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

Prashastha Mishra[1], Howard D. Rundle[2,3], and Aneil F. Agrawal[1,3]

[1]Department of Ecology & Evolutionary Biology, University of Toronto, Toronto, Ontario, Canada

[2]Department of Biology, University of Ottawa, Ottawa, Ontario, Canada; E-mail: hrundle@uottawa.ca

[3]HDR and AFA contributed equally to this work.

Abstract

Many genes are differentially expressed between males and females and patterns of sex-biased gene expression (SBGE) vary among species. 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 (MCabs), or allowed mate competition in either small, simple (MCsim) or larger, structurally more complex (MCcom) mating environments. Consistent with SBGE being the product of sex-specific selection, 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*,

Introduction

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

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

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

Experimental evolution offers a powerful means to directly test whether a change in mating system 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).

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

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

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

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

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

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

142

143 **Methods**

144 *Samples for RNAseq*

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

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

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

168 After 104 generations of selection, samples for RNAseq were obtained as follows. Flies were reared
169 under a controlled density of 40 larvae per vial on a benign yeast-agar food (no salt). Adults emerged
170 eight days after hatching and were collected under light CO_2 (< 20 seconds) as virgins (within 8 hours of
171 emergence) and then held in single-sex vials at low density (10 flies per vial). Two days later, flies were
172 processed. For whole body samples, flies were transferred under light CO_2 (< 20 sec) to microcentrifuge
173 tubes (10 flies per tube). A few minutes later (and well after awakening from CO_2) tubes were flash
174 frozen in liquid nitrogen and then stored at -80 C. For head samples, the same procedure was used
175 except after flash freezing, tubes were vortex shaken for ca. 10 s to separate heads from bodies. Heads
176 were transferred to a new microcentrifuge tube (8-10 heads per tube) and then stored at -80 C. RNA
177 extraction was performed using ThermoFisher PicoPure RNA Isolation Kit. Paired-end (100 bp)
178 sequencing was performed using Illumina NovaSeq S4.

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

182 *Differential Expression between Mating Treatments*

183 Differential expression was analysed between each pair of mating treatments, separately for each sex
184 and tissue. Each RNAseq file was aligned to the *D. melanogaster* reference genome (dos Santos et al.,
185 2015) using STAR v2.7 (Dobin et al., 2013) with default parameters. The resulting alignment files were
186 processed with *htseq-count* to obtain gene-level read counts for each sample (Anders et al., 2015). The R
187 package *DESeq2* (Love et al., 2014) was used to estimate differential expression between pairs of mating
188 treatments. For all genes averaging >50 reads across all replicates, we estimated the $\log_2$ fold change in
189 expression between treatments (henceforth, 'treatment effect'). For any genes thus tested, *DESeq2*
190 yields an adjusted p-value by applying Benjamini and Hochberg corrections for multiple testing. A gene
191 was designated to have significant differential expression between treatments if the treatment effect
192 was accompanied by an adjusted p-value < 0.1. Such genes are called 'Treatment Differentially
193 Expressed genes' or 'TDE genes'.

194

195 *Sex-Biased Gene Expression*

196 We used an external dataset (Osada et al., 2017) to characterise genes with respect to sex-biased gene
197 expression for our analyses. This choice for an external dataset is not critical for our purposes; though
198 expression in this external dataset undoubtedly differs somewhat from our own, variation among genes
199 in SBGE tends to be much larger than variation in SBGE for a gene across studies (e.g., genes that are
200 male- or female-biased in one study are generally male- or female-biased in other studies, though they
201 may vary quantitively the magnitude of bias). Even across species separated by millions of years, among-
202 gene SBGE is strongly correlated (Zhang et al. 2007).

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

210

211 *Differential Isoform Usage Analysis*

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

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

226

227 *Identification of Gonad-Specific Genes*

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

235 $$\mathrm{GSI_j} = \frac{\mathrm{FPKM_{gonads,j}}}{\Sigma_i \mathrm{FPKM_{i,j}}},$$

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

239

240 **Results**

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

242 We first examined the frequency of SBGE within each mating treatment. The frequency of genes with
243 significant sex bias in expression was similar across treatments in whole bodies and in heads (Table 1).
244 Though we found statistically significant variation in the frequency of SBGE among treatments in the
245 whole body, these differences are not drastic. This is not surprising because, on a short
246 microevolutionary time, one would not expect large scale expression divergence that would cause genes
247 to change categories of SBGE (especially given that SBGE is fairly consistent across species; Zhang et al.
248 2007). However, this does not preclude the possibility of many subtle, quantitative changes in
249 expression.

250

251

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.

Mating treatment	Sample	Sex-biased gene expression		Sex-specific splicing	
		Percent of genes with SBGE	p-value	Percent of genes with SSS	p-value
MCabs	Body	88.4% (10357/11717)	**3.4×10^{-5}**	49.8% (3614/7250)	0.097
MCsim		87.6% (10266/11717)		48.1% (3484/7250)	
MCcom		87.2% (10222/11717)		49.0% (3552/7250)	
MCabs	Head	9.8% (913/9356)	0.56	4.2% (316/7547)	**$<2.2 \times 10^{-16}$**
MCsim		10.4% (973/9356)		13.6% (1023/7547)	
MCcom		10.5% (978/9356)		10.4% (785/7547)	

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 evolve 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 female body samples (again ignoring the MCabs - MCsim comparison due to the low total number of TDE genes). Both of these patterns hold to some degree in heads too: in those comparisons with statistically significant differences, TDE genes are more common among sex-biased than unbiased genes and TDE genes are more common among male- than female-biased genes.

A possible proximate mechanism for the body results is a change in the relative sizes of gonads (Mank, 2017). We repeated the analysis presented in Table 2 after excluding genes with highly gonad-specific expression. The results remain similar (Table S2). While this does not rule out the possibility that changes in gonads play an important role in expression divergence among treatments, the fact that the patterns are not weakened after removal of gonad-specific genes—along with the existence of the patterns in heads—suggests that there is more to the underlying mechanisms behind expression divergence than solely a change in gonad size.

We analysed the TDE genes for significant enriched biological processes or functions using a gene ontology enrichment test (Eden et al., 2009), but failed to find any significant associations. However, among the 574 TDE genes in total, we found 16 genes that putatively code for seminal fluid proteins (SFPs), identified in a recent study of the seminal fluid proteome (Wigby et al., 2020). One of these 16 putative SFP genes (CG42807) was divergently expressed in two pairwise comparisons (MCabs vs MCcom, MCsim vs MCcom).

298 Table 2: Results of Fisher's exact tests for the proportion of treatment differentially expressed (TDE)
299 genes in each sex bias category. Values in parentheses represent the fraction of TDE genes out of all
300 genes in the sex bias category.

301

Sample	Comparison	Percent TDE genes		p-value (UB vs SB)	Percent TDE genes		p-value (MB vs FB)
		UB	SB		FB	MB	
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)	**$<2.2 \times 10^{-16}$**
Male body	MCabs vs MCsim	0.5% (9/1699)	2.5% (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}**
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
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

302

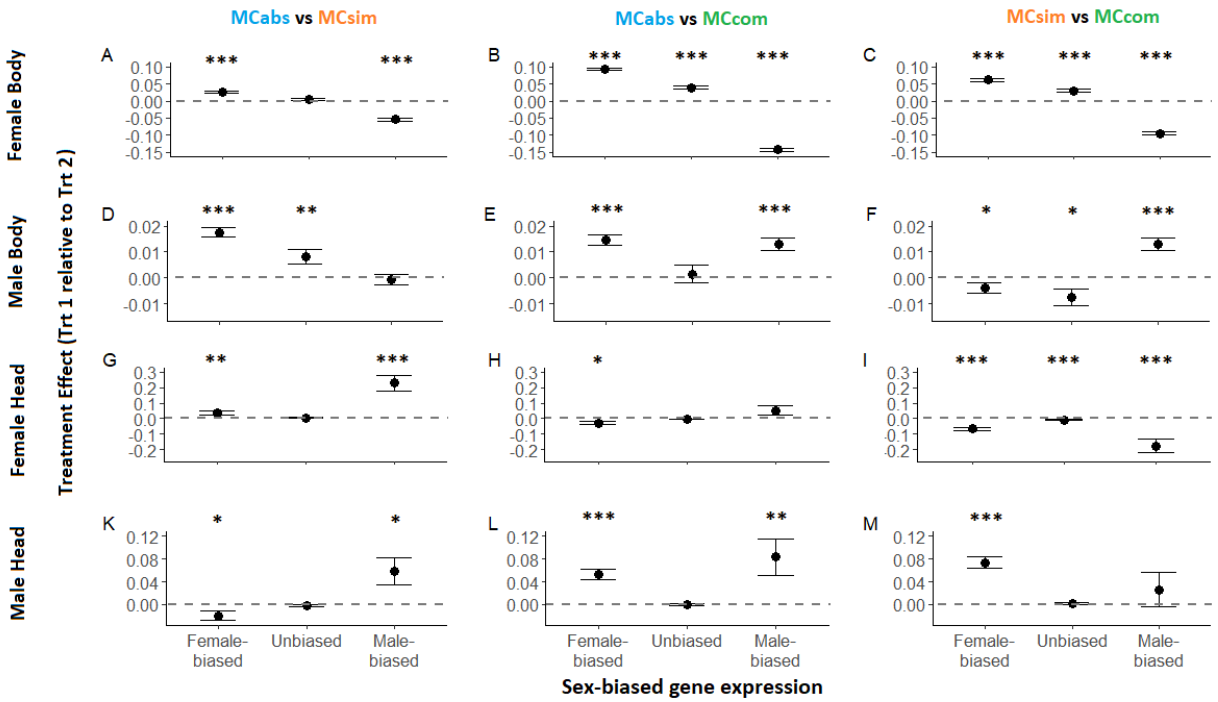
303

304

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

306 In the previous section we tested whether different types of genes (with respect to sex-bias) are more
307 likely to diverge, but we did not test for patterns in the direction and magnitude of divergence. In their
308 study, Hollis et al. (2014) predicted that, relative to polygamy, expression would be feminized in
309 populations evolving in monogamy, i.e., female-biased genes evolve to be upregulated in monogamy,
310 while male-biased genes become downregulated. Similar patterns are predicted in both female and
311 male tissues due to the presumed shared genetic architecture that constrains dimorphism under any
312 given mate competition regime (Prasad et al. 2007; Hollis et al. 2014).
313
314 We evaluated expression divergence in our populations from this Hollis et al. perspective by examining
315 divergence separately for female- and male-biased genes, focusing on the contrasts of our monogamy
316 treatment (MCabs) with each the two polygamy treatments (MCsim, MCcom). To visualise and test for
317 patterns of transcriptomic feminization/masculinization, we used the 'treatment effect' for each gene as
318 a measure of expression change, defined as the logarithmic ratio of expression in treatment 1 relative to
319 treatment 2. A positive treatment effect in a male-biased gene, for instance, would imply that its
320 expression is relatively masculinized in treatment 1; for female-biased genes, a positive treatment effect
321 would similarly imply relatively feminized expression in treatment 1. (We included unbiased genes for
322 completeness but these are not pertinent to the Hollis et al. prediction.) Results (Fig. 1) suggest that the
323 relationship between treatment effect and sex bias is inconsistent across treatment pairs, sexes and
324 sample types (head/body). Matching the Hollis et al. (2014) prediction, there is an overall feminization
325 of the female transcriptome in MCabs relative to the other two treatments, with a significant increase in
326 expression of female-biased genes and a significant decrease in expression of male-biased genes in
327 MCabs (Fig. 1A-B). In male bodies (Fig. 1D-E), the net changes are smaller, but more importantly, the
328 pattern is different. Though female-biased genes have increased expression in MCabs relative to the
329 other two treatments (similar to the female body result), male-biased genes are not significantly
330 reduced in expression in MCabs relative to MCsim and, in fact, have significantly increased expression in
331 MCabs relative to MCcom (i.e., opposite the prediction). A further layer of complication is added when
332 examining the heads, where the results differ between female and male samples and in neither sex is
333 there a consistent pattern of the predicted feminization of MCabs (Fig 1G-H, K-L).
334

335 Fig. 1 depicts mean treatment effects in discrete, and somewhat arbitrarily-bounded categories of sex
336 bias. We also performed LOESS regressions of treatment effect against continuously varying sex bias to
337 discern any patterns missed by treating variation in sex-biased gene expression as discrete. These
338 regressions (Fig. S2) show that the relationship between treatment effect and sex bias can vary within a
339 sex bias category, a nuance not captured in the discrete version of the plots. However, the overall sign
340 of mean treatment effect for a sex bias category is largely consistent in the two analyses.
341
342 As another approach to evaluating the Hollis et al. prediction, we looked for evidence of net changes in
343 feminization/masculinization via a different method using only the TDE genes. In female bodies, there is
344 a significant net feminization of TDE genes in MCabs relative to MCcom (Fig. S3), similar to the results
345 depicted in Fig. 1 and matching the Hollis et al. prediction. In male bodies, there is a significant net
346 masculinization of TDE genes in MCabs relative to MCcom (Fig. S3), opposite to the Hollis et al.
347 prediction. In most other contrasts, there is no significant net feminization or masculinization.
348
349
350
351
352
353



N

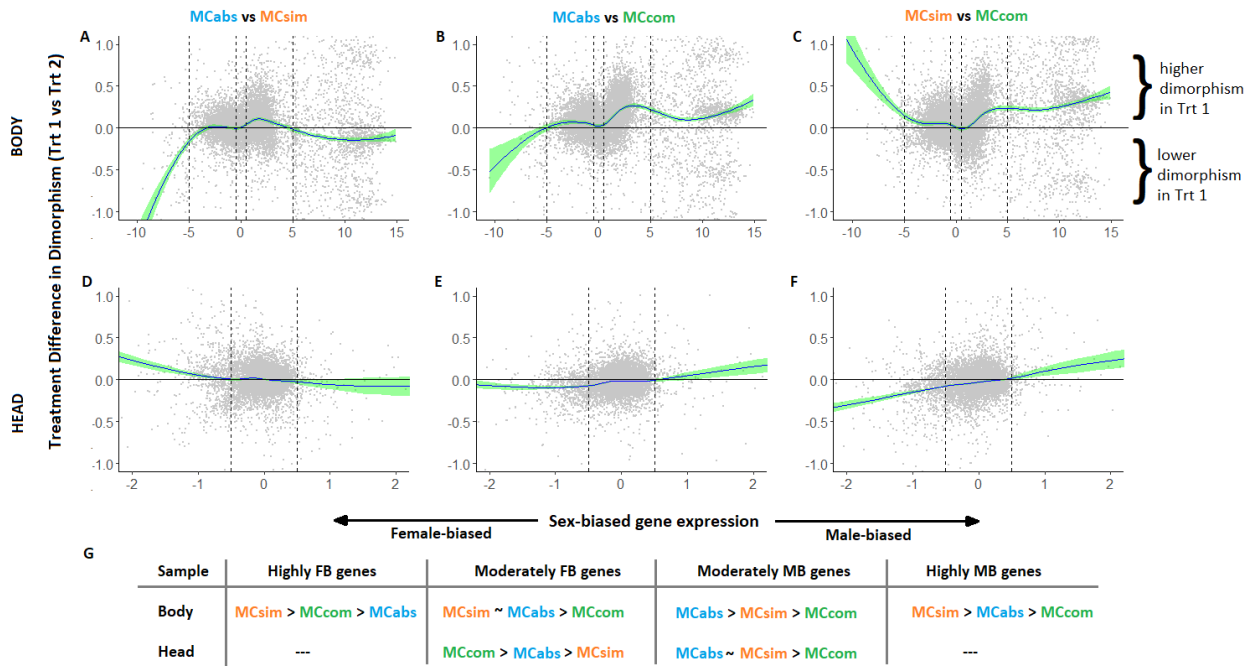
Sample	Female-biased genes	Male-biased genes
Female Body	MCabs > MCsim > MCcom	MCcom > MCsim > MCabs
Male Body	MCabs > MCcom > MCsim	MCsim ~ MCabs > MCcom
Female Head	MCcom > MCabs > MCsim	MCcom ~ MCabs > MCsim
Male Head	MCsim > MCabs > MCcom	MCabs > MCsim ~ MCcom

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.

Changes in degree of sexual dimorphism

In the previous section we examined expression changes for each sex separately, but these within-sex changes are not directly informative of changes in sexual dimorphism. Here we investigate differences

368 between treatments in the degree of expression dimorphism across genes with different levels of sex-
369 bias as determined from an external dataset (see Methods). We used LOESS regressions to evaluate how
370 treatment differences in 'local average dimorphism' varied with sex-bias (where local average refers to
371 the difference in dimorphism averaged across a local range of sex-bias). Patterns were complex (Fig. 2)
372 though treatment differences in local average dimorphism did tend to be larger for sex-biased than
373 unbiased genes. Among the sex-biased genes, the magnitude and even direction of treatment
374 differences in local average dimorphism varied between, but also within, sex-bias categories. In bodies,
375 local average dimorphism is highest in MCsim for both highly female- and male-biased genes, but
376 patterns differ for moderately-biased genes (summarized in Fig. 2G). In heads, where phenotypic sexual
377 dimorphism is not as marked, changes in local average dimorphism are detectable despite the relatively
378 low number of sex-biased genes. Female-biased genes are, on average, most dimorphic in MCcom, but
379 male-biased genes are least dimorphic in MCcom. Analyses excluding gonad-specific genes yielded
380 nearly identical results.
381
382



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. The sex bias 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.

Differential splicing amongst mating treatments

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

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

Discussion

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

If a history of sex-differential selection arising from mate competition is responsible for pre-existing SBGE, then we expect that selection on genes that are initially sex-biased is more likely to be sensitive to

430 a change in mate competition than selection on unbiased genes. Thus, we predicted that sex-biased
431 genes would be more likely to diverge among mate competition treatments than unbiased genes. The
432 results largely supported this prediction (Table 2); all seven of the comparisons in which there was a
433 significant difference were in the predicted direction (though not all comparisons yielded a significant
434 difference).

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

443 We also compared the relative frequency of DE genes in male- versus female-biased genes. Past studies
444 have shown that male-biased genes evolve more rapidly than female-biased genes, both in DNA
445 sequence (Meisel, 2011; Zhang et al., 2004) and gene expression (Allen et al., 2018; Meiklejohn et al.,
446 2003), though some studies describe more mixed results (Whittle & Johannesson, 2013; Yang et al.,
447 2016). Adaptive evolution in response to sexual selection has been invoked as an explanation (Ellegren
448 & Parsch, 2007), with some studies suggesting that male-biased genes are targeted more often by
449 strong positive selection (Pröschel et al., 2006; Zhang et al., 2004; but see Singh and Agrawal 2023)
450 presumably resulting from reproductive interactions. Our manipulation of mate competition resulted in
451 more divergence in expression of male- than female-biased genes (Table 2): in the six comparisons
452 where there was a significant difference, male-biased genes had a higher propensity for expression
453 divergence than female-biased genes. In some sense, this matches with fitness data showing stronger
454 signatures of local adaptation to the mate competition environment in males than females (Yun et al.
455 2019), suggesting greater phenotypic divergence in males than females. However, Table 2 contrasts
456 male- and female-biased genes within each sex, rather than contrasting divergence of males versus that
457 of females. There are not obvious differences in the proportion of significantly diverged genes between
458 male and female samples. This apparent discrepancy could be reconciled if the expression divergence of
459 male-biased genes has greater phenotypic and fitness consequences in males than females.

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

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

477 Because selection in enforced monogamy can differ from selection under mate competition in a variety
478 of ways, other details could become important to how divergence occurs. These three studies differ in
479 various ways which could, in principle, contribute to the among-study heterogeneity in results (e.g.,
480 species studied, nature of starting variation, food, temperature, density and population size, and
481 maintenance procedures (Li Richter & Hollis 2021)). One potentially important aspect is the precise
482 nature of mate competition, which can take many forms (e.g., there is no one true form of “polygamy”
483 for a “monogamy vs. polygamy” contrast). Our study directly shows that differences in how mate
484 competition occurs has major consequences for expression evolution. Within the context of our
485 experiment, where most other factors are not varied among treatments, the transcriptional differences
486 between our two different mate competition treatments (i.e., MCsim vs. MCcom) are generally as large
487 as those between either one of these with the enforced monogamy treatment (MCabs).

488 Though selection arising from mate competition is almost certainly an important driver of SBGE (Parsch
489 & Ellegren, 2013), there is little direct evidence (Harrison et al. 2014). It seems intuitive that, in the

490 absence of mate competition, expression dimorphism would become reduced. For example,
491 monogamous bird species are often less dimorphic with respect to plumage (and other traits) than non-
492 monogamous species, and presumably this reflects a reduction in expression dimorphism for genes
493 affecting such traits. Yet, it is unclear what the prediction for genome-wide expression dimorphism
494 should be as intralocus conflict on expression can easily exist under monogamy given that males and
495 females play different roles in reproduction and offspring rearing, even if overt traits such as plumage
496 do not experience divergent selection.

497 While the underlying motivation for the studies of Hollis et al. (2014) and Veltsos et al. (2017) was based
498 around sex-biased gene expression, neither attempted to examine how enforced monogamy changed
499 expression dimorphism per se. We examined how the average treatment effect on expression
500 dimorphism varied across local ranges of sex bias as determined in LOESS regressions. We found
501 differences in 'local average dimorphism' among mating regimes. These changes in local average
502 dimorphism tend to occur to a greater extent among sex-biased than unbiased genes, and more so in
503 bodies, which have much more sex-biased gene expression than heads (Fig. 2). However, we did not find
504 a consistent reduction in local average dimorphism between the enforced monogamy treatment and the
505 two treatments with mate competition; while some categories of sex-biased genes became less
506 dimorphic under monogamy, others became more so (Fig. 2). For example, relative to MCsim, MCabs
507 evolved reduced local average dimorphism of strongly sex-biased genes but increased dimorphism of
508 moderately male-biased genes in body samples. However, one aspect of expression was consistent with
509 the intuition of reduced dimorphism under monogamy: there was a notable reduction in the frequency
510 of genes with sex-specific splicing in heads of MCabs relative to either of the other two treatments,
511 though not in bodies (Table 1). While our experiment is the result of more than 100 generations of
512 selection, this is a relatively short period relative to other evolutionary time scales (e.g., sister species);
513 perhaps a more consistent reduction in expression dimorphism would be evident after enough time.
514 Nonetheless, this study shows that, though expression dimorphism readily evolves among mate
515 competition environments, enforced monogamy does not necessarily cause a rapid and consistent
516 reduction in dimorphism.

517 The most striking change in local average dimorphism occurred between the two treatments involving
518 mate competition, with MCsim showing greater dimorphism in bodies than MCcom across all sex bias
519 categories (Fig. 2C). Relative to MCcom, the MCsim environment is one in which intersexual
520 interactions, including mating, occur more frequently (Yun et al. 2017, 2019); exposure to males is more

521 harmful to females in the simple than complex environment and MCsim males have evolved to be more
522 harmful (Yun et al. 2017, 2021). Thus, it is reasonable to infer that interlocus sexual conflict is relatively
523 more important in MCsim than MCcom. However, this does not provide an obvious explanation for the
524 observed difference in expression dimorphism, as dimorphism is thought to evolve in response to intra-,
525 rather than interlocus, conflict.

526 In this study, we have treated each gene's expression as a trait (i.e., a measurable property) and
527 examined patterns in how these 'traits' change in response to an evolutionary manipulation of mate
528 competition. Just as with traditional traits (i.e., morphological, behavioral, or physiological phenotypes),
529 multiple possible genetic mechanisms could underly each change and a single genetic change could
530 affect multiple traits. At one (unlikely) extreme, every gene expression change could be due to a change
531 in that gene's *cis*-regulatory region. At the other extreme, a single genetic change could affect
532 expression levels of many genes, possibly by changing the size of different organs or relative abundance
533 of cell types within organs (Stewart et al. 2010). The truth likely lies somewhere between these two
534 extremes. In an evolutionary manipulation of mating system in *Drosophila pseudoobscura*, Wiberg et al.
535 (2021) found that genomically diverged sites were enriched near sites with expression divergence. That
536 result would not be expected if a very small number of genetic changes underlay most expression
537 differences, but it also does not imply that most expression changes are due to individual *cis*-regulatory
538 changes.

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

551

Acknowledgements

We thank George Sandler for doing the RNA extractions. This work was supported by the Natural Sciences and Engineering Research Council of Canada (AFA & HDR).

558 **Literature Cited**

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

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

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

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

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

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

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

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

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

577 Dean, R., A. E. Wright, S. E. Marsh-Rollo, B. M. Nugent, S. H. Alonzo, and J. E. Mank. 2017. Sperm
578 competition shapes gene expression and sequence evolution in the ocellated wrasse. Mol. Ecol.
579 26:505–518.

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

582 dos Santos, G., A. J. Schroeder, J. L. Goodman, V. B. Strelets, M. A. Crosby, J. Thurmond, D. B. Emmert,
583 W. M. Gelbart, and FlyBase Consortium. 2015. FlyBase: introduction of the *Drosophila*

584 *melanogaster* Release 6 reference genome assembly and large-scale migration of genome
585 annotations. Nucleic Acids Res. 43:D690–7.

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

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

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

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

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

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

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

600 Holland, B., and W. R. Rice. 1999. Experimental removal of sexual selection reverses intersexual
601 antagonistic coevolution and removes a reproductive load. Proc. Natl. Acad. Sci. U. S. A. 96:5083–
602 5088.

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

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

607 Lande, R. 1980. Sexual dimorphism, sexual selection, and adaptation in polygenic characters. Evolution
608 34:292–305. JSTOR.

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

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

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

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

618 MacPherson, A., L. Yun, T. S. Barrera, A. F. Agrawal, and H. D. Rundle. 2018. The effects of male harm
619 vary with female quality and environmental complexity in *Drosophila melanogaster*. Biol. Lett.
620 14:20180443.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

656 Witt, E., N. Svetec, S. Benjamin, and L. Zhao. 2021. Transcription factors drive opposite relationships
657 between gene age and tissue specificity in male and female *Drosophila* gonads. Mol. Biol. Evol.
658 38:2104–2115.

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

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

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

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

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

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

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

674

675

676

677

678

679