*. Biol. Lett.
690 14:20180443.

691 Martin, O. Y., and D. J. Hosken. 2003. Costs and benefits of evolving under experimentally enforced
692 polyandry or monogamy. Evolution 57:2765–2772.

693 Meiklejohn, C. D., J. Parsch, J. M. Ranz, and D. L. Hartl. 2003. Rapid evolution of male-biased gene
694 expression in *Drosophila*. PNAS 100:9894 –9899.

695 Osada, N., R. Miyagi, and A. Takahashi. 2017. Cis- and trans-regulatory effects on gene expression in a
696 natural population of *Drosophila melanogaster*. Genetics 206:2139–2148.

697 Parker D.J., J. Bast, K. Jalvingh, Z. Dumas, M. Robinson-Rechavi, and T. Schwander. 2019. Sex-biased
698 gene expression is repeatedly masculinized in asexual females. Nat. Comm. 10:4638.

699 Parsch, J., and H. Ellegren. 2013. The evolutionary causes and consequences of sex-biased gene
700 expression. Nat. Rev. Genet. 14:83–87.

701 Partridge, L., and K. Fowler. 1990. Non-mating costs of exposure to males in female *Drosophila*
702 *melanogaster*. J. Insect Physiol. 36:419–425.

703 Pointer, M. A., P. W. Harrison, A. E. Wright, and J. E. Mank. 2013. Masculinization of gene expression is
704 associated with exaggeration of male sexual dimorphism. PLoS Genet. 9:e1003697.

705 Prasad, N. G., S. Bedhomme, T. Day, and A. K. Chippindale. 2007. An evolutionary cost of separate
706 genders revealed by male-limited evolution. Am. Nat. 169:29–37.

707 Pröschel, M., Z. Zhang, and J. Parsch. 2006. Widespread adaptive evolution of *Drosophila* genes with sex-
708 biased expression. Genetics 174:893–900.

709 Rowe, L., and H. D. Rundle. 2021. The alignment of natural and sexual selection. Annu. Rev. Ecol. Evol.
710 Syst. 52:499–517.

711 Singh, A., and A. F. Agrawal. 2022. Sex-specific variance in fitness and the efficacy of selection. Am. Nat.
712 199: 587-602.

713 Singh, A., and A. F. Agrawal. 2023. Two forms of sexual dimorphism in gene expression in *Drosophila*
714 *melanogaster*: their coincidence and evolutionary genetics. Mol. Biol. Evol. 40: msad091.

715 Stewart, A. D., A. Pischedda, and W. R. Rice. 2010. Resolving intralocus sexual conflict: genetic
716 mechanisms and time frame. J. Hered. 101:S94–9.

717 Stuglik, M. T., W. Babik, Z. Prokop, and J. Radwan. 2014. Alternative reproductive tactics and sex-biased
718 gene expression: the study of the bulb mite transcriptome. Ecol. Evol. 4:623–632.

719 Veltsos, P., Y. Fang, A. R. Cossins, R. R. Snook, and M. G. Ritchie. 2017. Mating system manipulation and
720 the evolution of sex-biased gene expression in *Drosophila*. Nat. Commun. 8:2072.

721 Whittle, C. A., and H. Johannesson. 2013. Evolutionary dynamics of sex-biased genes in a hermaphrodite
722 fungus. Mol. Biol. Evol. 30:2435–2446.

723 Wiberg, R. A. W., P. Veltsos, R. R. Snook, and M. G. Ritchie. 2021. Experimental evolution supports
724 signatures of sexual selection in genomic divergence. Evol Lett 5:214–229.

725 Wigby, S., N. C. Brown, S. E. Allen, S. Misra, J. L. Sitnik, I. Sepil, A. G. Clark, and M. F. Wolfner. 2020. The
726 Drosophila seminal proteome and its role in postcopulatory sexual selection. Philos. Trans. R. Soc.
727 Lond. B Biol. Sci. 375:20200072.

728 Wigby, S., and T. Chapman. 2004. Female resistance to male harm evolves in response to manipulation
729 of sexual conflict. Evolution 58:1028–1037.

730 Witt, E., N. Svetec, S. Benjamin, and L. Zhao. 2021. Transcription factors drive opposite relationships
731 between gene age and tissue specificity in male and female *Drosophila* gonads. Mol. Biol. Evol.
732 38:2104–2115.

733 Yang, L., Z. Zhang, and S. He. 2016. Both male-biased and female-biased genes evolve faster in fish
734 genomes. Genome Biol. Evol. 8:3433–3445.

735 Yun, L., A. F. Agrawal, and H. D. Rundle. 2021. On male harm: How it is measured and how it evolves in
736 different environments. Am. Nat. 198:219–231.

737 Yun, L., M. Bayoumi, S. Yang, P. J. Chen, H. D. Rundle, and A. F. Agrawal. 2019. Testing for local
738 adaptation in adult male and female fitness among populations evolved under different mate
739 competition regimes. Evolution 73:1604–1616.

740 Yun, L., P. J. Chen, K. E. Kwok, C. S. Angell, H. D. Rundle, and A. F. Agrawal. 2018. Competition for mates
741 and the improvement of nonsexual fitness. Proc. Natl. Acad. Sci. U. S. A. 29:201805435.

742 Yun, L., P. J. Chen, A. Singh, A. F. Agrawal, and H. D. Rundle. 2017. The physical environment mediates
743 male harm and its effect on selection in females. Proc. Biol. Sci. 284:20170424.

744 Zhang, Y., D. Sturgill, M. Parisi, S. Kumar, and B. Oliver. 2007. Constraint and turnover in sex-biased gene
745 expression in the genus *Drosophila*. Nature 450:233–237.

746 Zhang, Z., T. M. Hambuch, and J. Parsch. 2004. Molecular evolution of sex-biased genes in *Drosophila*.
747 Mol. Biol. Evol. 21:2130–2139.

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