Expression divergence in response to sex-biased selection

Karl Grieshop[1,2,3*†], Michelle J. Liu[2,* †], Ryan S. Frost[2], Matthew P. Lindsay[2], Malak Bayoumi[2], Martin I. Brengdahl[4], Ruxandra I. Molnar[5] and Aneil F. Agrawal[2,*]

[†]These authors contributed equally

[1] School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ, UK

[2]Department of Ecology and Evolutionary Biology, University of Toronto, Toronto, Ontario, M5S 3B2 Canada

[3]Department of Molecular Biosciences, The Wenner-Gren Institute, Stockholm University, 10691 Stockholm, Sweden

[4]IFM Biology, Linköping University, 581 83 Linköping, Sweden

[5]Department of Molecular Genetics, University of Toronto, Toronto, ON M5S 1A8 Canada

ORCIDs:

https://orcid.org/0000-0001-8925-5066 (KG)

https://orcid.org/0009-0002-8270-1451 (MJL)

https://orcid.org/0009-0002-8698-5711 (RF)

https://orcid.org/0000-0002-1052-7274 (MIB)

https://orcid.org/0000-0001-9123-3261 (MPL)

https://orcid.org/0009-0005-0646-0838 (MB)

https://orcid.org/0000-0003-2496-2733 (AFA)

*Corresponding authors:

k.grieshop@uea.ac.uk (KG)
mchelle.liu@mail.utoronto.ca (MJL)
a.agrawal@utoronto.ca (AFA)

Abstract

It remains debated whether greater degrees of sexual dimorphism would evolve if not for intersexual genetic constraints. Here we used experimental evolution to partially break the intersexual genetic constraint in *Drosophila melanogaster* to test for the presence of sexually antagonistic genetic variation and characterize the evolution of sexual dimorphism in gene expression. In six replicate populations of 1000 flies, a dominant marker (*DsRed*) on Chromosome 2 was used to force a "*Red*" pool of genetically variable chromosomes through exclusive father-to-son inheritance, while a complimentary pool of "*NonRed*" chromosomes was inherited primarily from mothers to daughters. After 100 generations, males carrying a *Red* chromosome copy exhibited greater mating success than males with only *NonRed* chromosomes, consistent with the accumulation of male-benefit/female-detriment sexually antagonistic alleles in the *Red* pool relative to *NonRed*. Differentially expressed genes between flies with and without *Red* chromosomes had on average higher intersexual genetic correlations (r_{MF}), as expected if such correlations represent a constraint to sex-specific adaptation under normal inheritance. If conflict hinders the evolution of further dimorphism, the transcriptomes of male-selected *Red* chromosomes were predicted to evolve to be "masculinized" relative to female-selected *NonRed* chromosomes. Consistent with this, splicing patterns in *Red* males (but not *Red* females) were masculinized relative to *NonRed* males. Contrastingly, gene expression levels were largely feminized in *Red* flies of both sexes compared to *NonRed*. We discuss alternative forms of intralocus sexual conflict that may explain these patterns.

Introduction

Differences in the ways that males and females maximize fitness can cause shared traits to experience sexually discordant or antagonistic selection. Despite this, most traits are expected to be genetically correlated between the sexes due to their shared gene pool, meaning that selection in one sex will cause a correlated response in the other (Lande 1980). If the intersexual genetic correlation (r_{MF}) for a given trait is strong and positive, it can constrain said trait from responding to sexually antagonistic selection and prevent both sexes from reaching their respective sex-specific optimum, a phenomenon known as intralocus sexual conflict (Lande 1980; Bonduriansky and Chenoweth 2009).

Sexual dimorphism is often thought to result from sexually antagonistic selection, although dimorphism can also evolve for other reasons (Lande 1980; Cheng and Houle 2020). Even when sexual dimorphism does reflect the response to sexually antagonistic selection, it remains debated whether the current degree of sexual dimorphism reflects resolved conflict, or whether higher levels of dimorphism in some traits would evolve if not due to intersexual genetic constraints. To address this debate, Prasad et al. (2007) used an approach first employed by Rice (1996, 1998) to experimentally restrict the inheritance of haplotype genomes in *Drosophila melanogaster* to only males (father-to-son transmission), thereby removing the constraint due to female-specific selection on a (normally) shared gene pool. After 25 generations, these evolved male-limited haplotypes conferred an increase of fitness in males but a reduction of fitness in females, presumably because male-benefit/female-detriment alleles were free to accumulate in the absence of female-specific selection that would (normally) constrain the response to male-specific selection (and logically, vice versa). Importantly, male-limited haplotypes, when expressed in either sex, caused a “masculinization” (i.e., trait values moved further in the male

direction of sexual dimorphism) in several sexually dimorphic traits such as body size, growth rate, and development time (Prasad et al. 2007), as well as wing size and shape (Abbott et al. 2010). These observations provide some evidence that the shared gene pool can represent an ongoing constraint in the evolution of traits experiencing sexually antagonistic selection for further dimorphism. Transcriptomics allows us to further explore this question with respect to thousands of gene expression traits simultaneously.

The expression of a gene can be considered as a quantitative trait and may also exhibit dimorphism between the sexes. The most commonly studied form of expression dimorphism is sex-differences in the level of expression of a given gene (known as sex-biased gene expression, "SBGE"). SBGE is common across the genome of various plant and animal taxa (Ellegren and Parsch 2007). Like other sexually dimorphic traits, the current magnitude of SBGE for a given gene may represent an evolutionary response to past sexual conflict over expression, but it is unclear whether conflict is fully resolved or whether there remains sexually antagonistic selection for further dimorphism in gene expression. One advantage in studying expression traits (as opposed to classical traits) is that the *cis* regulatory regions of genes identified through these studies are obvious candidates for regions of sexually antagonistic polymorphisms that can be subject to further investigation. Given these considerations, transcriptomics has been increasingly implemented in more recent studies of sexual antagonism. One such study was done by Abbott et al. (2020), who employed a similar experimental design as Prasad et al. (2007) to implement 50 generations of male-limited evolution on a pool of X chromosomes *in D. melanogaster* and test for evidence of ongoing sexual conflict. Consistent with prediction, they found a masculinization in expression profiles of flies carrying a copy of the male-limited X (i.e., upregulation of male-biased genes and downregulation of female-biased genes). However, it is

97 unclear how broadly their conclusions apply considering that X chromosomes exhibit distinct
98 inheritance, dosage compensation (Meiklejohn et al. 2011), and distribution of sex-biased genes
99 (Parisi et al. 2003; Sturgill et al. 2007) relative to the rest of the genome.

100 Besides sexual dimorphism in the level of a gene's expression, the sexes can also differ in
101 how a gene is spliced. Sex-specific splicing (SSS; also referred to as "sexually dimorphic
102 isoform usage") refers to quantitative differences between the sexes in the relative abundance of
103 alternative isoforms produced for a given gene. Similar to SBGE, genes displaying SSS are
104 widespread across the genome (McIntyre et al. 2006; Telonis-Scott et al. 2009; Blekhman et al.
105 2010; Naftaly et al. 2021) and may have evolved to alleviate the conflict caused by sex-
106 differences in selection (Rogers et al. 2021). The link between SSS and sexual conflict is
107 relatively underexplored, though previous studies which investigated SBGE and SSS found that
108 their co-occurrence is underrepresented, possibly implying that they represent different routes in
109 the resolution of sexual conflict (Rogers et al. 2021; Singh and Agrawal 2023).

110 Here we infer how the shared gene pool of the sexes may constrain the evolution of
111 dimorphic gene regulation, both with respect to expression levels and isoform usage. To relax
112 constraints imposed by a shared gene pool, we used experimental evolution in *D. melanogaster*
113 to divide ~50% of autosomal genes (i.e., Chromosome 2) into two separate gene pools. In six
114 replicate populations of 1000 flies, a genetically variable pool of copies of Chromosome 2, each
115 marked with a dominant fluorescent marker (*DsRed*; hereafter "*Red*" pool), was experimentally
116 forced to undergo male-limited father-to-son inheritance (i.e., Y-like inheritance). Concurrently, a
117 complimentary "*NonRed*" pool of chromosomes was disproportionately inherited from mother-
118 to-daughter (i.e., X-like inheritance). This separation of genes pools is expected to allow sex-
119 specific adaptation that would have previously been constrained under normal autosomal

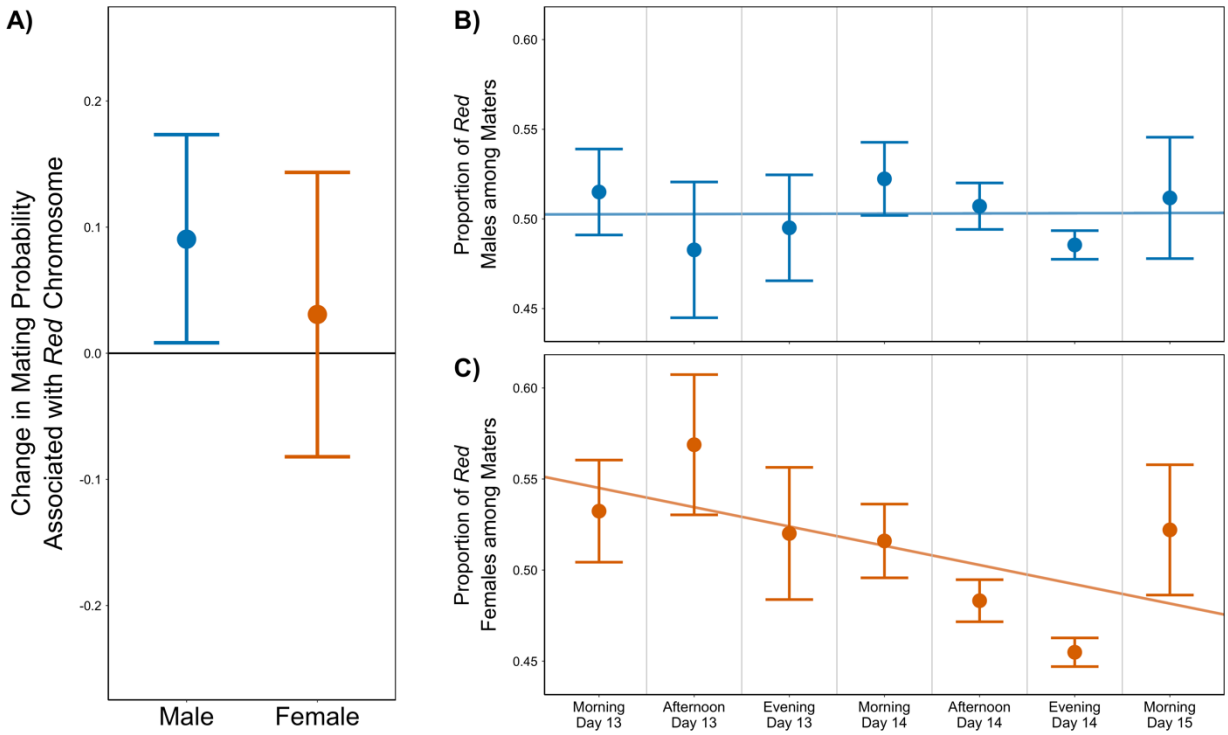
120 inheritance. Thus, divergence between *Red* and *NonRed* gene pools should reveal pre-existing
121 sexually antagonistic variants segregating in the common ancestral population.
122 We first provide evidence for ongoing sexual antagonism by comparing the effect of *Red*
123 vs. *NonRed* chromosomes on mating success, a major component of male fitness. We then
124 investigate divergence in the levels of gene expression and isoform usage between *Red* and
125 *NonRed* chromosomes to test predictions regarding the characteristics of sexually antagonistic
126 genes. Finally, we assess the directionality of the expression changes in carriers vs. non-carriers
127 of the male-limited *Red* chromosome with respect to existing sexual dimorphism. If the shared
128 genome is a constraint in the evolution of further dimorphism, we expect that evolution in a
129 male-limited pool will result in a masculinization of gene expression in individuals carrying a
130 copy of the *Red* chromosomes.

Results

Mating Assays

Six replicate populations of 1000 flies each were subject to experimental evolution wherein a dominant marker, *DsRed*, was used to enforce male-limited (Y-like) inheritance on a pool of 500 genetically diverse marked "*Red*" copies of Chromosome 2, and X-like inheritance on the complementary set of 1500 genetically diverse wildtype "*NonRed*" copies of Chromosome 2, for ~100 generations (details in Materials and Methods). If the shared gene pool of the sexes prevents each sex from reaching their optimum fitness, then restricting the inheritance of *Red* Chromosome 2 copies to only males should lead to the accumulation of male-beneficial variants in this chromosome pool. As mating success is a major component of male fitness, we assessed the mating success rates of males carrying a copy of a *Red* chromosome (*DsRed*/+, "*Red*" males) relative to those who did not (+/+, "*NonRed*" males) over a two-day period that aligned with the "interaction" period of the experimental evolution regime. *Red* males had a greater chance of being observed in copulation than *NonRed* males ($P \approx 0.01$; Fig. 1A). The proportion of *Red* males among maters was relatively constant across the duration of the two-day mating assay (Pearson's product-moment correlation $r = 0.001$, $P \approx 0.98$; Fig. 1B).

148



149

Figure 1. The effect of male-selected *Red* chromosomes on mating propensity of males and females. Panel **(A)** shows the estimated change in the probability of being observed in a successful mating event given that an individual carries vs. does not carry a *Red* chromosome. Panels on the right display the average proportion of *Red* individuals among the total number of observed maters within each observation window for **(B)** males and **(C)** females. Error bars indicate standard error of the mean. "Day" refers to the number of day(s) after eggs were first laid. The slope of the proportion of mating males carrying a *Red* chromosome over time windows (B) is not statistically different from zero, whereas the corresponding slope is significantly negative for females (C; $P < 0.05$, Pearson's product-moment correlation). Mating activity peaked on the evening of Day 14 (Fig. S6), which immediately preceded the time which would correspond to the key oviposition period during which eggs laid were allowed to contribute to the next generation in the normal maintenance schedule (i.e., Morning of Day 15/Day 1 of the subsequent generation).

150

151

152 Though the primary goal of the mating assay was to assess the effect of the male-selected
153 *Red* chromosomes on male mating success, the assay design also allowed us to investigate the
154 effect of *Red* chromosomes in females. There was no difference in mating success of *Red* vs.
155 *NonRed* females ($P \approx 0.5$; Fig. 1A). This is perhaps not surprising given that mating success is
156 not thought to be as important to female fitness as it is to male fitness. Intriguingly, however, the
157 proportion of *Red* females among all mating females decreased significantly over the duration of
158 the mating assays until their usual oviposition period (i.e., the morning of Day 15; Pearson's
159 product-moment correlation $r = -0.09$, $P \approx 0.04$; Fig. 1C). These results are consistent with two
160 non-mutually exclusive phenomena: i) male-limited selection on *Red* chromosomes having
161 caused a correlated response in female mating propensity (i.e., *Red* females mate sooner), which
162 could be maladaptive to females (Fowler and Partridge 1989; Travers et al. 2015), and ii) female-
163 biased selection on *NonRed* chromosomes having caused adaptation in the timing of mating
164 behavior surrounding oviposition. While we did not measure female fecundity, it is plausible that
165 the increased matings of *NonRed* females nearer to the timing of their oviposition day (Fig. S6)
166 would have conferred higher fecundity to *NonRed* relative to *Red* females as male seminal fluids
167 transmitted during matings can cause a temporary increase in egg laying rate (Chapman et al.
168 2003).
169
170 *Differential Expression Analysis*
171 Because *Red* chromosomes were selected only in males while their *NonRed* counterparts
172 experienced selection disproportionately in females, expression differences between *Red* and
173 *NonRed* genotypes are expected to be enriched for genes under sexually antagonistic selection.
174 We measured gene expression in *Red* and *NonRed* flies of both sexes originating from the six

replicate populations which had evolved for ~100 generations under the experimental evolution regime. We performed differential gene expression analysis between genotypes with and without *Red* chromosomes to identify candidate targets of sexually antagonistic selection on expression. We identified 385 differentially expressed (DE) candidate genes which significantly differed in the magnitude of gene expression between *Red* and *NonRed* females from our Experimental populations (5% FDR; genes near the *DsRed* marker are excluded). Due to higher variance in *Red* vs. *NonRed* effect sizes among male samples, only 15 genes met the equivalent statistical threshold in males. Instead, a more liberal criterion was used to define DE genes from the *Red* vs. *NonRed* male comparison by selecting the 350 genes with the greatest magnitude of expression differences.

Though different genes were identified from males and females (only 40 genes overlap), the *Red* vs. *NonRed* effect of DE genes identified from one sex were positively correlated with their effect in the other sex (Pearson's correlation $r = 0.64$, $P < 0.01$ and $r = 0.66$, $P < 0.01$ for DE genes identified from males and those identified from females, respectively; Fig. S7), as expected given that the intersexual correlation in level of expression tends to be strongly positive for most genes in this species (Griffin et al. 2013; Singh and Agrawal 2023). In comparison, the correlations between *Red* vs. *NonRed* effects of DE candidate genes when measured in Experimental samples vs. in Controls were considerably weaker ($r = -0.06$ and $r = 0.2$ for DE genes identified from males and females, respectively; Fig S7). The correlations in effects across sexes of Experimental samples and the relative lack of correlation in effects between Experimental and Control samples suggest that expression differences in DE genes can be largely attributed to the experimental evolution and are not merely measurement error. In the sections below we describe the analyses featuring the combined set of DE genes identified from

198 males and females (695 genes in total), though corresponding analyses performed separately for
199 DE genes identified from males and those identified from females are included in the
200 Supplementary Material.

201 DE candidate genes comprised 5% (695/13612) of total transcripts examined and were
202 not evenly distributed across chromosomes. Unsurprisingly, these genes were overrepresented
203 (6.4%) on Chromosome 2 (i.e., the manipulated chromosome) relative to Chromosome 3 (4.2%;
204 Fisher's exact $P < 0.01$) and to the X chromosome (4.3%; Fisher's exact $P < 0.01$). The excess of
205 DE genes on the focal chromosome along with the presence of some DE genes on non-focal
206 chromosomes implies that both *cis*- and *trans*- regulatory effects contributed to expression
207 differences between *Red* and *NonRed* genotypes. Only one GO term relating to mRNA
208 degradation (GO:1990726) was significantly associated with DE genes upregulated in *Red*
209 relative to *NonRed* samples, but a wider variety of GO terms were enriched for genes that were
210 downregulated (Table S2). While it is difficult to draw meaningful inferences from these GO
211 enrichments, some of the enriched GO terms are intriguing in the context of sexually
212 antagonistic selection (e.g., reproductive process, locomotion, as well as several others relating
213 to neuromuscular functions and components). When comparing our set of DE genes with
214 candidate sexually antagonistic genes identified from past studies, we found moderate and
215 marginally significant overlap with candidates identified in Innocenti and Morrow (2010;
216 Fisher's exact $P \approx 0.06$; Table S3), and statistically significant overlap with genes enriched in
217 candidate sexually antagonistic SNPs identified by Ruzicka et al. (2019; Fisher's exact $P < 0.05$;
218 Table S3).

219 We next investigate the relationship between sexual antagonism and sex-biased gene
220 expression (SBGE) using our set of DE candidate genes. The "Twin Peaks" model proposed by

Cheng and Kirkpatrick (2016) predicted that unbiased genes and those with extreme sex-bias (e.g., sex-limited expression) are least likely to experience sex-differences in selection due to its apparent absence or resolution, respectively. Thus, ongoing intralocus sexual conflict is thought to be disproportionately found at genes with intermediate levels of sex-biased expression. We tested whether this prediction is reflected in our data by examining the distribution of DE genes with respect to their estimated sex bias in total expression as determined from an external data set (see Materials and Methods). Specifically, if intermediately sex-biased genes are most likely to experience ongoing intralocus sexual conflict, then such genes should be enriched for the *Red*/*NonRed* DE genes.

In line with the prediction of Cheng and Kirkpatrick (2016), we found enrichment of DE genes among intermediately male-biased genes (defined from the external data set as: $1 < \log_2$FC Male:Female < 5; Fig. 2, Fig. S8). In contrast to prediction, DE genes were relatively underrepresented among intermediately female-biased category ($-5 < \log_2$FC Male:Female < -1). To further examine the "Twin Peaks" model, we re-analysed the data in a manner similar to that of Cheng and Kirkpatrick (2016), without binning but rather using their continuous scale of sex-bias, $\Delta = (m - f)/(m + f)$, where m and f are male and female expression levels, respectively. The results remain similar in that *Red*/*NonRed* DE genes were overrepresented among intermediately male-biased genes but underrepresented among female-biased genes (Fig. S9).

240

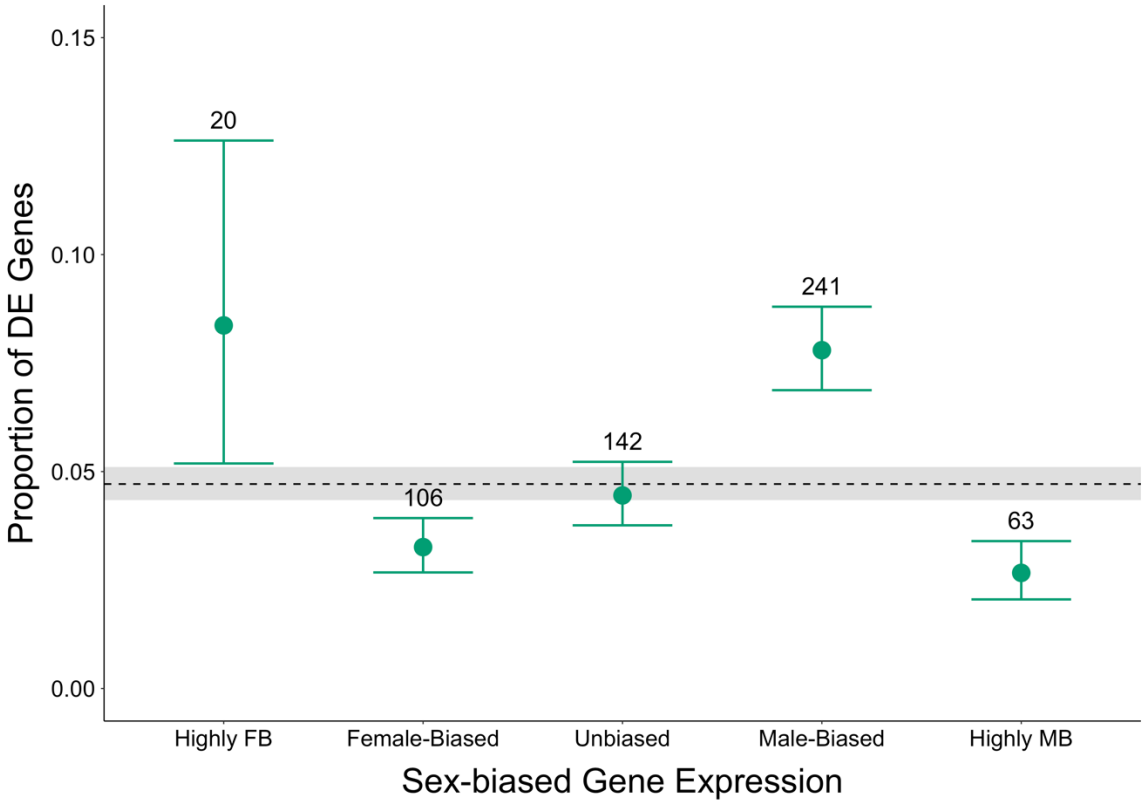


Figure 2. Frequency of differentially expressed DE genes across categories of sex bias in gene expression. Genes were classified into sex-bias categories based on expression from an external data set. The dashed line represents the overall proportion of DE genes out of all transcripts regardless of sex-bias, with 95% confidence interval (CI) shaded in grey. Points represent the point estimate for the fraction of all DE genes falling into each sex-bias category: Highly Female-biased (FB; $\log_2 FC < -5$; N = 239), Female-biased ($-5 < \log_2 FC < -1$; N = 3253), Unbiased ($-1 < \log_2 FC < 1$; N = 3191), Male-biased ($1 < \log_2 FC < 5$; N = 3091), and Highly Male-biased (MB; $\log_2 FC > 5$; N = 2364). Error bars represent 95% CIs calculated using the Clopper-Pearson method, and numbers above each bar indicate the number of DE genes in each bin.

241

242

The intersexual genetic correlation (r_{MF}) for any trait impedes the ability of that trait to evolve independently in one sex from the other. Consequently, traits—including gene expression traits—with high r_{MF} may experience a prolonged period of unresolved sexual conflict if they are subjected to sexually antagonistic selection (Lande 1980; Griffin et al. 2013). Given this rationale, we predicted that genes with high r_{MF} for their expression would be more likely to be experiencing the constraint ongoing sexual conflict and thus respond to selection when released from that constraint by sex-associated gene pools. To test this prediction, we compared the average r_{MF} (determined from a previously published data) for DE genes with that of the remaining genes assayed (hereafter "background genes").

On average, r_{MF} was significantly higher for DE genes compared to background genes (Fig. 3, Fig. S10). However, above we reported that male-biased genes were overrepresented among DE genes, and previous work reported that r_{MF} is elevated among male-biased genes (Singh and Agrawal 2023). To ensure that these relationships were not confounding our observation of higher r_{MF} for DE genes, we compared the average r_{MF} for DE and background genes in each SBGE category separately. Our conclusion for this analysis remains the same: r_{MF} was on average higher for DE genes compared to background genes, and significantly so for the comparison within unbiased and male-biased categories (Fig. 3).

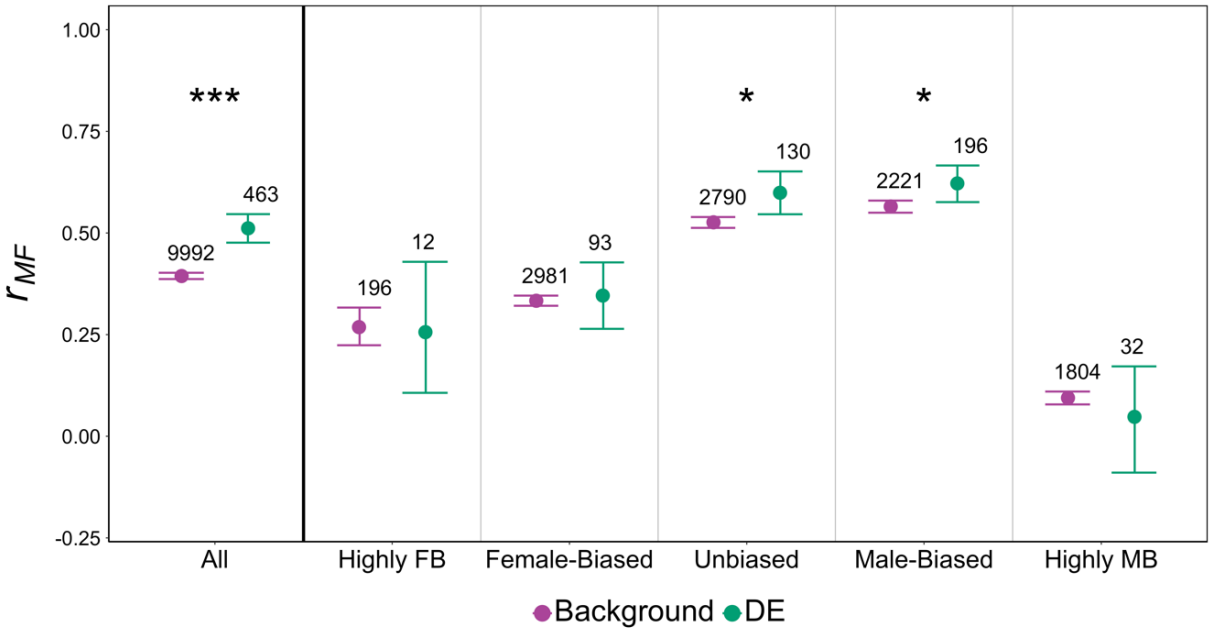


Figure 3. Average strength of intersexual genetic correlation (r_{MF}) in expression of differentially expressed (DE) and background genes. The leftmost panel shows the result of the analysis unstratified by sex-bias. The remaining panels display results stratified by sex-bias: Highly Female-biased (FB; $\log_2FC < -5$), Female-biased ($-5 < \log_2FC < -1$), Unbiased ($-1 < \log_2FC < 1$), Male-biased ($1 < \log_2FC < 5$), and Highly Male-biased (MB; $\log_2FC > 5$); genes were assigned to sex-bias category based on an external data set. Asterisks represent a significant difference ($P < 0.05$*, $P < 0.01$**, $P < 0.001$***, permutation test) between DE and background genes. Error bars indicate 95% bootstrapped CIs, and numbers above each bar represents the total number of genes represented by each dot. Note that only a small number of DE genes could be compared for highly sex-biased categories, thus results are included for completeness, but confidence intervals should be regarded with caution.

261

262

263 The average expression level of a gene is another covariate that positively correlates with
264 r_{MF} (Singh and Agrawal 2023) and could therefore drive our observed association. To ensure that
265 our observation of higher r_{MF} for DE genes was not driven by differences in expression levels
266 between DE and background genes, we repeated the comparison of r_{MF} between DE and
267 background genes by binning our data set into three average expression level categories (low,
268 medium, high). Again, DE genes displayed significantly higher r_{MF} compared to background
269 genes in all three comparisons (Fig. S11).
270
271 *Differential Splicing Analysis*
272 The relative usage of different isoforms is another gene expression trait that may be under
273 sexually antagonistic selection. Though we are constrained by the limitations of short-read
274 RNAseq data for assessing differences in splicing profiles, we identified 36 (0.31%; N=11539)
275 genes with evidence of differential splicing between *Red* and *NonRed* samples (10% FDR; 14
276 genes originated from *Red* and *NonRed* male comparison, and 24 genes from the female
277 comparison; 2 genes overlapped between both comparisons). As for the DE genes, the regions in
278 and around these differentially spliced (DS) genes are likewise expected to be enriched for
279 candidate sexually antagonistic polymorphisms. Consistent with the analysis of DE genes, DS
280 genes were disproportionately located on the manipulated Chromosome 2 (0.7%) relative to
281 Chromosome 3 (0.1%; Fisher's exact $P < 0.01$) and the X chromosome (0.2%; Fisher's exact $P <$
282 0.05). GO analysis did not reveal any terms enriched for this set of genes. We also found no
283 significant overlap of DS genes with sets of candidate sexually antagonistic genes identified in
284 Innocenti and Morrow (2010) nor in Ruzicka et al. (2019; Table S3).

285 Like other forms of dimorphism, sex-specific splicing (SSS)—sexual differences in the
286 isoform usage—may have evolved due to a history of sexually antagonistic selection, yet it is
287 unclear if the existence of SSS represents a complete resolution or whether such genes are likely
288 experiencing ongoing conflict. Though we found few DS genes overall, they were
289 overrepresented among genes with SSS compared to those without (Fisher's exact $P < 0.01$;
290 Table S4), suggesting that current levels of SSS represent an incomplete resolution. In addition,
291 DS genes were enriched among DE genes (Fisher's exact $P < 0.01$; Table S5).
292
293 *Direction of Sexual Dimorphism in the Effect of* Red *Chromosomes*
294 In this section we examine the effect of the experimentally evolved *Red* and *NonRed*
295 chromosomes on the overall expression profiles (regardless of the significance status of
296 individual genes) of males and females. If sexually dimorphic traits are undergoing antagonistic
297 selection for further dimorphism but are normally constrained by a shared gene pool, it has been
298 argued that allowing selection to occur only on one sex should relax this constraint and cause
299 trait values to evolve further in the direction of that sex (Prasad et al. 2007; Abbott et al. 2010;
300 Abbott et al. 2020). In the context of our experiment, this logic predicts that the male-selected
301 *Red* chromosomes, relative to the *NonRed* chromosomes, should have an expression profile
302 shifted further in the male direction (i.e., a "masculinization" of expression *sensu* Hollis et al.
303 2014). With respect to expression levels, this amounts to reduced expression of female-biased
304 genes and increased expression of male-biased genes.
305 We first consider expression in females. Contrary to prediction, we observe a pattern of
306 "feminization" in *Red* compared to *NonRed* females, with an average upregulation of female-
307 biased genes and downregulation of male-biased genes (Fig. 4, orange points; Table S7). As in

308 females, males also showed increased expression of female-biased genes and decreased
309 expression of male-biased genes in *Red* relative to *NonRed* samples (Fig. 4, blue points; Table
310 S7). (Examining only DE candidate genes resulted in a qualitatively similar pattern in the
311 direction of expression changes; Fig. S12.) A notable exception to the apparent "feminization"
312 was the increased expression of highly male-biased genes in *Red* compared to *NonRed* males
313 (Fig. 4; note that most highly male-biased genes could not be assessed in females due to low
314 expression). We looked for evidence of whether this distinctive upregulation of highly male-
315 biased genes was driven by an increase in testes size in *Red* males. On the contrary, the genes
316 that were amongst the top 5%, 10%, or 25% upregulated transcripts were less testis-specific
317 (calculated from an external data set) relative to those that were less or not upregulated in that
318 highly male-biased category (permuted $P < 0.001$ for all three tests; Table S6).
319

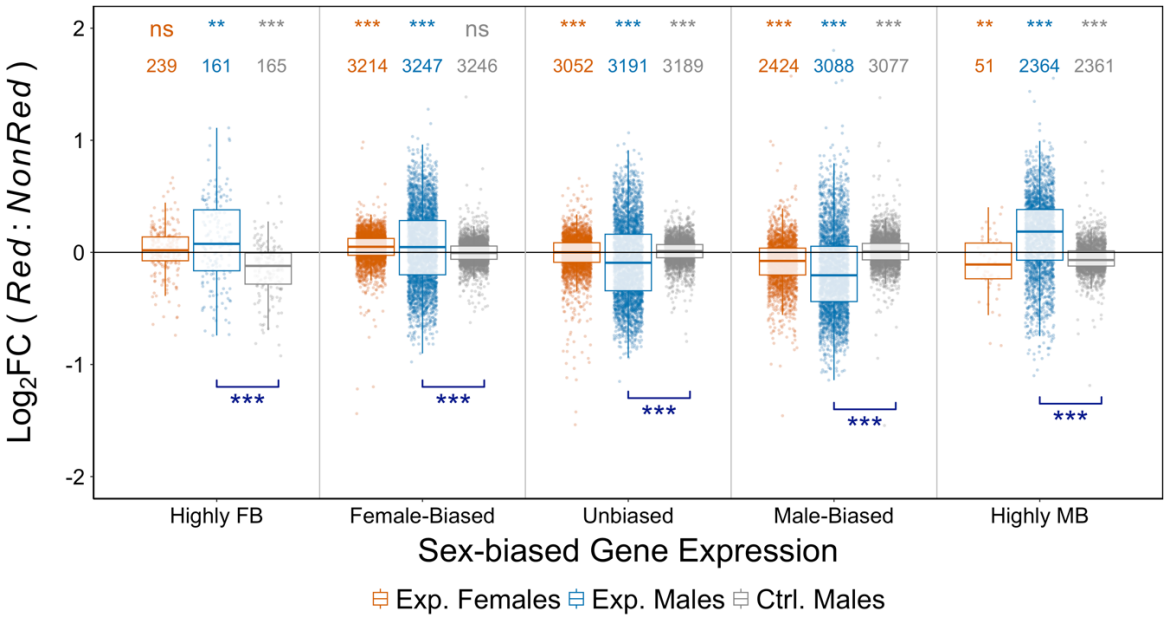


Figure 4. *Red* vs. *NonRed* changes in expression, stratified by sex-bias category: Highly Female-biased (FB; $\log_2FC < -5$), Female-biased ($-5 < \log_2FC < -1$), Unbiased ($-1 < \log_2FC < 1$), Male-biased ($1 < \log_2FC < 5$), and Highly Male-biased (MB; $\log_2FC > 5$); genes were assigned to sex-bias category based on an external data set. Log_2FC *Red* vs. *NonRed* changes were estimated from three types of samples: females from the Experimental populations (orange), males from the Experimental populations (blue), and males from the Control populations (grey). Positive (negative) values indicate higher (lower) expression in *Red* samples compared to *NonRed*. The number above each boxplot denotes the number of genes. Asterisks shown at the top of the figure represent significant deviation from zero ($P < 0.05$*, $P < 0.01$**, $P < 0.001$***, two-tailed permutation test). Asterisks shown at the bottom indicate significance ($P < 0.05$*, $P < 0.01$**, $P < 0.001$***, two-tailed permutation test) from the comparison of the effects of *Red* chromosomes in Experimental vs. Control males. No samples from Control female were collected.

Next, we consider the direction of difference in splicing patterns between *Red* and *NonRed* samples. To do so, we created a sexual splicing index, ϕ, to quantify the splicing profile of a gene in a focal sample type in relation to that in reference female vs. reference male splicing profiles, with $\phi = -1$ and $\phi = +1$ indicating maximal similarity to the reference female and male profile, respectively. Female and male reference splicing profiles were based on an external data set and ϕ was calculated for a set of 2036 genes that had consistent SSS profiles across several additional external data sets.

The splicing profiles of males and females from the Experimental populations were largely concordant with extant sexual dimorphism; that is, most genes in Experimental males (females) were more similar in their splicing profiles to the reference male (female) estimate than the reference splicing profile of the opposite sex. Our main interest is in the difference between *Red* and *NonRed* samples within each sex. On average, *Red* males had more strongly positive (i.e., more "male-like") values of ϕ than their *NonRed* counterparts (Fig. 5, Table S8; $P < 0.01$, paired *t*-test). Contrastingly, despite the relatively small difference, the splicing profiles of *Red* females were slightly, but significantly, more "feminized" relative to *NonRed* females (Fig. 5, Table S8; $P < 0.05$, paired *t*-test).

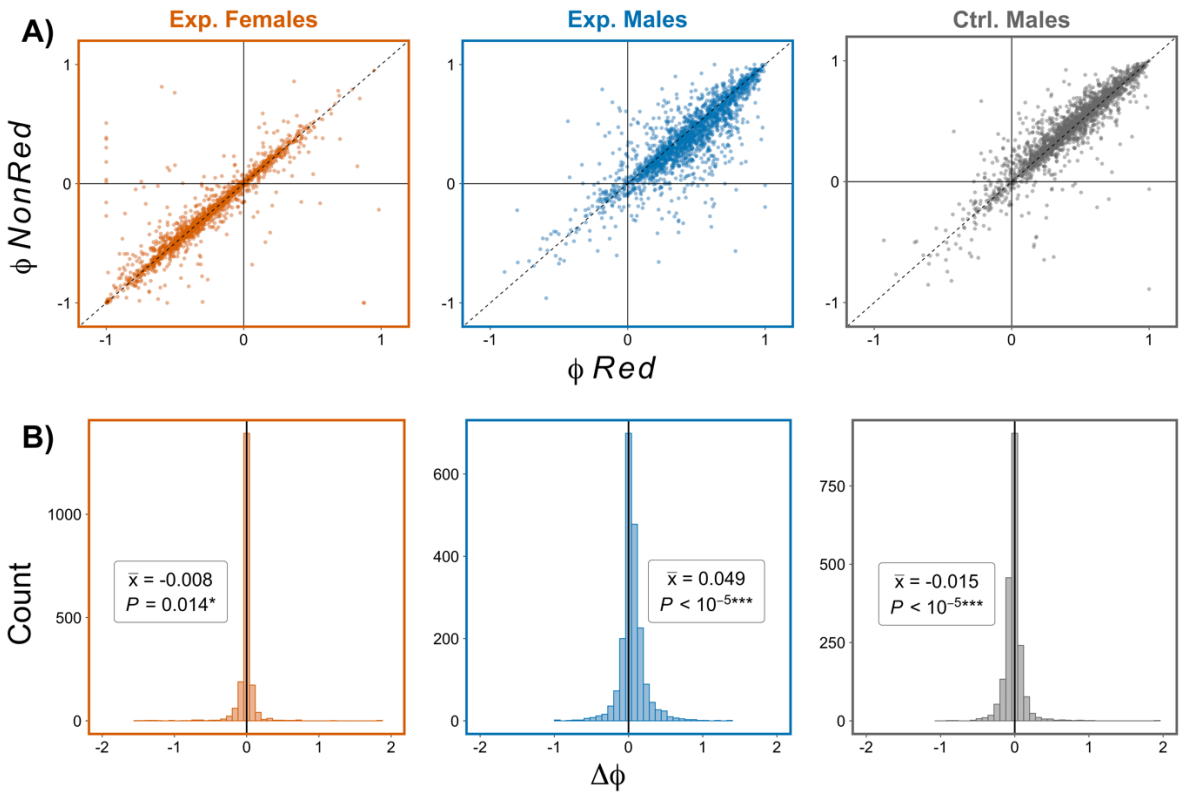


Figure 5. **Splicing profiles of *Red* and *NonRed* samples in relation to reference profiles for male and female as measured via the sexual splicing index, ϕ.** $\phi = -1$ and $\phi = +1$ indicate maximal similarity of splicing profile to that in a reference female and male, respectively. **(A)** Points represent ϕ for each gene (N=2036) calculated in *Red* (x-axis) and *NonRed* (y-axis) samples for females and males from Experimental populations, as well as males from Control populations. Points above or below the dashed ($x = y$) line represent genes that are "feminized" or "masculinized", respectively, in *Red* relative to *NonRed* samples. **(B)** Distribution of $\Delta\phi = \phi_{Red} - \phi_{NonRed}$ for each of the three sample types. $\overline{\Delta\phi} > 0$ ($\overline{\Delta\phi} < 0$) indicate an average "masculinization" ("feminization") of isoform profiles of *Red* relative to *NonRed* samples. *P*-values are from paired *t*-tests.

343 *Results not due to the* DsRed *marker itself*

344 Approximately 30 generations prior to measuring expression, a "Control" population was
345 established from each Experimental population. In Control populations, *Red* and *NonRed*
346 chromosomes could recombine with one another and there was no experimental manipulation to
347 associate sex with the *DsRed* marker. RNA-Seq data was obtained from *Red* and *NonRed* males
348 from the Control populations at the same time as for Experimental populations. At the level of
349 individual genes, only one gene was significantly differentially expressed between *Red* and
350 *NonRed* Control males, and two genes showed significant alternative splicing (these genes were
351 excluded from the respective DE/DS candidate sets in the preceding analyses).

352 Control males showed statistically significant but relatively small differences between
353 *Red* and *NonRed* samples in overall total gene expression (Fig. 4, Table S7) and in splicing
354 profiles (Fig. 5, Table S8). Importantly, the effects of *Red* vs. *NonRed* effects on total gene
355 expression and relative isoform usage in males of the Experimental populations were much
356 larger in magnitude and in the opposite direction from effects seen in Controls. Thus, these
357 results indicate that the difference between *Red* and *NonRed* chromosomes in the Experimental
358 population is not due to the effect of the *DsRed* marker itself.

359

360 *Effect Sizes of Experimental Evolution in* Red *vs* NonRed *Chromosomes*

361 The differences between *Red* and *NonRed* chromosomes in the Experimental populations could
362 be driven by evolution within the pool of *Red* chromosomes or within the pool of *NonRed*
363 chromosomes, or both. Though we do not have data on the ancestral chromosome pools, we
364 compared the transcriptomic differences between males from Experimental vs. Control
365 populations, performed separately for *Red* and *NonRed* males.

366 The expression difference of Experimental vs. Control for most genes was in the same
367 direction in both *Red* and *NonRed* males (7,857/12,023 ≈ 66%; 36% (29%) upregulated
368 (downregulated) in Experimental relative to Control; Fig. S13). For these congruently expressed
369 genes, differences between Experimental vs. Control were of larger magnitude on average in
370 *NonRed* than *Red* ($P < 0.01$, paired *t*-test), with 86% of point estimates being larger for *NonRed*
371 than *Red* contrasts (Fig. 6A). We also detected greater Experimental vs. Control differences with
372 respect to splicing profiles in *NonRed* males than in *Red* males, (Fig. 6B; $P < 0.01$, paired *t*-test).
373 These results suggest that the divergence between *Red* and *NonRed* chromosomes within the
374 Experimental populations were more strongly driven by evolutionary changes that occurred
375 within the *NonRed* (rather than *Red*) pool of chromosomes.
376

377

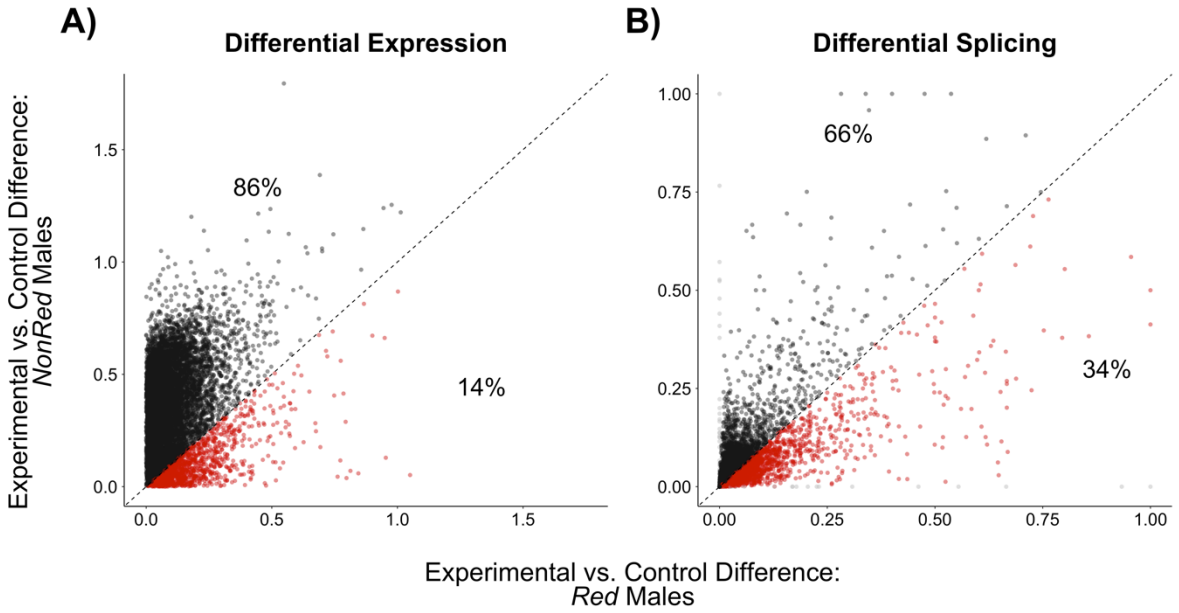


Figure 6. Effect sizes of the experimental evolution on males with (*Red*) and without (*NonRed*) the manipulated *Red* chromosomes. Each point represents the estimated change in expression trait of a gene between Experimental and Control for *Red* (x-axis) and *NonRed* (y-axis) males. (**A**) Estimated effect sizes on total gene expression, measured as the absolute value of the $\log_2$FC between expression in Experimental and Control males of each genotype. Only genes with the same direction of expression change in *Red* and *NonRed* samples are shown for simplicity of interpretation (i.e., same sign for $\log_2$FC values, 66% of all genes; all genes shown in Fig. S13). (**B**) Estimated effect sizes on alternative splicing, measured as percent dissimilarity in splicing profiles ($d_{g,C}$, Eq.1; where g is genotype *Red* or *NonRed*) between Experimental and Control males for *Red* and *NonRed* samples. Points in red ($x > y$) indicate genes with greater magnitude of difference between Experimental and Control in *Red* males, and points in black ($x < y$) indicate those with greater magnitude of change in *NonRed* males. The percentage of genes above and below the diagonal is indicated.

378

379 **Discussion**

380 The shared gene pool is expected to cause intralocus sexual conflict over homologous traits that
381 are selected in different directions in males and females. Rice (1996, 1998) was the first to
382 demonstrate evidence of this conflict by manipulating the transmission of haploid genomes to
383 only *D. melanogaster* males, thus removing the effect of selection in females and causing
384 adaptation to favour higher male fitness at the expense of female fitness (see also Rice 1992). In
385 this study, we used a similar approach to relax the intersexual genetic constraints on a major
386 autosomal chromosome (i.e., Chromosome 2) in *D. melanogaster*. We expect chromosomes in
387 the *Red* gene pool to evolve towards the male-optimum as a result of male-limited (Y-like)
388 transmission, while those in the *NonRed* gene pool which experienced selection
389 disproportionately in females (through X-like transmission) should evolve towards the female
390 optimum. In line with this prediction, we observed that males carrying a *Red* chromosome
391 displayed higher mating success relative to males carrying only *NonRed* chromosomes,
392 suggesting that male-benefit and/or female-benefit sexually antagonistic alleles have
393 accumulated in the male-selected *Red* or female-selected *NonRed* pool, respectively.

394 While divergence in mating success—a key fitness component—of *Red* and *NonRed*
395 males provides evidence for ongoing sexual conflict occurring in the ancestral population, we
396 sought to gain insight into potential targets of this conflict at a lower level. To this end, we used
397 RNA-Seq data collected from whole fly bodies to scan the transcriptome for those genes that
398 have diverged in expression in response to the relaxation of conflict in our experiment; such
399 genes are potential targets of sexually antagonistic selection. However, it is prudent to recognize
400 that this approach alone cannot be used to pinpoint the direct targets of selection for several
401 reasons, one being that we do not know the degree of independence for each gene's change in

402 expression. Although much of regulatory variation is due to *cis* mechanisms, *trans* changes
403 controlled by only one or a small number of genetic factors can contribute substantially to the
404 expression of many genes (Wittkopp et al. 2008; Blows et al. 2015; Osada et al. 2017).
405 Furthermore, potential allometric differences between *Red* and *NonRed* flies can contribute to
406 changes in levels of whole-body expression. Finally, not all changes in expression are necessarily
407 the outcome of adaptive divergence as linked selection can be an important factor in "evolve &
408 resequence" experiments (Kofler and Schlötterer 2014). Nonetheless, we expect that expression
409 divergence between *Red* and *NonRed* gene pools in our experiment should be enriched, relative
410 to the background, in genuine targets of intralocus sexual conflict. It was not our goal to identify
411 individual genes but rather to detect characteristic patterns among the genes that diverge in our
412 experiment.

413 Others have previously attempted to identify targets of sexually antagonistic selection
414 through studying variation in gene expression. Innocenti and Morrow (2010) identified genes
415 differing in expression among 15 lines of *D. melanogaster* which varied along an axis of sexual
416 antagonism (e.g., some genotypes conferred high male fitness and low female fitness whereas
417 others were the reverse). An alternative way of identifying candidate genes is to look directly at
418 genomic variation. Ruzicka et al. (2019) identified candidate sexually antagonistic SNPs through
419 a genome wide association study from 203 fly haplotypes. That we detected some overlap
420 between our candidates and those in past studies (Table S3)—despite differences in population
421 history and methods as well as power limitations in each study—adds confidence that true
422 positives exist among the candidates in each of these studies.

423 One of the more intriguing findings of Ruzicka et al. (2019) is the evidence that sexually
424 antagonistic SNPs were more likely to be maintained as polymorphisms both within and among

species, hinting at long-term balancing selection occurring at loci under conflict. We made a preliminary attempt to look for evidence of balancing selection by comparing candidates vs. background genes with respect to Tajima's *D* (using values from Singh and Agrawal (2023) which were calculated using 4-fold degenerate sites) but found no difference either when DE and DS candidate genes were considered together or separately (permuted $P > 0.1$). Notably, Ruzicka et al. (2019) found an enrichment of sexually antagonistic candidates among nonsynonymous sites and argued that structural variants, rather than expression variants (as would pertain to our work), may be more likely to be subjected to the type of long-term balancing selection that can leave distinctive population genetic signatures.

In examining the properties of the candidate genes from our own study, we first considered sex-biased gene expression. While dimorphic expression can evolve for multiple reasons (Lande 1980; Houle and Cheng 2021), sexually antagonistic selection is an obvious one. Even if sexual antagonism was responsible for dimorphic expression, it is unclear whether genes with sex-biased expression are subject to ongoing sexually antagonistic selection or whether current levels of dimorphic expression represent a full resolution of past conflict. Conversely, genes with unbiased expression may not experience antagonistic selection or may simply have been constrained from evolving dimorphism despite sexual antagonism. A model by Cheng and Kirkpatrick (2016) predicted that genes with intermediate levels of sex-bias would be the most likely targets of ongoing antagonistic selection. They found that genes with intermediate levels of female- or male-biased expression were enriched among the candidates identified by Innocenti and Morrow (2010) in *D. melanogaster*. Our findings, however, revealed a different pattern in which genes with moderate male-biased expression were overrepresented in our set of DE candidates but moderately female-biased genes were underrepresented. Although the reasons for

448 this pattern are unclear, it suggests that sexual antagonism is more strongly associated with male-
449 biased genes than female-biased genes. This pattern is also somewhat reflected in the result
450 presented by Cheng and Kirkpatrick (2016): though both moderately male- and female- biased
451 genes were overrepresented among the sexually antagonistic candidates identified in *D.*
452 *melanogaster*, the pattern of overrepresentation was considerably stronger for male- than female-
453 biased genes (their Fig. 2). Perhaps contrary to our current findings and Cheng and Kirkpatrick
454 (2016), Tajima's *D* was reduced among male- and female-biased genes compared to unbiased
455 genes in several bird species (Wright et al. 2019), and reduced only among male-biased genes in
456 guppies (Wright et al. 2018). However, we caution that a reduced Tajima's *D* could reflect a
457 higher rate of selective sweeps rather than indicate reduced sexual antagonism, per se.

458 The occurrence of ongoing sexual conflict implies that the current degree of dimorphism
459 is not optimal (i.e., one or both sexes are not at their respective optima). If the shared gene pool
460 is a constraint in the evolution of further dimorphism, then manipulating selection to favour one
461 sex over the other should allow traits to evolve more towards that sex's fitness optimum. For
462 instance, male-limited genome-wide evolution resulted in a "masculinization" of several
463 dimorphic phenotypic traits in *D. melanogaster* (Prasad et al. 2007; Abbott et al. 2010), i.e., traits
464 evolved in the male direction of existing dimorphism. Similarly, female-limited evolution of the
465 X chromosome resulted in a feminization (Lund-Hansen et al. 2020).

466 The first study to apply to these types of ideas to expression traits was Hollis et al.
467 (2014). They used enforced monogamy, rather than a manipulation of inheritance, in an attempt
468 to weaken selection on males, thus biasing evolution towards the female optimum. Indeed, Hollis
469 et al. (2014) observed evidence for the feminization of the *D. melanogaster* transcriptome after
470 65 generations of evolution under monogamy. However, other studies which took on similar

471 approaches failed to reproduce the same result. For instance, Veltsos et al. (2017) reported that
472 expression tend to be masculinized in monogamous *Drosophila pseudoobscura* populations
473 relative to polygamous populations. Similarly, Mishra et al. (2024) found complex patterns of
474 expression divergence between *D. melanogaster* populations evolved under monogamy and
475 those evolved in environments where mate competition occurred. The inconsistencies in these
476 results are perhaps unsurprising as enforced monogamy alters selection on both sexes in multiple
477 ways beyond a simple weakening of selection on males (Mishra et al. 2024). Consequently, a
478 manipulation of mating systems is not the most reliable way to experimentally alleviate
479 intralocus sexual conflict.

480 Abbott et al. (2020) used a more direct method to eliminate sexual conflict through male-
481 limited transmission of the X chromosome to study the constraint in the evolution of expression
482 dimorphism. Consistent with the prediction of masculinization, male-limited X chromosome
483 evolution resulted in male-biased genes being significantly upregulated, and female-biased genes
484 being significantly downregulated. Opposite to Abbott et al. (2020), we observed an overall
485 downregulation of male-biased genes and upregulation of female-biased genes in male-selected
486 *Red* autosomes relative to female-selected *NonRed* autosomes. This pattern fits neither the
487 predicted outcome that would indicate the absence of intralocus sexual conflict (i.e., no response
488 to the separation of gene pools; Fig. 7A), nor the predicted masculinization of the *Red* gene pool
489 that would indicate the occurrence of conflict for further sexual dimorphism (Fig. 7B).

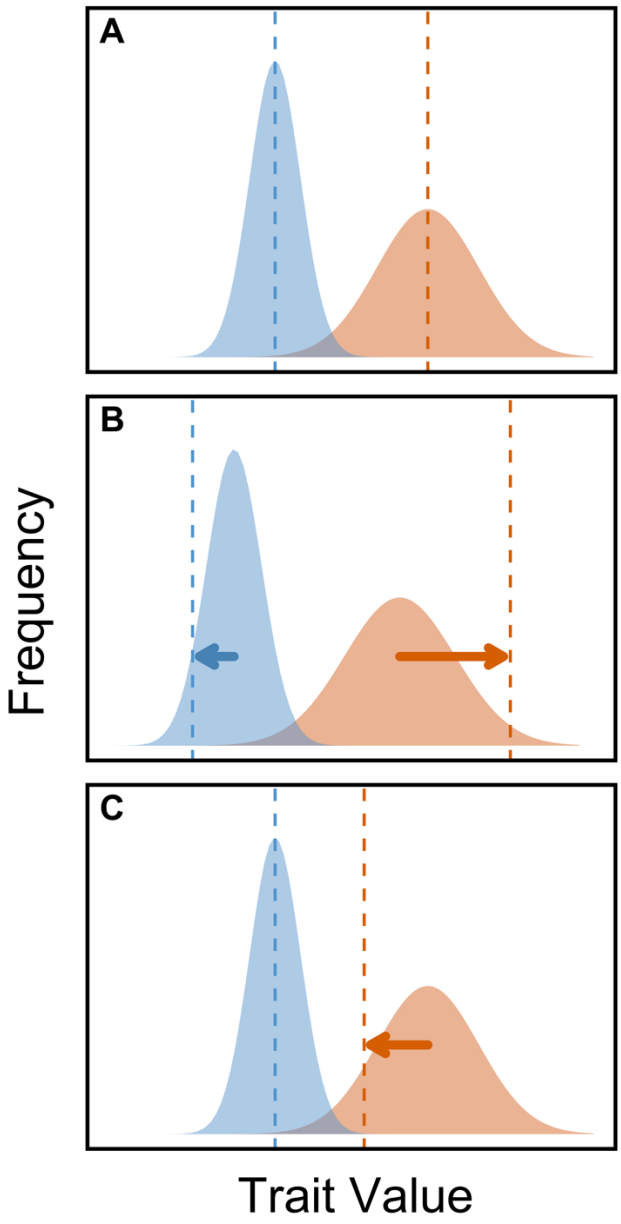


Figure 7. Hypothetical predictions for the relationship of sexual dimorphism and intralocus sexual conflict. Blue and orange represent male and female trait distributions, respectively (r_{MF} is assumed to be large and positive). Dashed lines represent the phenotypic optimum of each sex (complete fitness distribution not shown). Arrows indicate the direction and magnitude of selection. In panel (**A**), there is no ongoing conflict and the trait displays the optimum degree of sexual dimorphism. In (**B**), there is sexual conflict owing to directional selection in both sexes for further sexual dimorphism. In (**C**), conflict is caused by directional selection for less dimorphism in females which would cause a correlated but maladaptive response in (i.e. males are selection to maintain the current trait value). The narrower distribution of males relative to females is intended to reflect a history of stronger selection in males resulting in the male mean being closer to its optimum in B and C.

Retrospectively, we considered other plausible forms of sexual conflict which can explain our result but are not highlighted as often as the classical prediction of constraint in the evolution of further sexual dimorphism (Fig. 7B). Another form of conflict, for example, is one where some degree of sexual dimorphism has previously evolved, but changes in the phenotypic optimum of one or both sexes lead to antagonistic selection for a lower degree of dimorphism (Fig. 7C; see also Zhu et al. 2023). This is analogous to two forces pushing against each other, as opposed to the more commonly known tug-of-war description of conflict. (These seemingly opposite scenarios are conceptually identical in that under both scenarios selection is operating in opposite directions between the sexes and a positive intersexual genetic correlation means that selection in one sex results in a maladaptive change in the other.) This alternative form of conflict, when eliminated, should result in evolution towards the phenotypic optimum of the opposite sex, consistent with our observation of masculinization of female-biased *NonRed* chromosomes relative to *Red* chromosomes with respect to expression magnitude. As depicted in Fig. 7B, if males were initially reasonably close to their optimum but females more substantially displaced, then this could explain why evolution appeared to occur primarily in *NonRed* rather than *Red* chromosome pools (as evidenced by the different magnitude of divergence seen between Experimental and Control flies within each pool, Fig. 6). One speculative explanation that could lead to this condition is if selection in males around the male optimum is strong during Lande's (1980) theorized “rapid phase” of adaptation before the onset of sexually antagonistic selection. It would then imply that the following “slow phase”, at which point the strength of selection in males and in females becomes similar and conflict ensues, should begin when the population is on average closer to the male than to the female phenotypic optimum. (Of course, other factors such as the lower population sizes and recombination rates experienced by the *Red*

514 pool relative to the *NonRed* pool may also contribute to the differences in their rates of
515 expression evolution.)
516 Although we cannot conclusively identify why our results qualitatively differ from those
517 of Abbott et al. (2020) with respect to the "masculinization/feminization" of expression
518 magnitude, we recognize two possibilities. First is that the discordance in patterns reflects
519 differences in population histories, which may result in differences in where the phenotypic
520 distributions of the ancestral populations lie relative to the sex-specific optima prior to the onset
521 of experimental evolution. In other words, the type of ongoing conflict (e.g., Fig. 7B vs. 7C) that
522 was most prevalent differed in the two distinct ancestral populations used in each study. Another
523 possibility is that the dynamics of sexual conflict on the X (the focus of Abbott et al. 2020) differ
524 from the conflict that occurs on the autosomes. There are known differences between X and
525 autosomes which are likely related to sexual conflict. For example, the *Drosophila* X is unusual
526 in gene content, being enriched for female-biased genes and depauperate for male-biased genes
527 relative to autosomes (Parisi et al. 2003; Meisel et al. 2012; Singh and Agrawal 2023). However,
528 this observation alone does not automatically lead to a prediction about the type of conflict
529 (*sensu* Fig. 7) featured on the X versus autosomes. (A third possibility is that there may be
530 important differences arising from experimental design; Abbott et al. (2020) noted the possibility
531 that their observed expression changes could be affected by adaptation to the major chromosomal
532 constructs inherent to the design of their experimental evolution.)
533 Compared to our analysis of differential gene expression, our results with respect to
534 differential splicing are more in line with the prediction of a masculinization in *Red* males
535 relative to *NonRed* males. The contrast between this pattern and the feminization that we
536 observed with respect to expression magnitude highlights that these two aspects of expression

537 need not evolve in concert and may experience different forms of sexual conflict. One surprising
538 aspect of the splicing results was that the *Red* vs. *NonRed* contrast was significantly positive in
539 males (indicating a masculinizing effect of *Red*) but significantly negative in females (indicating
540 a feminizing effect of *Red*). This is an obvious contradiction of expectation if splicing profiles
541 were previously constrained by positive genetic correlations between the sexes (i.e., the *Red* vs.
542 *NonRed* contrast should have similar effects when measured in each sex, as seen in the
543 differential expression results, Fig. 4). However, this apparent feminization of *Red* splicing
544 profiles observed in females is tempered by two facts. First, though the effect in females was
545 statistically significant, the magnitude of the effect was very small compared to males (Fig. 5).
546 Second, while the effect of *Red* in females is slightly negative, the point estimate of the mean in
547 females is less negative (i.e., more masculinized) than the corresponding estimate in Control
548 males. (Unfortunately, data is only available for Control males but not Control females, so this
549 comparison is non-ideal.) We also stress that the metric ϕ used to compare the directionality of
550 splicing changes collapses what is truly a multi-dimensional trait (isoform profile) down to a
551 univariate measure and this may result in some unexpected outcomes, especially when effects are
552 weak (as in females). Ideally, to determine differences in splicing patterns, one would directly
553 measure the expression of isoforms derived from a gene, not rely on indirect inferences via read
554 counts of exons and splice junctions as we have done here. This cannot be reliably achieved
555 using short-read RNA-Seq data, but future analyses using long-read sequencing data could
556 potentially mitigate this issue and allow a more accurate analysis of alternative splicing in males
557 and females.

558 The results reported here not only provide further evidence that the shared gene pool of
559 the sexes can act as an adaptive constraint in the evolution of traits under sexually antagonistic

560 selection, but also suggest a form of conflict that is different from what is often described by the
561 classical models of sexual antagonism. Here we also present a novel analysis of sexual conflict
562 through looking at changes in relative isoform usage, which represents the first experimental
563 evolution test for the link between sexual conflict and sex-specific splicing. Though better
564 technologies will undoubtedly provide greater resolution, we were able to observe a significant
565 response of splicing profiles to a separation of gene pools and find evidence that the net direction
566 of the divergence of *Red* vs. *NonRed* chromosomes (masculinization) differs from that observed
567 for expression magnitude (feminization), at least for males.

568 **Materials and Methods**

569 *Base population and fly maintenance*

570 The experimental population originated from the Dahomey base population originally collected
571 from modern-day Benin, West Africa, in 1970. All flies were maintained on standard *Drosophila*
572 yeast-sugar-agar media, on a 12:12 hr light:dark cycle, at 25°C and 50% relative humidity unless
573 otherwise stated.

574

575 *Experimental evolution*

576 The second autosome of *D. melanogaster* contains ~40% of the genes in their genome. Because
577 there is no meiotic recombination in male *D. melanogaster* it is possible to force male-limited
578 inheritance of whole autosomes by tracking a dominant marker. In six replicate populations of
579 N=1000 flies, we used the dominant marker *DsRed* on position 48C of Chromosome 2R to force
580 a pool of 500 genetically diverse second autosomes of an outbred laboratory stock population of
581 *D. melanogaster* (see above) to be inherited exclusively from fathers to sons (genealogically like
582 Y chromosomes), leaving the complementary set of 1500 second autosomes to be inherited
583 genealogically like X chromosomes. Hence, carriers of the *DsRed* marker ("*Red*" flies) were
584 heterozygous *DsRed*/+ and "*NonRed*" flies were homozygous +/+.

585 A detailed description of the experimental evolution protocol is provided in Supplemental
586 Text S1; only the main points of the protocol are presented here. The basic protocol consisted of
587 seeding 10 bottles (per population) containing 40 mL of standard media sprinkled with live yeast
588 with 50 *Red* males and 50 *NonRed* females that had eclosed on the 12th day of a generational
589 cycle. These N=1000 adults were allowed to interact (court, choose, mate, remate, etc.) until the
590 morning of the 15th day ("Day 1" of next generation) before being "flipped" onto new

591 oviposition substrate (new bottles of standard media) for a four-hour egg-laying period (seeding
592 the "main" bottles), and then flipped onto new media again for another four-hour egg-lay period
593 in the afternoon (seeding the "back-up" bottles). Eleven days later ("Day 12"), the emerging
594 offspring from the ten bottles were mixed in a plexiglass cage and sampled to culture 10 bottles
595 with *Red* males and *NonRed* females. This maintenance cycle was repeated for at least 97
596 generations prior to the collection of the data presented in this paper.

597 The exclusion of *Red* females eliminates female-specific selection from the evolution of
598 *DsRed*-marked chromosomes, but also removes recombination. A small fraction of each
599 population underwent slightly altered protocols to allow for a low level of recombination on *Red*
600 chromosomes, both between one another and between *Red* and *NonRed* chromosomes. (Because
601 *NonRed* homozygote females are used in the main cross each generation, *NonRed* chromosomes
602 recombine with one another at a higher rate). The expected time a sampled allele spends in either
603 males or females or on *Red* or *NonRed* chromosomes can be estimated by simulation. For
604 example, an allele that is recombination distance $r = 0.3$ from the *DsRed* marker and sampled
605 from a *Red* chromosome at generation 98 (approximately the time when samples were collected
606 for RNAseq) is expected to have spent 86.1% of the time on *Red* chromosomes and 86.7% of the
607 time in males. This allele would have experienced 0.6 recombination exchanges between *Red*
608 chromosomes, 0.83 between *Red* and *NonRed* chromosomes, and 2.5 between *NonRed*
609 chromosomes. In contrast, a homologous allele sampled from a *NonRed* chromosome is expected
610 to have spent 4.5% of the time on *Red* chromosomes, 36.1% of the time in males, and
611 experienced 0.06 recombination exchanges between *Red* chromosomes, 0.41 between *Red* and
612 *NonRed* chromosomes, and 18.3 between *NonRed* chromosomes. See Table S1 for further
613 information on expected histories of alleles sampled from *Red* or *NonRed* chromosomes.

614 At Generation 68, from each Experimental population, a Control population of ~1000
615 flies was established and maintained on the same maintenance cycle with one major difference:
616 there was no sorting each generation to associate the *DsRed* marker with sex. In the Control
617 populations, *Red* and *NonRed* chromosomes recombined freely, erasing any accumulated
618 differences between them. (A full description of the establishment of the Controls is given in the
619 Supplementary Text S1.)
620
621 *Mating success assay*
622 In each of three generations (99, 101, and 103), we assayed mating success in *Red* and *NonRed*
623 flies of both sexes by placing roughly 1500 unsorted adult flies leftover from culturing a
624 replicate Experimental population (i.e., on Day 12 of its usual 14-day schedule) into each of four
625 plexiglass mating cages (N=12 cages per Experimental population in total). Cages contained 8
626 petri dishes with 7ml of standard media sprinkled with live yeast. Half of these plates were
627 changed for fresh media on the afternoon of Day 14. Observations were done during the 12-hour
628 light cycle in the mornings, afternoons, and evenings of Day 13-14 (i.e., the usual interaction
629 period) as well as the morning of Day 15 (i.e., just after flies would usually be flipped to new
630 oviposition substrate to lay the eggs that would be the source of the subsequent generation under
631 the normal protocol). Each cage was observed for a 90-second period at 30-minute intervals in
632 the mornings and evenings (0:30 – 3:30 and 5:30 – 8:30 Zeitgeber time after lights on,
633 respectively), and at 15-minute intervals in the afternoons (3:30 – 5:30 Zeitgeber time) when
634 peak mating activity was initially expected (Sakai and Ishida 2001). Copulating pairs were
635 aspirated from the cage into empty vials, frozen at -20°C then scored later that day for genotype
636 (*Red* or *NonRed*) and sex and categorized as "mated" individuals. A sample of at least 200

637 randomly chosen flies which had not been observed in copulation were collected at the end of the
638 trial (categorized as "unmated"), frozen at -20°C and scored later that day for genotype and sex.
639 A total of 55471 flies were scored for this assay.

640 A mixed-effects binomial regression with the probit link function was fit to the data using
641 the *R* package *lme4* (v1.1-35.1; Bates et al. 2015) with mating status as the response variable and
642 genotype (*Red* or *NonRed*), sex, and their interaction as fixed effects. A random effect term for
643 genotype nested within population replicate was also included in the model. The mating
644 probability of *Red* and *NonRed* flies, respectively, was inherently linked to the total number of
645 flies collected (i.e., the observed mating pairs and the arbitrary number of unmated flies sampled
646 at the end), which varied by mating cage. To account for this covariate, the *offset* argument for
647 the *glmer* function was specified as the ratio of mated flies to unmated flies scored for each given
648 sex within a given cage. Given a significant genotype × sex interaction term in the initial model,
649 the male and female data were subsequently fitted separately to a mixed-effects probit regression
650 with genotype as a fixed effect, genotype as a random effect nested within population replicate,
651 and the *offset* specified as the within-cage ratio of mated to unmated flies of the given sex.
652

653 *RNA sample preparation and extraction*

654 Fly samples for RNA sequencing were generated by allowing ~500 *NonRed* females housed with
655 *Red* males to lay eggs on grape-agar lay plates (with a thin layer of yeast paste) for 1-2 hours,
656 picking first-instar larvae 24 hours later, and seeding three vials (containing 7 mL of standard
657 media) per replicate population with 60 first-instar larvae. Larvae from Experimental and
658 Control populations 4 and 5 were picked on generation 97, populations 2 and 3 on generation 98,
659 and populations 1 and 6 on generation 102. These flies were reared to adulthood under the

660 abiotic conditions above and collected as virgins by clearing vials on the morning of peak
661 emergence and collecting newly emerged offspring over the next four-hour period. Virgins were
662 sorted for sex and *DsRed*, and three samples (two representing back-ups) per category were
663 generated by placing 10 flies of the appropriate type into a labeled 1.5 mL centrifuge tube, flash-
664 frozen in a dry-ice-and-ethanol bath, and stored at -80°C for later RNA extraction. There were
665 six sample categories: *Red* Experimental males, *Red* Experimental females, *NonRed*
666 Experimental males, *NonRed* Experimental females, *Red* Control males, and *NonRed* Control
667 males. For each sample category, one biological replicate per replicate population was subject to
668 RNA extractions using a TRIzol™ reagent protocol and sequenced on an Illumina NovaSeq S4
669 platform (100 bp paired-end reads) at an average of 80 million reads per sample (N=36 samples).
670

671 *RNA-Seq data processing*

672 The *Drosophila melanogaster* reference genome (BDGP6.28, release 102; dos Santos et al.
673 2015) was indexed using *genomeGenerate* in *STAR* (v2.7.9a; Dobin et al. 2013) with the --
674 *genomeSAindexNbases* set to 12. Paired-end RNA-Seq reads were aligned to the indexed
675 reference genome using *STAR* in two-pass mode to improve accuracy and the identification of
676 splice junctions. The resulting aligned BAM files were then sorted by query name using *SortSam*
677 in *GATK* (v4.2.3.0-1; Van der Auwer and O'Connor 2020). The original Fastq files of each
678 sample were converted to BAM format using *FastqToSam* in *GATK* and then merged with the
679 aligned BAM to generate a single comprehensive BAM file using *MergeBamAlignment* in
680 *GATK*. Duplicate reads in merged BAM files were marked using *MarkDuplicates* in *GATK* to
681 reduce PCR amplification bias, and these duplicate-marked BAM files were re-sorted by query
682 name, again, using *SortSam* in *GATK*.

683

684 *Differential Expression Analysis*

685 *Htseq-count* (v0.13.5; Anders et al. 2015) was used to generate a TSV file for each sample
686 containing raw read counts per *FlyBase* gene ID contained in the reference *D. melanogaster*
687 genome annotation (release 6.28; dos Santos et al. 2015). Raw read counts were then analyzed
688 using *DESeq2* (v.1.38.3; Love et al. 2014). Pairwise contrasts of $\log_2$ fold-change between
689 *DsRed*/+ and +/+ samples (i.e., "*Red*" and "*NonRed*" samples, respectively) were estimated
690 separately for Experimental females, Experimental males, and Control males. Genes averaging
691 <10 reads across replicates were excluded from the analysis. We also excluded genes on
692 Chromosome 4 and on the Y chromosome, and genes within a region of 1 Mb of the approximate
693 location of the *DsRed* marker on the right arm of Chromosome 2 (2R:10700000-11875000,
694 BDGP6.28, release 102; dos Santos et al. 2015).

695 In the Experimental *Red* vs. *NonRed* female comparison, differentially expressed (DE)
696 candidate genes were defined as those with significant differential expression between *Red* and
697 *NonRed* samples (5% FDR). Due to the high variation of effect sizes among male samples, only
698 15 of the genes tested in Experimental males (N=13515) showed statistical significance. As our
699 goal was to examine properties of potential targets of divergent selection, which is more usefully
700 performed with a larger number of candidates, we instead applied a less stringent criterion in
701 defining DE genes by selecting 350 genes with the greatest magnitude of expression differences
702 ($|\log_2 FC|$) between Experimental *Red* and *NonRed* males.

703 Gene Ontology (GO) analysis for DE genes was done separately for those upregulated
704 and those downregulated in *Red* vs. *NonRed* samples using *g:Profiler* (Kolberg et al. 2023), with
705 the custom background set defined as the remaining genes assessed in our samples but not

706 differentially expressed. The chromosomal distribution of DE genes was analysed using the *D.*
707 *melanogaster* BDGP6.28 release 102 annotation (dos Santos et al. 2015), excluding
708 Chromosome 4 and the Y chromosome. For analyses examining *Red* vs. *NonRed* expression
709 divergence in relation to sex-biased gene expression (SBGE), we used estimates of $\log_2$FC
710 Male:Female expression from an external *D. melanogaster* population data set (Mishra et al.
711 2022) as a measure of SBGE to avoid potential circularity concerns. Note that while the
712 magnitude of SBGE of a given gene varies somewhat across studies (e.g., due to genotype,
713 rearing conditions), this "within-gene" variation is small compared to the variation across genes,
714 which is the relevant issue with respect to our usage. SBGE values from the external data set are
715 indeed highly correlated with SBGE values calculated from our Experimental flies (Pearson's
716 correlation $r = 0.91$, $P < 10^{-5}$) and, more generally, SBGE values in *Drosophila* are even quite
717 strongly correlated among species separated by millions of years of evolution (Zhang et al.
718 2007). For analyses examining *Red* vs. *NonRed* expression divergence in relation to the
719 intersexual genetic correlation in expression, we used r_{MF} values estimated by Singh and
720 Agrawal (2023) based on whole-body expression of samples from 185 lines from the *Drosophila*
721 *melanogaster* Genetic Reference Panel (Mackay et al. 2012) measured by (Huang et al. 2015).
722
723 *Differential Splicing Analysis*
724 We used aligned BAM files generated from *STAR* (v2.7.9a; Dobin et al. 2013) described above
725 as the input for *QoRTs* (v1.3.6; Hartley and Mullikin 2015), which quantifies the number of reads
726 mapping to each exon and splice junction derived from the reference *D. melanogaster* genome
727 annotation (release 6.28; dos Santos et al. 2015). We then used the *R* package *JunctionSeq*
728 (v.3.11; Hartley and Mullikin 2016) to statistically test for differences in relative exon or splice

729 junction expression between *Red* and *NonRed* samples. As with the differential expression
730 analysis, we performed the *Red* vs. *NonRed* differential splicing analysis separately for
731 Experimental males, Experimental females, and Control males.

732 DS candidate genes from the *Red* vs. *NonRed* Experimental male comparison and the
733 Experimental female comparison were defined as those with significant differences in their gene-
734 wise relative isoform usage after applying a 10% FDR correction. GO analysis and analysis on
735 the chromosomal distribution of DS genes were done in the same manner as the analyses of DE
736 genes described above. We also ran *JunctionSeq* on RNA-Seq data from Mishra et al. (2022;
737 PRJNA1173240) to compare splicing profiles between males and females and assign the sex-
738 specific splicing (SSS) status of each gene, which allowed us to analyze whether our DS genes
739 were more likely to occur among SSS or non-SSS genes.

740 To analyze the direction of differential splicing between *Red* and *NonRed* samples
741 relative to existing sexual dimorphism, we first needed to establish a reference female- and male-
742 like splicing pattern for any dimorphic gene and then develop a metric to compare the degree of
743 difference of splicing profiles in our samples to these reference types. Short-read RNAseq data
744 limits the resolution of splicing profile; though our measure of differences in splicing profiles
745 (described below) is coarse-grained, it is unbiased with respect to our goals. We begin by
746 describing how we quantified "splicing profile" and then the metric we used for measuring the
747 difference between two profiles.

748 We utilized the raw counts obtained from *QoRTs* to quantify the relative expression of
749 exons and splice junctions within a given gene for each sample. Counts were first normalized
750 using *DESeq2*'s geometric normalization method to account for variations in library size across
751 samples (Love et al. 2014). The within-gene relative expression of each exon and splice junction

752 (i.e., each *QoRTs* counting bin) was then derived by dividing the sum of normalized counts
753 across all samples of a given type by the global across-sample expression of the gene (i.e., sum
754 of normalized counts across all counting bins for a gene). Only reads that mapped to known
755 exons or spliced junctions present across all data sets considered were included in the analysis.
756 For each pairwise contrast between two sample types, the magnitude of difference in splicing
757 profiles of a given gene was calculated as:

758 $$d_{x,y} = 1 - \sum_{i=1}^{n} \min\,(x_i, y_i)$$

759 (1)

760 where n represents the number of counting bins for a given gene, and x_i (y_i) refers to the relative
761 expression of exon or junction i in sample type x (y).

762 To determine if *Red* (relative to *NonRed*) genotypes had been masculinized with respect
763 to expression profile, we needed to identify dimorphically spliced genes and then establish their
764 "reference" male and female splicing profiles. We did so using external data sets. First, using the
765 expression data of Mishra et al. (2022), we identified genes with significant SSS (FDR < 0.05).
766 For these genes, we then measured the relative read counts for each counting bin (as described
767 above), separately for male and female samples in the Mishra et al. (2022) data set, which we
768 took as the reference splicing profiles for each sex. To help ensure we were examining genes
769 where male and female expression profiles were consistently different (i.e., dimorphism is not
770 population-specific), we filtered genes using additional data sets (Mishra et al. 2024)
771 representing fly populations evolved in three different mating regimes. Specifically, we retained
772 only those genes where the male splicing profile in each of the three other data sets had a smaller
773 difference from the reference male profile (using Eq.1) than from the female reference profile,
774 and vice versa for the female splicing profile. (Note that estimates for sexual dimorphism in

splicing profiles ($d_{m,f}$) were highly correlated between our Experimental populations and the four external populations considered; average Pearson's correlation $r = 0.79$; $P < 0.01$ across all data sets.) After filtering, we retained 2036 genes for the analysis described below.

We used Eq.1 to estimate the difference in splicing profiles of *Red* or *NonRed* samples vs. the reference male profile ($d_{g,M}$, where g is *Red* or *NonRed* gentoype) and the reference female profile ($d_{g,F}$) for each of the 2036 genes. To analyze whether the overall splicing difference is biased towards the direction of a specific sex, we then calculated the sexual splicing index:

$$\phi = \frac{d_{g,F} - d_{g,M}}{d_{g,F} + d_{g,M}} \tag{2}$$

where $\phi = -1$ and $\phi = +1$ indicates maximal similarity to the reference female and male profile, respectively. We used the difference in ϕ between *Red* and *NonRed* samples to indicate whether splicing profiles in *Red* samples were on average "masculinized" ($\phi_{Red} - \phi_{NonRed} > 0$) or "feminized" ($\phi_{Red} - \phi_{NonRed} < 0$) relative to *NonRed*.

Data Availability

RNA-Seq reads have been deposited in the Sequence Read Archive (SRA) under the BioProject Accession Number [**INSERT BIOPROJECT NUMBER HERE FOLLOWING ACCEPTANCE**]. All code and data for analyses and figures are documented in the following GitHub repository: https://github.com/mchlleliu/MaleLimitedEvo

Acknowledgements

This research was supported by the Natural Sciences and Engineering Research Council (to AFA), the Swedish Research Council (2018-06775 to K.G.), and the University of Toronto's Faculty of Arts and Science (Postdoctoral Fellowship to K.G.).

References

Abbott JK, Bedhomme S, Chippindale AK. 2010. Sexual conflict in wing size and shape in Drosophila melanogaster. *Journal of Evolutionary Biology* 23:1989–1997.

Abbott JK, Chippindale AK, Morrow EH. 2020. The microevolutionary response to male-limited X-chromosome evolution in Drosophila melanogaster reflects macroevolutionary patterns. *Journal of Evolutionary Biology* 33:738–750.

Anders S, Pyl PT, Huber W. 2015. HTSeq—a Python framework to work with high-throughput sequencing data. *Bioinformatics* 31:166–169.

Bates D, Mächler M, Bolker B, Walker S. 2015. Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software* 67:1–48.

Blekhman R, Marioni JC, Zumbo P, Stephens M, Gilad Y. 2010. Sex-specific and lineage-specific alternative splicing in primates. *Genome Research* 20:180.

Blows MW, Allen SL, Collet JM, Chenoweth SF, McGuigan K. 2015. The Phenome-Wide Distribution of Genetic Variance. *The American Naturalist* 186:15–30.

Bonduriansky R, Chenoweth SF. 2009. Intralocus sexual conflict. *Trends in Ecology & Evolution* 24:280–288.

Chapman T, Bangham J, Vinti G, Seifried B, Lung O, Wolfner MF, Smith HK, Partridge L. 2003. The sex peptide of Drosophila melanogaster: Female post-mating responses analyzed by using RNA interference. *Proc Natl Acad Sci U S A* 100:9923–9928.

Cheng C, Houle D. 2020. Predicting Multivariate Responses of Sexual Dimorphism to Direct and Indirect Selection. *The American Naturalist* 196:391–405.

Cheng C, Kirkpatrick M. 2016. Sex-Specific Selection and Sex-Biased Gene Expression in Humans and Flies. *PLoS Genet* 12:e1006170.

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

dos Santos G, Schroeder AJ, Goodman JL, Strelets VB, Crosby MA, Thurmond J, Emmert DB, Gelbart WM, the FlyBase Consortium. 2015. FlyBase: introduction of the Drosophila melanogaster Release 6 reference genome assembly and large-scale migration of genome annotations. *Nucleic Acids Research* 43:D690–D697.

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

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

831 Griffin RM, Dean R, Grace JL, Rydén P, Friberg U. 2013. The Shared Genome Is a Pervasive
832 Constraint on the Evolution of Sex-Biased Gene Expression. *Molecular Biology and*
833 *Evolution* 30:2168–2176.

834 Hartley SW, Mullikin JC. 2015. QoRTs: a comprehensive toolset for quality control and data
835 processing of RNA-Seq experiments. *BMC Bioinformatics* 16:224.

836 Hartley SW, Mullikin JC. 2016. Detection and visualization of differential splicing in RNA-Seq
837 data with JunctionSeq. *Nucleic Acids Res* 44:e127.

838 Hollis B, Houle D, Yan Z, Kawecki TJ, Keller L. 2014. Evolution under monogamy feminizes
839 gene expression in Drosophila melanogaster. *Nat Commun* 5:3482.

840 Houle D, Cheng C. 2021. Predicting the Evolution of Sexual Dimorphism in Gene Expression.
841 *Mol Biol Evol* 38:1847–1859.

842 Huang W, Carbone MA, Magwire MM, Peiffer JA, Lyman RF, Stone EA, Anholt RRH, Mackay
843 TFC. 2015. Genetic basis of transcriptome diversity in Drosophila melanogaster.
844 *Proceedings of the National Academy of Sciences* 112:E6010–E6019.

845 Innocenti P, Morrow EH. 2010. The Sexually Antagonistic Genes of Drosophila melanogaster.
846 *PLOS Biology* 8:e1000335.

847 Kofler R, Schlötterer C. 2014. A Guide for the Design of Evolve and Resequencing Studies.
848 *Molecular Biology and Evolution* 31:474–483.

849 Kolberg L, Raudvere U, Kuzmin I, Adler P, Vilo J, Peterson H. 2023. g:Profiler—interoperable
850 web service for functional enrichment analysis and gene identifier mapping (2023
851 update). *Nucleic Acids Research* 51:W207–W212.

852 Lande R. 1980. Sexual Dimorphism, Sexual Selection, and Adaptation in Polygenic Characters.
853 *Evolution* 34:292–305.

854 Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for
855 RNA-seq data with DESeq2. *Genome Biology* 15:550.

856 Lund-Hansen KK, Abbott JK, Morrow EH. 2020. Feminization of complex traits in Drosophila
857 melanogaster via female-limited X chromosome evolution. *Evolution* 74:2703–2713.

858 Mackay TFC, Richards S, Stone EA, Barbadilla A, Ayroles JF, Zhu D, Casillas S, Han Y,
859 Magwire MM, Cridland JM, et al. 2012. The Drosophila melanogaster Genetic Reference
860 Panel. *Nature* 482:173–178.

861 McIntyre LM, Bono LM, Genissel A, Westerman R, Junk D, Telonis-Scott M, Harshman L,
862 Wayne ML, Kopp A, Nuzhdin SV. 2006. Sex-specific expression of alternative transcripts
863 in Drosophila. *Genome Biol* 7:R79.

Meiklejohn CD, Landeen EL, Cook JM, Kingan SB, Presgraves DC. 2011. Sex Chromosome-Specific Regulation in the Drosophila Male Germline But Little Evidence for Chromosomal Dosage Compensation or Meiotic Inactivation. *PLOS Biology* 9:e1001126.

Meisel RP, Malone JH, Clark AG. 2012. Disentangling the relationship between sex-biased gene expression and X-linkage. *Genome Res.* 22:1255–1265.

Mishra P, Barrera TS, Grieshop K, Agrawal AF. 2022. Cis-regulatory variation in relation to sex and sexual dimorphism in Drosophila melanogaster. https://www.biorxiv.org/content/10.1101/2022.09.20.508724v1, last accessed November 2, 2023

Mishra P, Rundle HD, Agrawal AF. 2024. The evolution of sexual dimorphism in gene expression in response to a manipulation of mate competition. *Evolution* 78:746–757.

Naftaly AS, Pau S, White MA. 2021. Long-read RNA sequencing reveals widespread sex-specific alternative splicing in threespine stickleback fish. *Genome Res.* 31:1486–1497.

Osada N, Miyagi R, Takahashi A. 2017. Cis- and Trans-regulatory Effects on Gene Expression in a Natural Population of Drosophila melanogaster. *Genetics* 206:2139–2148.

Parisi M, Nuttall R, Naiman D, Bouffard G, Malley J, Andrews J, Eastman S, Oliver B. 2003. Paucity of Genes on the Drosophila X Chromosome Showing Male-Biased Expression. *Science* 299:697–700.

Prasad NG, Bedhomme S, Day T, Chippindale AK, Arnqvist AEG, Whitlock EMC. 2007. An Evolutionary Cost of Separate Genders Revealed by Male-Limited Evolution. *The American Naturalist* 169:29–37.

Rice WR. 1992. Sexually Antagonistic Genes: Experimental Evidence. *Science* 256:1436–1439.

Rice WR. 1996. Sexually antagonistic male adaptation triggered by experimental arrest of female evolution. *Nature* 381:232–234.

Rice WR. 1998. Male fitness increases when females are eliminated from gene pool: Implications for the Y chromosome. *Proc Natl Acad Sci U S A* 95:6217–6221.

Rogers TF, Palmer DH, Wright AE. 2021. Sex-Specific Selection Drives the Evolution of Alternative Splicing in Birds. *Molecular Biology and Evolution* 38:519–530.

Ruzicka F, Hill MS, Pennell TM, Flis I, Ingleby FC, Mott R, Fowler K, Morrow EH, Reuter M. 2019. Genome-wide sexually antagonistic variants reveal long-standing constraints on sexual dimorphism in fruit flies. *PLOS Biology* 17:e3000244.

Sakai T, Ishida N. 2001. Circadian rhythms of female mating activity governed by clock genes in Drosophila. *Proceedings of the National Academy of Sciences* 98:9221–9225.

Singh A, Agrawal AF. 2023. Two Forms of Sexual Dimorphism in Gene Expression in Drosophila melanogaster: Their Coincidence and Evolutionary Genetics. *Molecular Biology and Evolution* 40:msad091.

Sturgill D, Zhang Y, Parisi M, Oliver B. 2007. Demasculinization of X chromosomes in the Drosophila genus. *Nature* 450:238–241.

Telonis-Scott M, Kopp A, Wayne ML, Nuzhdin SV, McIntyre LM. 2009. Sex-Specific Splicing in Drosophila: Widespread Occurrence, Tissue Specificity and Evolutionary Conservation. *Genetics* 181:421–434.

Travers LM, Garcia-Gonzalez F, Simmons LW. 2015. Live fast die young life history in females: evolutionary trade-off between early life mating and lifespan in female Drosophila melanogaster. *Sci Rep* 5:15469.

Van der Auwer GA, O'Connor BD. 2020. Genomics in the Cloud: Using Docker, GATK, and WDL in Terra. 1st ed. O'Reilly Media, Inc.

Veltsos P, Fang Y, Cossins AR, Snook RR, Ritchie MG. 2017. Mating system manipulation and the evolution of sex-biased gene expression in Drosophila. *Nat Commun* 8:2072.

Wittkopp PJ, Haerum BK, Clark AG. 2008. Regulatory changes underlying expression differences within and between Drosophila species. *Nat Genet* 40:346–350.

Wright AE, Fumagalli M, Cooney CR, Bloch NI, Vieira FG, Buechel SD, Kolm N, Mank JE. 2018. Male-biased gene expression resolves sexual conflict through the evolution of sex-specific genetic architecture. *Evol Lett* 2:52–61.

Wright AE, Rogers TF, Fumagalli M, Cooney CR, Mank JE. 2019. Phenotypic sexual dimorphism is associated with genomic signatures of resolved sexual conflict. *Mol Ecol* 28:2860–2871.

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

Zhu C, Ming MJ, Cole JM, Edge MD, Kirkpatrick M, Harpak A. 2023. Amplification is the primary mode of gene-by-sex interaction in complex human traits. *Cell Genomics* 3:100297.