Article

# Two forms of sexual dimorphism in gene expression in *Drosophila melanogaster*: their coincidence and evolutionary genetics

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**Abstract**

Sex differences in gene regulation is a proximate mechanism by which evolution can resolve sexually antagonistic phenotypic selection. One form of differential gene regulation is sex-biased gene expression (SBGE) in which the sexes differ in the amount a gene is expressed. Less attention has been given to sexual dimorphism in isoform usage, i.e., sex-specific splicing (SSS). In whole body expression in *Drosophila melanogaster*, we find a negative association between SSS and SBGE, possibly suggesting these are alternate routes to resolving sexual antagonistic selection. However, in heads, both forms of expression dimorphism are much less frequent and the association between them is positive. We evaluate whether expression dimorphism contributes to the heterogeneity among genes in $r_{mf}$, the intersexual genetic correlation in whole body expression that constrains the extent to which a gene's expression can evolve independently between the sexes. We find lower $r_{mf}$ values for genes with than without SSS. Though male-biased genes are known to have greater evolutionary divergence in expression, we find they have higher $r_{mf}$ values than female-biased genes (except genes with extreme male-bias). Genes with expression dimorphism are likely to have experienced a history of sex differences in selection and this may leave signatures in their population genetic statistics. SSS is associated with reduced values of Tajima's *D* and elevated *Direction of Selection* (*DoS*) values, suggestive of higher rates of adaptive evolution. Though *DoS* is highly elevated for genes with extreme male bias, *DoS* otherwise tends to decline from female-biased to unbiased to male-biased genes.

## Introduction

In sexual populations, the sexes employ different reproductive strategies to maximize fitness and as result, shared phenotypes often exhibit divergent, sex-specific trait optima. Because the sexes largely share a genome, the genetic basis of such traits is often the same between the sexes. Thus, genetic variation underpinning such shared traits will often experience divergent, sexually antagonistic selection resulting in an evolutionary conflict between the sexes termed 'intra-locus sexual conflict' (Arnqvist and Rowe 2005; Bonduriansky and Chenoweth 2009). Intra-locus sexual conflict has been well documented in a variety of plant (e.g., Hersh et al. 2015; Zemp et al. 2016; Delph et al.2022) and animal (e.g., Arnqvist and Rowe 2005; Cox and Calsbeek 2009) species and may be an important mechanism in maintaining genetic variation (Kidwell et al. 1977; Rice 1984). Ultimately, intra-locus sexual conflict is thought to be resolved through the evolution of sexual dimorphism (Lande 1980; Rice 1984).

Gene expression can be viewed as a trait in its own right or as a proximate mechanism affecting 'traditional' traits (e.g., morphological, behavioural, or physiological). Over the last ~15 years, there has been a strong interest in investigating sexual dimorphism in gene expression, typically called 'sex-biased gene expression', SBGE (Ellegren and Parsch 2007; Parsch and Ellegren 2013; Mank 2017). SBGE refers to sex differences in the abundance of all transcripts mapping to a given gene. In addition to dimorphism in total expression of a gene, the sexes may differ in other aspects of the transcriptome. Here we examine SBGE as well as 'sex-specific splicing' (SSS), which here refers to quantitative differences in the relative usage of different isoforms between the sexes. Because the function of proteins and other gene products depends on the specific exons retained in mRNA transcripts after transcription, usage of different isoforms has the potential to greatly expand the functional repertoire of the genome, allowing a single gene to take on different functional roles across developmental stages (e.g., Gibilisco et al. 2016), tissue types (e.g., Barberan-Soler and Zahler 2008; Telonis-Scott et al. 2009; Revil et al. 2010; Ramani et al. 2011; Tan et al. 2013), and abiotic environments (Long et al. 2013; Jakšić and Schlötterer 2016).

Across the sexes, differential isoform usage (SSS) could allow the sexes to express their optimal trait values through the production of different gene products despite sharing a genome. Widespread SSS has been documented in humans (Trabzuni et al. 2013) and other primates (Blekhman et al. 2010) as well as in birds (Rogers et al. 2020). Several microarray and RNA-seq studies across *Drosophila* species have also shown that SSS is widespread (e.g., McIntyre et al. 2006; Telonis-Scott et al. 2009; Chang et al. 2011), tissue-specific (Telonis-Scott et al. 2009), and evolutionarily conserved (Gibilisco et al. 2016).

Here, we further explore SBGE and SSS using publicly available RNA-seq data from two tissue types (head and body tissue) of males and females of adult *Drosophila melanogaster.* As SBGE and SSS can represent different forms of sex-differential gene regulation, we are interested in their relationship. Resolution or

91 mitigation of intra-locus sexual conflict could require the evolution of both SBGE and SSS; on the other
92 hand, SSS and SBGE may serve as alternative routes to mitigating sexual conflict so that the evolution of
93 one form of sex-differential regulation obviates selection for the other. We investigate the co-occurrence of
94 SSS and SBGE, finding SSS is less common among sex-biased than unbiased genes in the body, though the
95 opposite pattern occurs in the head.

96 For any quantitative trait—including gene expression—a strong intersexual genetic correlation ($r_{mf}$)
97 constrains the potential for that trait to evolve independently between the sexes. Expression levels for most
98 genes are positively genetically correlated across the sexes, yet the strength of the intersexual genetic
99 correlation varies considerably among genes (Griffin et al. 2013; Allen et al. 2018). What factors contribute
100 to this heterogeneity in $r_{mf}$ values? It has been predicted that dimorphic traits will tend to have lower $r_{mf}$
101 values than traits lacking dimorphism (Lande 1980; Bonduriansky and Rowe 2005). Using *Drosophila*
102 *melanogaster*, Griffin et al. (2013) were the first to examine variation in gene expression $r_{mf}$ values across
103 the genome, finding—as predicted—that $r_{mf}$ values tended to be lower in sex biased than unbiased genes.
104 However, in their analysis, Griffin et al. (2013) did not distinguish on basis of the direction of the bias, i.e.,
105 male- versus female-biased genes. One might predict that male-biased genes would have lower $r_{mf}$ values
106 (Allen et al. 2018) given the observation that male-biased genes tend to diverge in their degree of sex bias
107 more rapidly than female-biased genes (Meiklejohn et al. 2003; Zhang et al. 2007). With the benefit of a
108 better dataset than was available to Griffin et al. (2013), we revisit these questions in *D. melanogaster*.
109 Further, we ask if any of the heterogeneity in $r_{mf}$ values among genes is related to SSS. Given that $r_{mf}$ is
110 expected to be reduced in traits with more dimorphism and that SSS represents another aspect of dimorphism
111 in expression, we also expect $r_{mf}$ would be lower, on average, in genes with SSS.

112 Genes with expression dimorphism are likely to have experienced a history of differential selection
113 between the sexes but the evolutionary consequence of differential selection are unclear. Compared to genes
114 without dimorphic expression, are dimorphic genes more likely to experience balancing selection or relaxed
115 selection or rapid adaptation? Because selection affects patterns of sequence diversity and divergence,
116 comparing population genetic statistics among genes that differ in the strength, direction, and form of
117 expression dimorphism may provide insights into selective forces acting on dimorphic genes (Meiklejohn et
118 al. 2003; Pröschel et al. 2006; Fraïsse et al. 2019, Sayadi et al. 2019, Wright et al. 2019). For example, male-
119 biased genes in *D. melanogaster* show signatures of higher rates of adaptive protein evolution than unbiased
120 or female-biased genes (e.g., Pröschel et al. 2006; Fraisse et al. 2018). We examine variation across the
121 genome in three population genetic statistics—relative levels of nonsynonymous diversity, Tajima's *D*, and
122 direction of selection (*DoS*)—and test for associations of these with SBGE and SSS.

123

124 **Results and Discussion**

125 Prevalence of Sex-Specific Splicing

126 We find evidence of extensive sex-specific splicing (SSS) among multi-exon genes expressed in both head
127 and body tissue of adult *Drosophila melanogaster*. Table 1 summarizes the results of our differential exon
128 usage analysis. Of multi-exon genes tested, 5375 and 790 genes expressed in adult body and head tissue
129 exhibit statistically significant SSS respectively which accounts for 57.5% and 10.2% of all genes tested in
130 bodies and heads respectively. Considering the union of genes expressed in either tissue, we detected SSS in
131 at least one of the tissues for 56% of genes. These data accord well with several previous studies that have
132 found evidence of widespread SSS both in this species (e.g., McIntyre et al. 2006; Telonis-Scott et al. 2009;
133 Chang et al. 2011) as well as other species of *Drosophila* (Gibilisco et al. 2016). McIntyre et al. (2006)
134 reported that 68% of all multi-transcript genes expressed in whole body tissue exhibited significant sex-bias
135 in the expression of one or more alternative isoforms in a microarray study using *D. melanogaster*. Similarly,
136 a microarray study in this species by Telonis-Scott et al. (2009) found that ~58% of multi-exon genes tested
137 in whole body tissue exhibited SSS.

138

139 **Table 1:** Summary of the differential exon usage analysis between the sexes.

| | Genes Tested | Genes with Sex-Specific Splicing (% of genes tested) |
|---|---|---|
| Head | 7772 | 790 (10.2%) |
| Body | 9346 | 5375 (57.5%) |
| [1]Head or Body | 9788 | 5488 (56.1%) |

140 [1]These data refer to the union of genes tested and exhibiting SSS found in either head, body or both.

141 Heads and bodies differ substantially in the frequency of SSS. Although the majority of genes
142 exhibiting SSS in head tissue also exhibited SSS in the body, there are many genes exhibiting SSS in body
143 tissue where we did not detect dimorphism in head tissue. 7330 genes were tested in both tissues. 4187
144 exhibit SSS in the body but only 677 (16%) of these also exhibit SSS in the head. Of the 7330, a total of 779
145 genes exhibit SSS in the head and 87% of these also exhibit SSS in the body. These results are consistent
146 with the findings of Telonis-Scott et al. (2009) who reported reduced levels of SSS in genes when expressed
147 in head tissue compared to carcass (i.e., head- and gonad-less bodies) and gonadal tissue.

148

149 Coincidence of two forms of sexually dimorphism in expression

150 Numerous studies have examined sex differences variation in total expression for a gene. Studies of sex-
151 biased gene expression (SBGE) have shown repeatedly that SBGE is highly tissue specific (e.g., Parisi et al.
152 2004; Yang et al. 2006; Dutoit et al. 2018); both our data and other studies (e.g., Telonis-Scott et al. 2009;
153 Chang et al. 2011) indicate that the same is true for sex differences in isoform usage. Because both of these
154 forms of sexual dimorphism are thought to create phenotypic dimorphism and help resolve intra-locus sexual
155 conflict, we are interested in understanding the relationship between SBGE and SSS.

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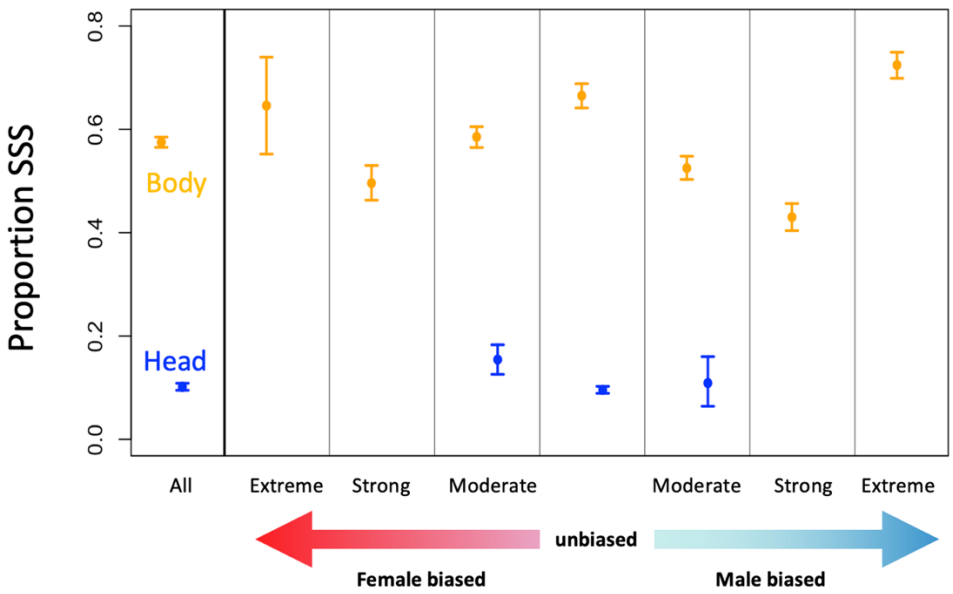


157

158 **Figure 1**. Proportion of genes exhibiting sex-specific splicing (SSS). The leftmost section shows results
159 irrespective of SBGE; the remaining sections show results stratified by SBGE. Points in orange and blue
160 represent reflect characterizations based on expression in body and head samples, respectively. Only three
161 levels of SBGE are shown with respect to head because very few genes fall into the other levels. Error bars
162 represent bootstrap 95% confidence intervals.

163

164 SBGE is widespread both in heads and bodies, though there is much greater variation among genes
165 in the strength of SBGE in bodies relative to heads (e.g., Innocenti and Morrow 2010; Baker et al. 2011;
166 Dutoit et al. 2018). To visualize the relationship between SSS and SBGE, we divided genes into bins
167 representing different levels of sex-bias using defined ranges of $\log_2$FC in gene expression (see Methods).
168 Within each bin, we calculated the proportion of genes exhibiting SSS. With respect to expression in the
169 body, SSS is more common amongst unbiased genes than sex-biased genes, except for those with extreme
170 sex-bias (Figure 1). This visual pattern is confirmed by the significantly negative quadratic SBGE term on

171 the probability of SSS ($z$ = -4.96; $p = 7 \times 10^{-7}$; Table S1). These fly body results are similar in this respect
172 with Rogers et al. (2020) who reported non-significant trends towards a negative relationship between SSS
173 and SBGE in gonadal tissue of three bird species.

174 The reduction in isoform dimorphism at highly sex-biased genes expressed in the body could be
175 because SBGE and SSS provide alternative routes to the resolution of sexual conflict and thus the evolution
176 of one reduces the selective pressure for the evolution of the other. On the other hand, the pattern goes in the
177 opposite direction with respect to expression in the head (Table S1, positive quadratic SBGE term, $z$ = 3.02;
178 $p$ = 0.0025); SSS is less common among unbiased genes than sex-biased genes (at least those that are
179 female-biased). One hypothesis for this latter pattern is that the resolution of intra-locus conflict may often
180 require both forms of expression dimorphism whereas those genes experiencing sexually concordant
181 selection will evolve neither, creating a positive association between SSS and SBGE. The difference in the
182 relationships observed in head and body cannot be explained by differences in which genes are expressed.
183 The patterns remain the same even if we consider only those genes expressed and tested for both SBGE and
184 SSS in both tissues (Fig. S2).

185 An alternative, more mundane, explanation is that the positive association of SBGE and SSS in
186 heads exists because of how we quantify gene/isoform expression (i.e., counting reads mapping to genes);
187 we may be biased towards finding a positive association between SBGE and SSS because we are observing
188 the same phenomenon in different ways. For example, if both males and females express a single isoform of
189 a gene at the same level but females also express an additional isoform that is not expressed by males, we
190 would detect both SBGE and SSS at this gene. However, this methodological bias must not be very strong as
191 there are many unbiased genes that exhibit SSS (670 in head and 1007 in body). Moreover, the association
192 between SBGE and SSS is negative in the body.

193 Given that the X chromosome spends a disproportionate amount of its time in females, and because
194 of its hemizygous expression in males, there has been considerable interest in whether it is enriched for
195 genes that experience sexually antagonistic selection contributing to intralocus conflict or contributing to
196 sexual dimorphism (Rice 1987; Frank and Patten 2020). This has motivated interest in examining the
197 location of genes exhibiting expression dimorphism. In the body data, there is no evidence of an enrichment
198 of genes exhibiting SSS on the X chromosome (Figure 2; permutation test: $p < 0.78$). In contrast, SBGE is
199 strongly associated with the X chromosome, with female-biased genes being overrepresented on the X
200 chromosome relative to unbiased genes whereas male biased genes are underrepresented on the X
201 chromosome (Figure 2; linear effect of SBGE on probability of being located on X: $z$ = -7.06; $p < 1.7 \times$
202 $10^{-12}$). This observation of a "demasculinization" of the X chromosome has been well documented in
203 previous studies of SBGE in *D. melanogaster* as well as several other species (Parisi et al. 2003; Ranz et al.

204 2003; Khil et al. 2004; Reinke et al. 2004; Sturgill et al. 2007; Meisel et al. 2012; Albritton et al. 2014),
205 though Figure 2 illustrates the continuous, rather than discrete, nature of the relationship.

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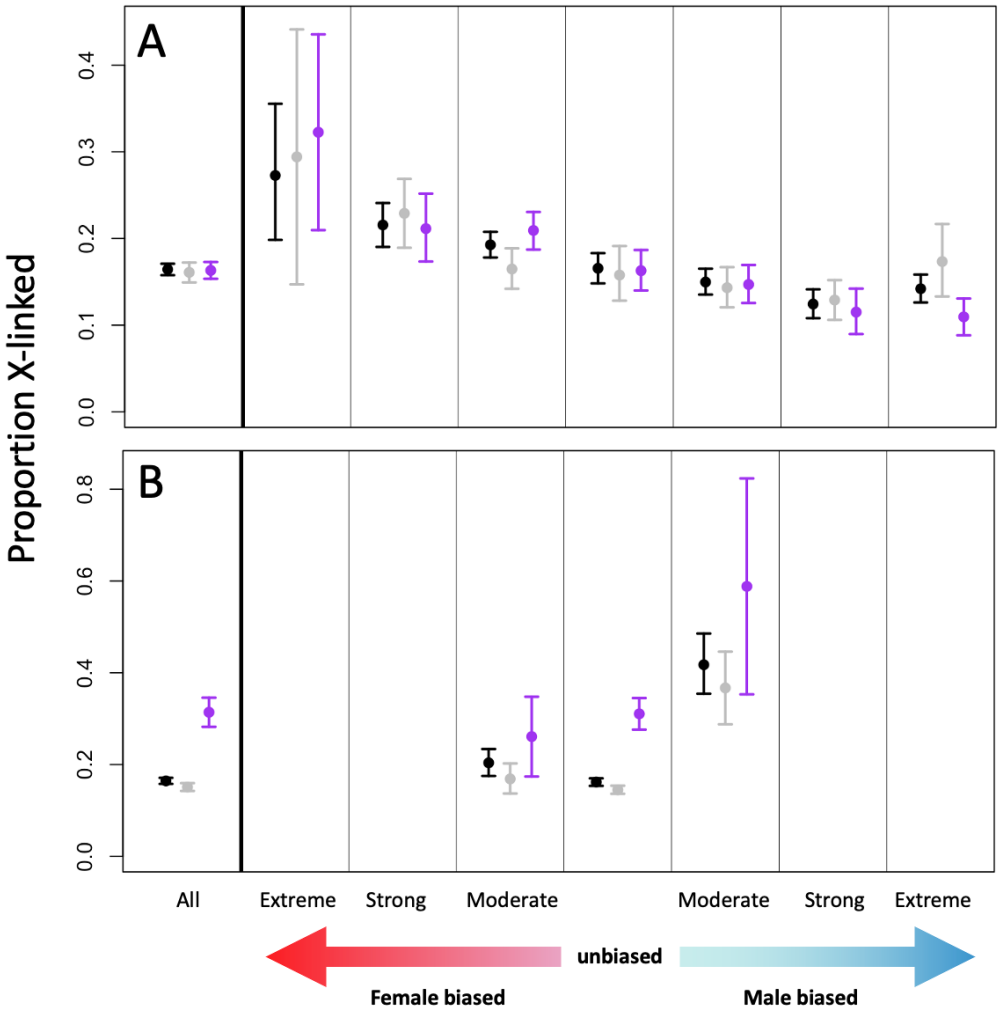


207

208 **Figure 2.** Proportion of X-linked genes with respect to SSS and SBGE as measured in (**A**) body and (**B**)
209 head. The leftmost section in each panel shows results irrespective of SBGE; the remaining sections show
210 results stratified by SBGE: extreme female bias ($\log_2 FC < -5$), strong female bias ($-5 \leq \log_2 FC < -2$ ),
211 moderate female bias ($-2 \leq \log_2 FC < -0.5$), unbiased expression ($0.5 \leq \log_2 FC < 0.5$), moderate male bias
212 ($0.5 \leq \log_2 FC < 2$), strong male bias ($2 \leq \log_2 FC < 5$), and extreme male bias ($\log_2 FC \geq 5$). Points in grey
213 and purple represent non-SSS and SSS genes, respectively; points in black are all genes irrespective of SSS
214 status (including genes that were not tested for SSS so the black points represent more genes than the
215 combined sum of genes represented by grey and purple points). Error bars are bootstrap 95% confidence
216 intervals.

217

218 These patterns are quite different for the head data where there is a significantly higher proportion of
219 X-linked genes exhibiting SSS relative to non-SSS genes (Figure 2; permutation test of SSS vs non-SSS

genes: $p < 10^{-4}$). With respect to SBGE, both male- and female-biased genes are overrepresented on the X relative to unbiased genes, with the strongest overrepresentation among male-biased genes (Figure 2; bootstrap 95% confidence intervals of proportion of X-linked genes for both male- and female-biased genes do not overlap with unbiased genes; see Meisel et al. (2012)). The over-representation of SSS genes on the X chromosome occurs primarily among unbiased genes rather than sex-biased genes, indicating that this pattern is not driven by a positive association between SSS and sex-biased expression.

Association of SSS and SBGE with average expression

It is useful to consider how our two focal aspects of expression (SSS and SBGE) are associated with two other important aspects of expression. We first consider a measure of the overall level of expression, measured as the log of FPKM averaged across a variety of tissues from larvae as well as adult males and females from the Fly Atlas 2 dataset (Krause et al. 2022). Average expression levels are higher for genes with versus without SSS (Fig. 3; permutation test: $p < 10^{-4}$ for both body and head). To some extent, this will be due to the greater power to detect SSS in genes that have higher expression. Regardless of the underlying reason, this association will be an important consideration when we later consider evolutionary characteristics of genes with SSS. In bodies, average expression is greater for unbiased than sex-biased genes (quadratic effect of SBGE on average expression is significantly negative: $z = -33.6$, $p < 2 \times 10^{-16}$). In heads, average expression tends to decline as we move from female-biased genes to unbiased genes to male-biased genes (linear effect of SBGE on average expression is significantly negative: $z = -22.9$, $p < 2 \times 10^{-16}$).

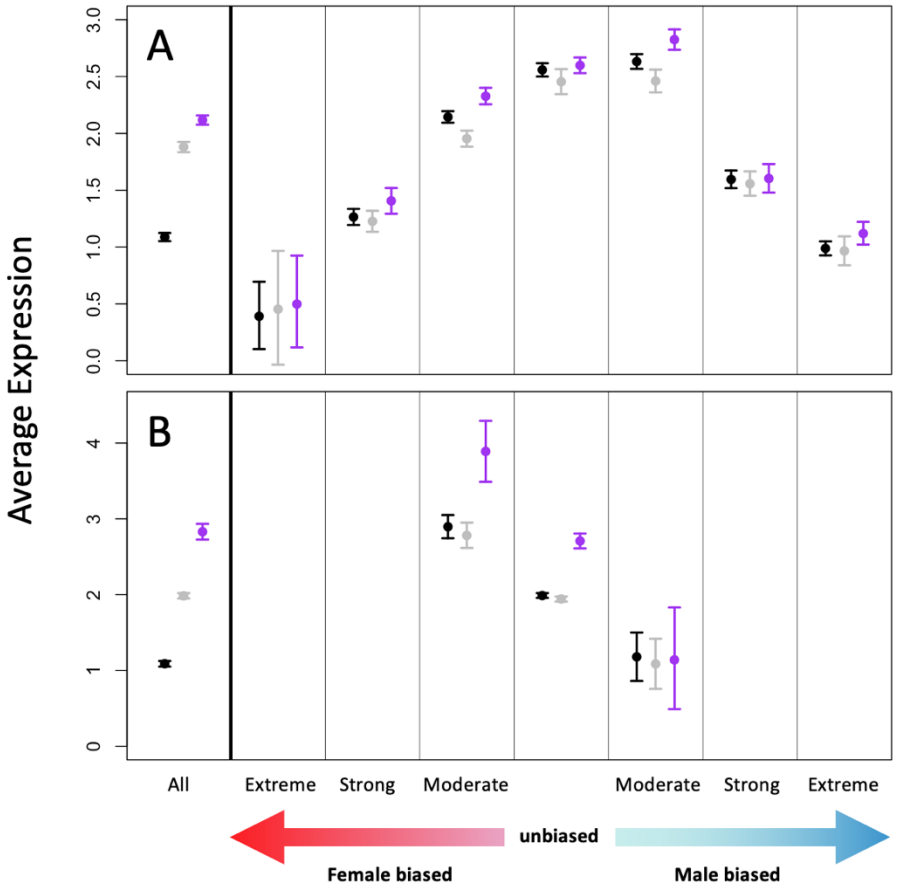


**Figure 3. Association of SSS and SBGE with average expression.** Expression level averaged across multiple tissues in both larval and adult tissues is shown with respect to SSS and SBGE in (**A**) body and (**B**) head. The leftmost section in each panel shows results irrespective of SBGE; the remaining sections show results stratified by SBGE: extreme female bias ($\log_2FC < -5$), strong female bias ($-5 \leq \log_2FC < -2$ ), moderate female bias ($-2 \leq \log_2FC < -0.5$), unbiased expression ($0.5 \leq \log_2FC < 0.5$), moderate male bias ($0.5 \leq \log_2FC < 2$), strong male bias ($2 \leq \log_2FC < 5$), and extreme male bias ($\log_2FC \geq 5$). Points in grey and purple represent non-SSS and SSS genes, respectively; points in black are all genes irrespective of SSS status (including genes that were not tested for SSS so the black points represent more genes than the combined sum of genes represented by grey and purple points). Error bars are bootstrap 95% confidence intervals.

<u>Association of SSS and SBGE with tissue specificity</u>

The other aspect of expression we consider is tissue specificity, based on the evenness of expression across multiple tissues in larva as well as adult males and females calculated using data from Fly Atlas 2 (Krause et al. 2022). Tissue specificity is higher for sex biased than unbiased genes (Fig. 4; quadratic effect of SBGE on tissue specificity is significantly positive: body $z = 51.2$, $p < 2 \times 10^{-16}$; head $z = 26.5$, $p < 2 \times 10^{-16}$). Tissue

258 specificity is lower for SSS than non-SSS genes (permutation test SSS vs non-SSS genes: body $p < 10^{-4}$;
259 head $p < 10^{-4}$).

260 Previous studies in *Drosophila* (Assis et al. 2012; Fraïsse et al. 2019) and other animals (Mank et al.
261 2008; Dean and Mank 2016) have also found that sex-biased genes have higher tissue specificity than
262 unbiased genes; this has been interpreted as sexual dimorphism evolving more readily when there is less
263 pleiotropic constraint on expression. Under this premise, one might expect the same for SSS. However, in
264 the body data, SBGE is associated with greater tissue specificity whereas SSS is associated with lower tissue
265 specificity. Similarly, Rogers et al. (2020) found that genes exhibiting SSS exhibited lower tissue specificity
266 in gene expression than non-SSS genes in birds.

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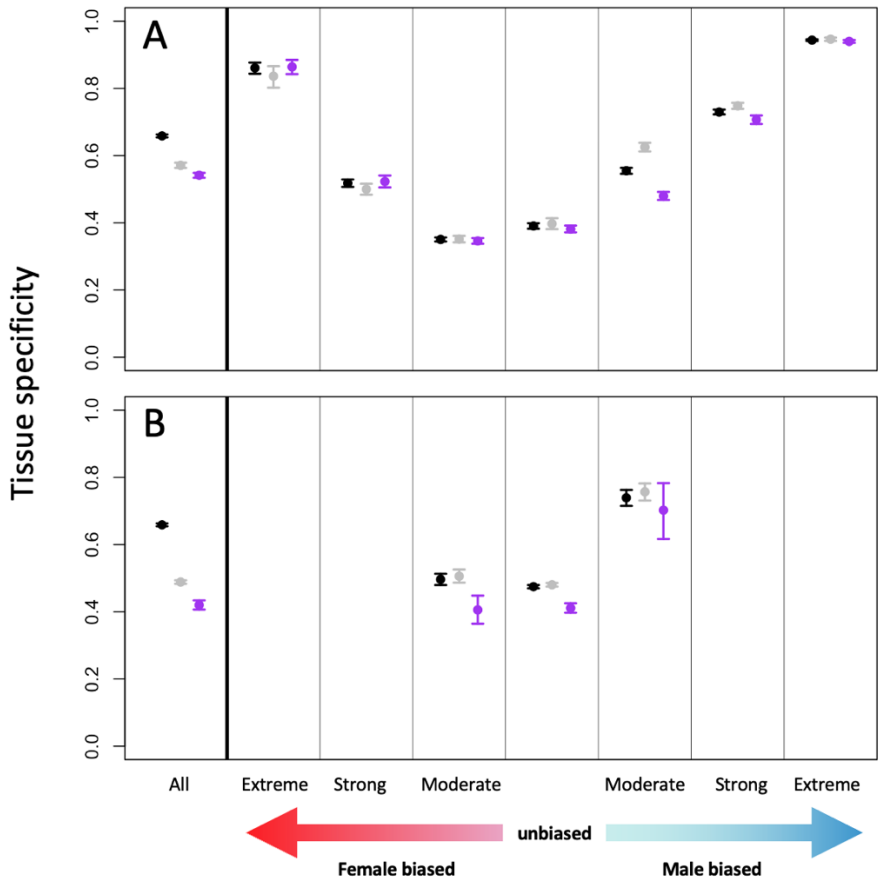


**Figure 4.** Mean tissue-specificity with respect to SSS and SBGE as measured in (**A**) body and (**B**) head. The leftmost section in each panel shows results irrespective of SBGE; the remaining sections show results stratified by SBGE: extreme female bias ($\log_2 FC < -5$), strong female bias ($-5 \leq \log_2 FC < -2$ ), moderate female bias ($-2 \leq \log_2 FC < -0.5$), unbiased expression ($0.5 \leq \log_2 FC < 0.5$), moderate male bias ($0.5 \leq \log_2 FC < 2$), strong male bias ($2 \leq \log_2 FC < 5$), and extreme male bias ($\log_2 FC \geq 5$). Points in grey and purple represent non-SSS and SSS genes, respectively; points in black are all genes irrespective of SSS status (including genes that were not tested for SSS so the black points represent more genes than the combined sum of genes represented by grey and purple points). Error bars are bootstrap 95% confidence intervals.

<u>Associations of SSS and SBGE with Evolutionary Properties</u>

We next examine whether SSS and SBGE are related to one quantitative genetic property ($r_{mf}$) and three population genetic properties (relative nonsynonymous diversity, Tajima's *D*, and *DoS*). We do this using SSS and SBGE as measured in the body, not head. The available data on $r_{mf}$ is based on whole body expression so it is appropriate to analyze variation in $r_{mf}$ in relation to body-based measures of SSS and SBGE. The analysis of the population genetic properties is motivated by the idea that genes with dimorphic

286 expression are more likely than non-dimorphic genes to have a history of sex differences in selection;
287 analysis of the population genetic properties may reveal insights into the nature of this selection. Our *a priori*
288 expectation is that dimorphism as measured from body, rather than head, samples is a better indicator of the
289 likelihood that a gene has experienced a history of differential selection between the sexes. For example,
290 many genes have dimorphic expression in body but not head and it is likely more reasonable for these
291 analyses to characterize those genes as dimorphic (and likely to have experienced a history of differential
292 selection between the sexes) than as non-dimorphic.

293 For each evolutionary genetic property, we examine associations with SSS and SBGE in two ways. First, we
294 examine simple associations of each evolutionary property with SSS and SBGE, which are straightforward
295 to represent graphically. This approach ignores other factors that could be important sources of variation in
296 the focal evolutionary property. Such factors could confound or obscure a relationship with SSS or SBGE if
297 they covary with SSS or SBGE. Consequently, we also use linear models in which we examine variation in
298 an evolutionary property as a function of SSS and SBGE as well as other relevant covariates. These other
299 covariates include X-linkage (i.e., X or autosomal) as well as several other aspects of expression: average
300 expression, tissue specificity, and summary measures of the across-tissue expression profile for each sex.
301 The latter are the values from the three leading principal components (PCs) of variation in across-tissue
302 expression profiles within each sex (i.e., 6 PCs total). In the analyzes of the population genetic properties, we
303 also included two other covariates that could affect the strength of linked selection: recombination rate and
304 gene length. As we show graphically, genes with extreme sex bias (particularly extreme male-bias) often
305 differ dramatically from other genes. We exclude these genes from all the linear models. The vast majority
306 of genes that could be regarded as "sex-limited", i.e., having very low read counts in one sex, fall into the
307 extreme sex-bias categories (especially the extreme male-bias category). Such genes may only experience
308 selection in only one sex and this may be an important reason for their unique properties.

309

310 *Intersexual genetic correlation*, $r_{mf}$

311 The extent to which a gene's expression level can evolve independently in one sex from the other is
312 mediated by the intersexual genetic correlation in expression level, $r_{mf}$. Genes with dimorphic expression are
313 predicted to have reduced $r_{mf}$. (This could be either because dimorphism evolves more easily for genes with
314 low $r_{mf}$ or because the process of evolving dimorphic expression results in an increased degree of
315 independence of how expression is regulated between the sexes, i.e., lower $r_{mf}$ evolves as a by-product of
316 increased expression dimorphism.) Previous work in *D. melanogaster* found that $r_{mf}$ values decline moving
317 from unbiased to increasingly sex-biased genes but did not distinguish between male- and female-biased
318 genes (Griffin et al. 2013). This distinction is important as male-biased genes diverge in their extent of

319 SBGE more rapidly female-biased genes (Meiklejohn et al. 2003; Zhang et al. 2007) and one potential
320 contributing factor could be a lower $r_{mf}$ in male-biased genes (Allen et al. 2018).

321 We find a striking relationship between $r_{mf}$ and SBGE, with female-biased genes having lower $r_{mf}$ than
322 unbiased genes (non-overlapping 95% confidence intervals in Fig. 5A). Moderately and strongly male-
323 biased genes have the highest average $r_{mf}$ values. In contrast, genes with extreme male bias have very low $r_{mf}$
324 values on average. A linear model that helps control for correlated variables indicates that $r_{mf}$ increases going
325 from female-biased to unbiased to male-biased genes (significant linear effect of SBGE in Table 2; note this
326 analysis excludes genes with extreme sex bias). In *Drosophila serrata*, $r_{mf}$ is also higher in male- than
327 female-biased genes (Allen et al. 2018). These results imply that the faster divergence in SBGE in male- than
328 female-biased genes occurs despite the higher $r_{mf}$ values of the former, presumably due to a higher rate in
329 changes in selection on expression of male- than female-biased genes.

330 For SSS to exist, there must already be some sex differences in how gene expression is regulated. Thus, one
331 might further predict that expression levels across sexes for genes with SSS are less likely to be strongly
332 genetically correlated. Consistent with this prediction, we find that $r_{mf}$ is lower, on average, for genes with
333 vs. without SSS (permutation test: $p < 10^4$; Fig. 5A). This effect of SSS remains significant in the linear
334 model with other covariates, including SBGE (Table 2). These results indicate that expression levels in the
335 two sexes should be able to evolve more independently in genes with SSS. This generates a prediction for
336 future studies that genes with SSS should tend to show higher rates of divergence in SBGE, assuming all else
337 equal.

338 Other aspects of expression are also significantly associated with $r_{mf}$ (Table 2). $r_{mf}$ is higher for genes with
339 higher average expression and genes with higher tissue specificity. The latter result seems counter intuitive
340 because one might expect a greater degree of genetic independence between the sexes (i.e., lower $r_{mf}$) for
341 genes expressed predominantly in highly dimorphic or sex-limited tissues (e.g., gonads, accessory glands,
342 spermatheca). However, it is worth remembering the analysis in Table 2 excludes genes with extreme sex
343 bias and thereby excludes many genes with high specificity to such tissues. Among the remaining genes,
344 genes with high tissue specificity will tend to be predominantly expressed in tissues that are typically less
345 dimorphic (e.g., heart, midgut, tubule, etc.) whereas the genes with low tissue specificity will include genes
346 that are expressed both in highly dimorphic tissues (e.g., gonads) as well as in other tissues. This may
347 explain the surprising positive association of tissue specificity with $r_{mf}$. In addition, $r_{mf}$ is associated with
348 aspects of the across-tissue expression profile in both sexes (as captured by the PC terms). This indicates that
349 genes with similar across-tissue expression profiles tend to have similar $r_{mf}$ values, possibly reflecting the
350 effects of allometry and tissue heterogeneity on whole body expression measures. The direction of
351 associations of $r_{mf}$ with the specific PCs appear consistent with our preceding comments. For example, $r_{mf}$ is

352 positively associated with male PC1 and with female PC2; the loading for testis expression is strongly
353 negative on M-PC1 and the loading for ovary expression is strongly negative on F-PC2. These patterns
354 indicate that $r_{mf}$ is lower in genes with greater gonad expression, matching intuition. Though these
355 associations of $r_{mf}$ with other aspects of expression are interesting, our focus here is on SSS and SBGE.
356 Within the limits of inference from linear modeling, our analysis indicates that SSS and SBGE are
357 significantly associated with $r_{mf}$ even after accounting for these other aspects of expression.

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359

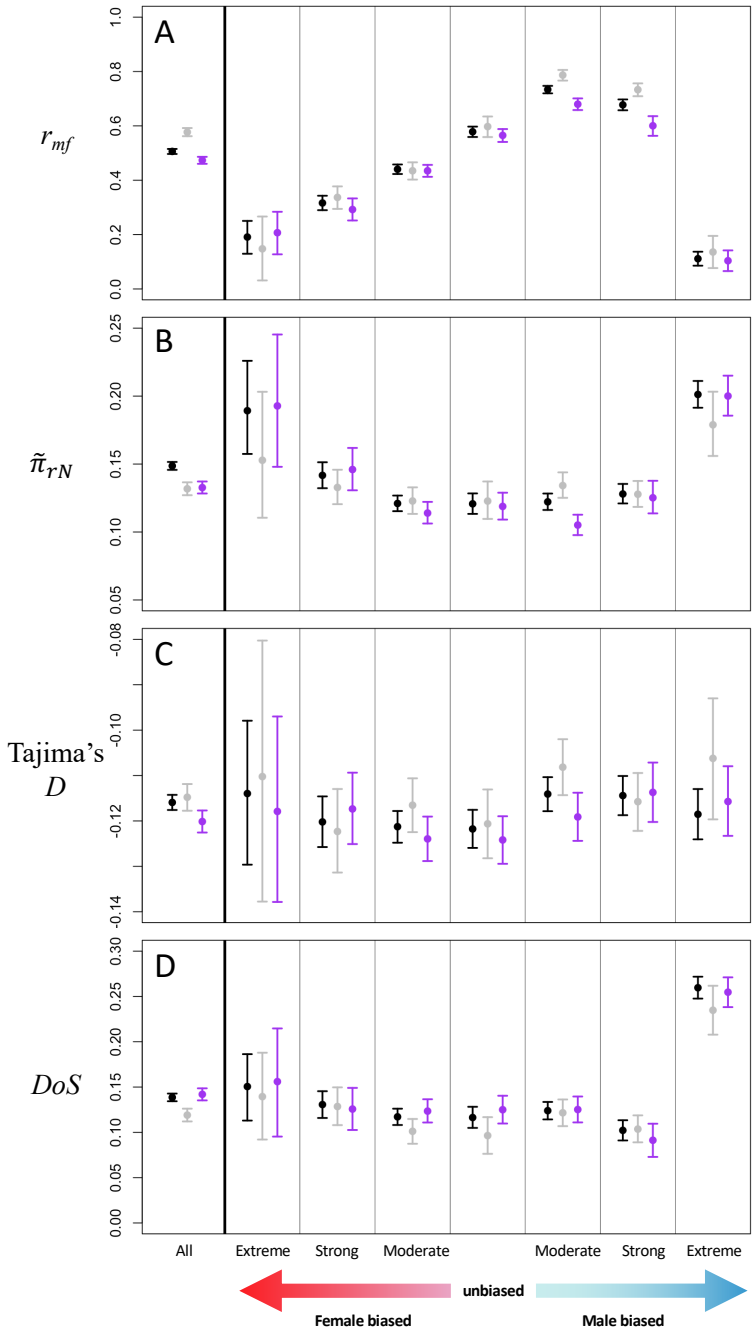


**Figure 5**. Four evolutionary genetic properties in relation to expression properties in the body. The leftmost section in each panel shows results irrespective of SBGE; the remaining sections show results stratified by SBGE: extreme female bias ($\log_2$FC < -5), strong female bias (-5 ≤ $\log_2$FC < -2 ), moderate female bias (-2 ≤ $\log_2$FC < -0.5), unbiased expression (0.5 ≤ $\log_2$FC < 0.5), moderate male bias (0.5 ≤ $\log_2$FC < 2), strong male bias (2 ≤ $\log_2$FC < 5), and extreme male bias ($\log_2$FC ≥ 5). Points in grey and purple represent non-SSS and SSS genes, respectively; points in black are all genes irrespective of SSS status (including genes that were not tested for SSS so the black points represent more genes than the combined sum of genes represented by grey and purple points). Error bars are bootstrap 95% confidence intervals.

371 **Table 2. Results from linear models examining genome-wide variation in $r_{mf}$,**
372 **$\tilde{\pi}_{rN}$, Tajima's *D* and *DoS***

| Model Term | $r_{mf}$ | $\tilde{\pi}_{rN}$ | Tajima's *D* | *DoS* |
|---|---|---|---|---|
| SSS | **-0.014**** <br> [-0.024, -0.004] | 0.001 <br> [-0.003, 0.005] | **-0.003*** <br> [-0.006, -0.0001] | **0.011***** <br> [0.005, 0.016] |
| SBGE | **0.041***** <br> [0.034, 0.048] | -0.002 <br> [-0.005, 0.001] | 0.000 <br> [-0.002, 0.003] | **-0.006**** <br> [-0.011, -0.002] |
| $SBGE^2$ | **-0.006***** <br> [-0.009, -0.003] | 0.000 <br> [-0.001, 0.001] | 0.000 <br> [-0.0001, 0.0004] | 0.000 <br> [-0.001, 0.001] |
| Avg Expression | **0.057***** <br> [0.050, 0.065] | **-0.011***** <br> [-0.014, -0.007] | **-0.004***** <br> [-0.007, -0.002] | **0.011***** <br> [0.005, 0.016] |
| Tissue Specificity | **0.250***** <br> [0.198, 0.300] | **0.051***** <br> [0.029, 0.073] | **0.027***** <br> [0.011, 0.042] | 0.008 <br> [-0.026, 0.040] |
| M-PC1 | **0.044**** <br> [0.037, 0.051] | -0.002 <br> [-0.005, 0.0005] | **0.003***** <br> [0.001, 0.005] | **-0.012***** <br> [-0.016, -0.008] |
| M-PC2 | **0.018***** <br> [0.009, 0.026] | 0.001 <br> [-0.002, 0.004] | **0.003**** <br> [0.001, 0.006] | -0.001 <br> [-0.006, 0.003] |
| M-PC3 | **0.021***** <br> [0.014, 0.027] | 0.001 <br> [-0.002, 0.003] | 0.000 <br> [-0.002, 0.002] | -0.003 <br> [-0.007, 0.001] |
| F-PC1 | 0.003 <br> [-0.006, 0.011] | 0.001 <br> [-0.002, 0.004] | 0.000 <br> [-0.002, 0.002] | -0.002 <br> [-0.007, 0.001] |
| F-PC2 | **0.013***** <br> [0.006, 0.019] | **0.003*** <br> [0.001, 0.006] | -0.001 <br> [-0.003, 0.001] | 0.001 <br> [-0.003, 0.005] |
| F-PC3 | -0.002 <br> [-0.007, 0.003] | 0.001 <br> [-0.002, 0.003] | 0.000 <br> [-0.002, 0.001] | 0.001 <br> [-0.002, 0.004] |
| X-linkage | **0.026***** <br> [0.015, 0.038] | **-0.007**** <br> [-0.012, -0.002] | **0.021***** <br> [0.018, 0.025] | **-0.030***** <br> [-0.037, -0.022] |
| log(recombination) | - | **-0.021***** <br> [-0.023, -0.018] | **-0.002**** <br> [-0.004, -0.001] | **0.022***** <br> [0.019, 0.025] |
| log(gene length) | - | **-0.022***** <br> [-0.028, -0.016] | **0.006**** <br> [0.002, 0.010] | **-0.027***** <br> [-0.036, -0.018] |

373 The numbers of genes used for the four analyses are $n$ = 4982, 4624, 4750, and 4761. For these analyses,
374 genes with extreme sex-bias ($\log_2 FC > |5|$) are excluded. The quadratic term, $SBGE^2$, is included to capture
375 effects of sex bias irrespective of the direction of the bias. Estimates in bold have significant permutation-
376 based $p$-values: * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$. 95% bootstrap confidence intervals are shown in
377 square brackets.

378

379 *Relative level of nonsynonymous diversity, $\tilde{\pi}_{rN}$*

380 A reduction in nucleotide diversity for nonsynonymous relative to synonymous variants is typically
381 interpreted as indicative of purifying selection on amino acid sequence variation, with lower nonsynonymous
382 diversity levels indicating stronger selection. We calculated $\tilde{\pi}_{rN} = \pi_N/(\pi_N + \pi_S)$ from 199 genotypes from
383 the DPGP3 (Lack et al. 2015, 2016). A simple inspection of relative levels of nonsynonymous diversity $\tilde{\pi}_{rN}$
384 reveals no clear difference between genes with versus without SSS (Fig. 5B). Genes with extreme sex-bias
385 have elevated levels of $\tilde{\pi}_{rN}$ (i.e., non-overlapping 95% confidence intervals in Fig. 5B), consistent with the

386 idea that such genes experience weaker net purifying selection because they are primarily selected in only
387 one sex (Pröschel et al. 2006; Dapper and Wade 2016, 2020). In the linear model, for which we exclude the
388 extreme sex-bias genes, there are no significant effects of SBGE or SSS.

389

390 *Tajima's* D

391 Tajima's *D* is a summary statistic that reflects information about the shape of the underlying genealogy,
392 which can be affected by the strength and form of selection at linked sites. As such, variation in Tajima's *D*
393 has been used to look for indirect evidence that genes with different expression properties are selected
394 differently (Sayadi et al. 2019; Wright et al. 2019). As shown in Fig. 5C, genes with SSS have lower values
395 of Tajima's *D* (permutation test: $p < 0.007$). There is no visually obvious relationship of Tajima's *D* with
396 SBGE (Fig. 5C). The results of the linear model are consistent with these findings for both SSS and SBGE
397 (Table 2).

398 The result that SSS and non-SSS genes differ with respect to Tajima's *D* suggests these two sets of
399 genes differ with respect to some aspect of selection. There is no evidence that there is a consistent
400 difference in the strength of purifying selection given that SSS and non-SSS genes do not differ with respect
401 to $\tilde{\pi}_{rN}$. This leaves open the possibility that the difference in Tajima's *D* could be because genes with SSS
402 are less likely to experience balancing selection or more likely to experience selective sweeps than genes
403 without SSS.

404

405 *Direction of Selection* DoS

406 An excess of divergence to polymorphism for nonsynonymous relative to synonymous variants can be
407 indicative of adaptive evolution (McDonald and Kreitman 1991). Based on this idea, Stoletzki and Eyre-
408 Walker (2011) devised the *DoS* metric for which positive values are indicative of adaptive evolution, with
409 more strongly positive values reflecting more frequent adaptive evolution. *DoS* is higher for genes with SSS
410 than those without (permutation of SSS vs non-SSS gene: $p < 0.006$; see also Table 2). With respect to
411 SBGE, *DoS* is substantially elevated for genes with extreme male-bias (Fig. 5D). Excluding the genes with
412 extreme sex-bias, the linear model detects a subtle but significant effect of SBGE on *DoS* (Table 2),
413 indicating a decline in *DoS* going from female-biased to unbiased to male-biased genes.

414 A potential concern in interpretating *DoS* is that large positive values could be primarily due to low
415 levels of nonsynonymous polymorphism, with divergence playing little role. However, divergence and
416 polymorphism components each contribute substantially to variation in *DoS* in this dataset. Moreover, if we

417 apply a linear model to just the divergence component of *DoS* (i.e, $D_N/(D_N + D_S)$), we again detect a positive
418 effect of SSS (Table S2). As with *DoS*, the divergence component of *DoS* declines from female-biased to
419 unbiased to male-biased genes (Table S2; this analysis again excludes genes with extreme sex bias, genes
420 with extreme male-bias have highly elevated values for the divergence component of *DoS,* Fig. S4).

421

422 *Other aspects of expression*

423 Though our focus is on SSS and SBGE, we note that each of the population genetic metrics have significant
424 associations with other aspects of expression (Table 2). For example, the linear model indicates $\tilde{\pi}_{rN}$ is lower
425 for more highly expressed genes. This is consistent with results from a variety of taxa, which is usually
426 interpreted as indicating stronger purifying selection on highly expressed genes (e.g., Carneiro et al. 2012;
427 Paape et al. 2012; Williamson et al. 2014; Ho et al. 2019). Though average expression is also associated with
428 higher values of *DoS*, this is likely a reflection of purifying selection rather than adaptation as average
429 expression has a significantly negative association with the polymorphism component of *DoS* but is not
430 associated with the divergence component of *DoS* (Table S2, Fig. S4; see also Fraïsse et al. 2019 and Huang
431 2022). The analysis of $\tilde{\pi}_{rN}$ also indicates that higher tissue specificity results in higher values of $\tilde{\pi}_{rN}$,
432 consistent with the idea of weaker purifying selection on genes with less pleiotropy (e.g., Osada 2007; Paape
433 et al. 2013).

434

435 Conclusions and further considerations

436 SSS and SBGE are two different ways to differentially regulate genes across the sexes and thus could be
437 mechanisms by which sexual conflict is resolved. Genes with SSS have lower values of the intersexual
438 genetic correlation in expression $r_{mf}$. This indicates that patterns of standing genetic variation are more
439 conducive to evolving greater sex differences in a gene's total expression (i.e., stronger SBGE) for genes
440 with SSS versus without, holding the amount of genetic variation constant. Thus, one might predict genes
441 with SSS to have higher levels of SBGE, which stands at odds with the observed negative association
442 between these two forms of expression dimorphism in the body (Fig. 1). This could be because when one of
443 these mechanisms evolves, it may negate the selective pressure to evolve the other (i.e., SSS genes are less
444 likely to be under antagonistic selection for total expression than non-SSS genes).

445 SSS and SBGE are each associated with some of the population genetic metrics we examined,
446 suggesting that genes with expression dimorphism tend to experience somewhat different selection than
447 non-dimorphic genes. Further, there are some differences between SSS and SBGE in how each are
448 associated with population genetic metrics, suggesting differences in selection between genes with these two

449 different forms of dimorphism. For example, SSS, but not SBGE, is associated with a reduction in Tajima's
450 *D*. An interpretation of the analyses Tajima's *D* and *DoS* together is that SSS genes experience more
451 adaptive evolution than genes without SSS. Perhaps when sexes use different isoforms, it is easier for
452 adaptation to occur in each sex because there is less pleiotropic constraint imposed by the other sex. In
453 contrast, the pattern with respect to SBGE (excluding genes with extreme sex-bias) is less clear. The *DoS*
454 analysis does not imply sex-biased genes experience more adaptive evolution than unbiased genes, though
455 this may be because adaptive evolution is modestly elevated among female-biased genes but modestly
456 reduced among male-biased genes relative to unbiased genes.

457 Such interpretations should be regarded with caution for several reasons. First, population genetic
458 metrics can be influenced by a multitude of factors and the interpretations above are not necessarily correct.
459 Second, the comparison of SSS and SBGE is complicated by the nature of how they are defined and
460 measured. SBGE has both a direction (i.e., negative to positive representing female- to male-biased) and a
461 magnitude. SSS, as measured here, simply indicates whether the sexes differ in their distribution of isoform
462 usage; there is no male/female direction. Magnitude only affects SSS status via the statistical power to detect
463 the difference, which also depends on other factors (e.g., expression level, sequencing depth). (Quantifying a
464 "magnitude" for SSS is complicated by the multitude of ways that isoform distributions can differ; but see
465 Rogers et al. 2020.)

466 An additional reason for caution is an unmeasured correlated variable could be responsible for the
467 reported associations of evolutionary properties with SBGE and SSS. For this reason, we incorporated
468 several plausibly important variables into our analyses to reduce this possibility. One aspect of this warrants
469 further discussion. Whole body samples represent a collection of tissue types and expression profiles differ
470 across tissues. Thus, expression differences between sexes can be due to either expression changes within
471 tissues (or cell types) between sexes or the relative contribution of each tissue within each sex (Stewart et al.
472 2010). We take the perspective that expression in whole body samples represents a coarse-grained level at
473 which dimorphism can be studied, while recognizing that several different biological levels could contribute
474 to this level of dimorphism. Examining dimorphism at this level we find that SBGE and SSS are associated
475 with several key evolutionary properties. A potential concern is that these associations are due to some other
476 feature of genes that is associated with these measures of expression dimorphism. For example, perhaps
477 genes predominantly expressed in particular tissues (e.g., gonads) evolve differently than other genes
478 expressed predominantly in other tissues (e.g., midgut) but genes in the former tissues tend to have different
479 levels of expression dimorphism measured at the whole body level than other genes (i.e., perhaps SBGE and
480 SSS are just crude indicators of a gene's among-tissue expression profile either because particular tissues
481 have more strongly dimorphic expression or because the relative sizes of particular tissues differs
482 considerably between sexes).

483 To mitigate this concern, our linear models explicitly included principal components (PCs) reflecting
484 variation in across-tissue expression profiles in both sexes. For every population genetic metric we
485 examined, one or more of the PC terms was significant, suggesting that genes with similar across-tissue
486 expression profiles experience, to some extent, similar population genetic pressures. However, the key point
487 with respect to our primary interest is that the effects of SBGE and SSS are significant even after accounting
488 for variation in across-tissue expression profiles. Further, statistical models without the PC terms yield
489 similar estimates for the effects of SBGE and SSS, providing no indication that considering variation in
490 across-tissue expression profiles has a substantial influence on the inferred effects of SBGE and SSS. While
491 it would be naïve to presume that the inclusion of the PCs in the analysis controls for all possible tissue
492 effects, our analyses show SBGE and SSS are associated with population metrics in ways that not readily
493 explained by tissue effects. Thus, the observed patterns with respect to SBGE and SSS are intriguing; an on-
494 going challenge is to better understand why they occur.

495

496 **Methods**

497 Obtaining RNA-seq Data and Read Mapping: We obtained publicly available raw RNA-seq data from the
498 DNA Data Bank of Japan Sequence Read Archive (Accession number: DRA002265). Detailed methods on
499 animal husbandry, RNA extraction and sequencing can be found in Osada et al. (2017). Briefly, males from
500 18 randomly chosen inbred lines of the *Drosophila* Genomic Resource Panel (DGRP; MacKay et al. 2012;
501 Huang et al. 2014) were crossed to females from a single inbred strain from a West African population. RNA
502 was extracted from heads and bodies (i.e., whole body minus heads) of 100 virgin F1 males and females
503 from each cross. RNA from female heads and bodies was obtained and sequenced from two replicates of
504 each of the 18 DGRP lines while only a single replicate was available for males. We therefore arbitrarily
505 retained sequence data only for female samples labelled as 'replicate 1'. Reads were inspected for quality
506 using *FastQC* (v0.11.9; Andrews 2010) and *MultiQC* (v0.8.3; Ewels et al. 2016). Reads were trimmed for
507 adapter content using *Trimmomatic* (v0.39; Bolger et al. 2014). RNA-seq reads were then mapped to the
508 *Drosophila melanogaster* reference genome (release 6.32; Dos Santos et al. 2015) using *STAR* (v2.7.1a;
509 Dobin et al. 2013) with default settings.

510 Sex-Specific Splicing: To identify a list of genes exhibiting SSS, we performed differential exon usage
511 analysis using the R package *JunctionSeq* (v1.17; Hartley and Mullikin 2016). Briefly, *JunctionSeq* splits
512 genes into annotated exons and splice junction 'counting bins' and counts the number of reads that overlap
513 each counting bin within an annotated gene. To test for differential exon usage between the sexes, a
514 generalized linear model (GLM) with logarithmic link function was fit at each gene feature (i.e., exon or
515 splice junction) to assess sex differences in the number of reads that map to a particular gene feature relative

516 to the total number of reads that map to the gene to which the feature belongs. Specifically, for each gene,
517 *JunctionSeq* fits the model

518 $$log(y_{ij}) = \mu + W_i + X_j + Z_{ij}$$

519 where $y_{ij}$ is the normalized count of reads from the $i^{th}$ sex mapping to the $j^{th}$ gene feature/counting bin. $\mu$ is
520 the experiment wide, mean normalized gene expression, $W_i$ reflects the expected number of reads mapping
521 to the focal gene in sex $i$, $X_j$ reflects the proportion of reads mapping to counting bin $j$, and $Z_{ij}$ is an
522 interaction term that reflects differences between the sexes in the proportion of reads mapping to counting
523 bin $j$. A significant effect of the interaction term was taken as evidence of SSS. To test the significance of the
524 interaction term, the above model was compared to a null model that was identical, but which omits the
525 interaction term.

526 *JunctionSeq* can test genes with at least two annotated exons. It applies a multiple-testing correction
527 method based on the Benjamini and Hochberg procedure (Benjamini and Hochberg 1995) to generate
528 adjusted *P*-values. Genes were considered to show evidence for sexual dimorphism in isoform usage if the
529 sexes exhibited statistically significant differential usage of a single exon (or more) within a gene using $p_{adj}$ <
530 0.05.

531 Ideally, genotype would be included in the model as a random effect but *JunctionSeq* does not allow
532 this. This is unlikely to be a major issue here. Our dataset is perfectly balanced with each genotype being
533 represented by only a single sample for each sex for each tissue (head and body data were analyzed
534 separately). More importantly, our goal is to identify a list of genes enriched for SSS for further analysis; our
535 focus is not on the accuracy of *p*-values of individual genes. As a simple check of the *JunctionSeq* pipeline,
536 we analyzed 10 datasets in which we switched the male/female labels for a randomly chosen half of the
537 genotypes. In these permuted data sets, no genes were identified as having SSS at $p_{adj} < 0.01$ whereas
538 thousands of genes are found in the true (unpermuted) data under the same criterion.

539 Sex-biased gene expression: We estimated differential gene expression between the sexes using the R
540 package *DESeq2* (v1.28.1; Love et al. 2014). Briefly, *DESeq2* estimates normalized read counts mapping to
541 genes on a $\log_2$ scale and uses a GLM framework to test for differential gene expression between
542 experimental conditions. Before performing differential gene expression analysis, we performed a 'pre-
543 filtering' procedure to remove genes that had very low numbers of reads mapping. Pre-filtering RNA-seq
544 data has been shown to substantially increase power in detecting differential gene expression by reducing the
545 burden of multiple testing but can also result in an increase in Type I error (i.e., increased false-positive rate)
546 (Bourgon et al. 2010). To balance these competing issues, we removed any genes that had fewer than an
547 average of 50 reads mapping from all 36 samples (i.e., 18 male and 18 female genotypes). We then estimated

548 SBGE as the $log_2$ fold change ($log_2FC$) in male to female gene expression for each annotated gene and
549 retained only genes that were tested for differential exon usage. In downstream analyses, we assigned each
550 gene to bins based on based on the log2FC in male to female gene expression.

551 For visual inspection of patterns, we assigned each gene to one of seven bins of predefined ranges of
552 $log_2FC$ in gene expression: extreme female bias ($log_2FC < -5$), strong female bias ($-5 \leq log_2FC < -2$ ),
553 moderate female bias ($-2 \leq log_2FC < -0.5$), unbiased expression ($0.5 \leq log_2FC < 0.5$), moderate male bias
554 ($0.5 \leq log_2FC < 2$), strong male bias ($2 \leq log_2FC < 5$), and extreme male bias ($log_2FC \geq 5$). Binning was
555 conducted separately with respect to expression in heads and bodies. A small number of genes for which the
556 standard error in $log_2FC$ was high (> 0.5) were excluded. The bins were used for graphical presentation of
557 results; as described below, our main analyses treat $log_2FC$ as a continuous variable. In heads, there were few
558 genes with $log_2FC > |2|$ so only the three central bins are shown in the figures. Distributions of $log_2FC$ for
559 each tissue are shown in Fig. S1.

560 Some genes could be regarded as "sex-limited" based on very low expression in one sex, though
561 there is no universally accepted definition. Operationally defining "sex-limited" as an average read count
562 across samples below 5 in only one sex, almost all such genes fall into the extreme sex-bias categories
563 (body: 959/963 = 99.6% [17 and 942 genes are extreme female- and male-biased, respectively], head: 2/2 =
564 100% [both are extreme female-biased]). Not all genes classified as having extreme sex-bias in the body
565 would be classified as sex-limited under this (arbitrary) definition; 79/96 = 82% of extreme female-biased
566 genes and 228/1170 = 19% of extreme male-biased genes would not qualify as "sex-limited" under this
567 definition even though the vast majority of genes with extreme sex bias have low to very low expression in
568 one sex. No analyses are based upon the classification of "sex-limited". Rather, we are simply alterting
569 readers that the "extreme sex-bias" categories include many genes that may be regarded as having sex-
570 limited expression.

571

572 <u>Average expression:</u> We used expression data from Fly Atlas 2 (Krause et al. 2020). For each gene,
573 expression values (measured as FPKM) were obtained from eight larval tissues and 24 adult tissues (12 per
574 sex). The larval tissues were central nervous system, midgut, hindgut, tubule, fat body, salivary gland,
575 trachea, and garland. The following 10 tissue types were used from both adult males and females: eye, brain,
576 ganglion, crop, midgut, hindgut, tubule, heart, fat body, and rectal pad. In addition, testis and accessory
577 glands were used from males and ovary and spermatheca from females. A "larval average" was calculated
578 across the eight larval tissues and an "adult average" across the 24 adult tissues; these mean of these two
579 values was used as a joint "larval-adult" average. The natural log of the larval-adult average was used for
580 analyses.

581 Tissue-specificity in expression: Using the expression data from Fly Atlas 2 (Krause et al. 2020), tissue
582 specificity was calculated as: $\tau = \sum_{i=1}^{30}(1 - \frac{\log(x_i)}{\log(x_{max})})/29$ where $x_i$ = 1 + the FPKM in tissue $i$ (Yanai et al.
583 2005). The 30 tissues used were all of those listed above but excluding rectal pad. (In exploring the Fly Atlas
584 2, we noticed patterns suggesting that the male and female values may be reversed. Tissue specificity values
585 are similar regardless of whether rectal pad is included or not.)

586 Across-tissue expression profiles: Separately for adults of each sex, we calculated for each gene a vector of
587 proportional expression across 11 tissues (as listed above, excluding rectal pad). The $i^{th}$ element of the vector
588 was expression in the $i^{th}$ tissue (FPKM) divided by the sum of that gene's expression values across all 11
589 tissues. Separately for each sex, we performed robust principal component analyses on the proportional data
590 using *pcaCoDa* (Templ et al. 2011). The first three principal components (PCs) for each sex were used in
591 downstream analyses. These PCs accounted for 79% and 71% of the variation in expression profiles in males
592 and females, respectively. Tissue loadings for each PC are given in Table S2.

593 Intersexual genetic correlation $r_{mf}$: Following Fraïsse et al. (2019), we calculated the intersexual genetic
594 correlation in gene expression from the data of Huang et al. (2015) who measured whole body gene
595 expression of 185 genotypes from the Drosophila Reference Genome Panel. Using *MCMCglmm* (Hadfield
596 2010), for each gene we fit the model $z_{ijk} = \mu + \phi_{jk} + e_{ijk}$ where $z_{ijk}$ is the normalized expression of the
597 $i$th replicate of sex $j$ of genotype $k$. $\mu$ is the mean expression. $\phi_{jk}$ is the random genetic effect of sex $j$ of
598 genotype $k$ and follows a bivariate Gaussian distribution characterized by sex-specific variances and the
599 intersexual covariance. $e_{ijk}$ is the residual. Models were fit using uninformative priors and run for a million
600 iterations after a burn-in period of 10,000 iterations with a thinning interval of 1000. From the resulting
601 MCMC posterior of sex-specific variances and intersexual covariances, a posterior of $r_{mf}$ was created and the
602 mean value used as the estimate of $r_{mf}$. We only considered $r_{mf}$ values for genes for which both sexes had
603 genetic variances > 0.001 and statistically significant ($p < 0.05$) in at least one sex based on results of Huang
604 et al. (2015; their Table S5). In the Supplementary Material we show another version of the main figure
605 using only genes for which the genetic variance was statistically significant in both sexes; the patterns are
606 very similar.

607 Population Genomic Data: We obtained whole genome sequences for 205 haploid genotypes of the DPGP3
608 *Drosophila* population which were originally collected from a wild population in Siavonga, Zambia (Lack et
609 al. 2015, 2016). FASTA files were obtained from the *Drosophila* Genome Nexus (DGN; Lack et al. 2015,
610 2016) website (https://www.johnpool.net/genomes.html) and were filtered to remove sites with evidence of
611 high relatedness and recent admixture using Perl scripts provided on the DGN website. Following Sohail et
612 al. (2017), we removed five genotypes that had extremely high or low numbers of variants. Additionally, we
613 removed a single genotype (ZI28) for which the length of the X chromosome in the FASTA file differed

614 from the other 204 genotypes. We then created a VCF file using the remaining 199 sequences with *SNP-sites*
615 (v2.5.1; Page et al. 2016). Coordinates for the VCF file were converted to release 6 of the *Drosophila*
616 reference genome using *UCSC liftOver* (Kent et al. 2002).

617 Estimating nucleotide diversity and Tajima's *D*: We obtained a GFF file for the *D. melanogaster* genome
618 from Ensembl (https://useast.ensembl.org/Drosophila_melanogaster/) and scored each coding site in the
619 genome as 4-fold (i.e., synonymous sites) or 0-fold (i.e., non-synonymous sites) degenerate across the
620 *Drosophila* genome using a Python script (script available at https://github.com/tvkent/Degeneracy). We
621 then parsed our VCF containing variant and invariant sites in the DPGP3 into two files that contained either
622 only 4-fold sites or only 0-fold degenerate sites. After excluding sites with data from fewer than 100 lines
623 and those with more than two alleles, we measured per site nucleotide diversity $\pi = 2p(1-p)$, where $p$ is
624 the estimated frequency of the reference base. Nonsynonymous diversity $\pi_N$ within a gene was measured as
625 the mean nucleotide diversity at 0-fold degenerate sites within that gene and synonymous diversity $\pi_S$ within
626 a gene as the mean nucleotide diversity at 4-fold degenerate sites within each gene. Our analysis focuses on a
627 measure of *relative* nonsynonymous diversity, $\tilde{\pi}_{rN} \equiv \pi_N/(\pi_N + \pi_S)$. We use this rather than the more
628 familiar ratio of $\pi_N/\pi_S$ because the latter has undesirable statistical properties, being highly sensitive to
629 sampling variation in $\pi_S$ and is undefined if $\pi_S = 0$. Our measure $\tilde{\pi}_{rN}$ is bounded between 0 and 1 and will
630 be similar to $\pi_N/\pi_S$ when $\pi_S >> \pi_N$, as is expected for typical genes.

631 Tajima's *D* (Tajima 1989) was estimated using 4-fold degenerate sites, accounting for the sample
632 size and number of 4-fold sites (both invariant and variant) considered for each gene (Walsh and Lynch
633 2020, p. 303); sites with data from a fewer than 100 lines were not considered. When sites varied in sample
634 size, we individually down sampled variant sites to the level of the site with the lowest sample size, then
635 calculated Tajima's *D*. Downsampling was performed 100 times per gene and the median value of Tajima's
636 *D* was used.

637 Direction of Selection (*DoS*): *DoS* (Stoletzki and Eyre-Walker 2011) is a metric that compares relative
638 nonsynonymous divergence to relative nonsynonymous polymorphism; in both cases, it is relative to the sum
639 of synonymous and nonsynonymous changes. Specifically, $DoS = D_N/(D_N + D_S) - P_N/(P_N + P_S)$ where $D_N$
640 and $P_N$ are the numbers of nonsynonymous interspecific differences and intraspecific polymorphisms,
641 respectively; $D_S$ and $P_S$ are defined analogously for synonymous changes. We used the *DoS* values reported
642 by Fraïsse et al. (2019). They calculated divergence with respect to *D. simulans*. They calculated
643 polymorphism from 197 haploid genomes of *D. melanogaster* genomes of the DPGP3. They excluded rare
644 variants (minor allele frequency < 5%) to reduce the influence of deleterious variants on *DoS*.

645 Statistical Analysis: For each of four evolutionary genetic properties of interest—$r_{mf}$, $\tilde{\pi}_{rN}$, Tajima's *D*, and
646 *DoS*—we used linear models to examine whether among-gene variation in the metric was predicted by the

647 following gene expression characteristics: SSS status, SBGE measured as $\log_2$FC, average expression, tissue
648 specificity, as well as three male PCs and three female PCs capturing major axes of variation in among-tissue
649 expression profiles. A quadratic term, $SBGE^2$, was included to capture effects of sex bias irrespective of the
650 direction of the bias. Genes with extreme sex-bias ($|\log_2 FC| > 5$) were excluded from these analyses because
651 visual inspection revealed that these genes had distinctly different values for several of the evolutionary
652 genetic properties of interest (see Results). For the results presented in the main text, SSS status was coded
653 as a numerical value: 2 (strong evidence of SSS: $p_{adj} < 0.01$), 1 (modest evidence: $0.2 < p_{adj} \leq 0.01$), or 0
654 (poor evidence: $p_{adj} \geq 0.2$).

655 All models included an indicator variable of whether the gene was autosomal or X-linked. In
656 addition, the models for each of the three population genomic metrics—$\tilde{\pi}_{rN}$, Tajima's *D*, and *DoS*—
657 included two other terms: log(recombination rate) and log(transcript length). Recombination is expected to
658 affect population genetic patterns by mediating the effects of linked selection. Transcript length is also
659 expected to affect linked selection as a given site in a long gene is expected to have more closely linked
660 targets of selection than a site in a short gene. Recombination values came from Fraïsse et al. (2019), who
661 obtained recombination measures from Chan et al. (2012).

662 The statistical support for model terms was assessed in two different ways. We obtained
663 permutation-based *p*-values using the *lmp* function from the *lmperm* package (Wheeler and Torchiano,
664 2016). We used perm = "Prob", Ca = 0.001, maxIter = $10^6$ and seq = FALSE for unique rather than
665 sequential sum of squares. Second, we used the *Boot* function from the *boot* package (Canty and Ripley
666 2020) on each linear model, followed by *confint* to obtain 95% bootstrap confidence intervals of model
667 terms. We specified method = "case" to sample over cases, which is considered a conservative approach. In
668 almost all cases, the two methods gave consistent results, i.e., when terms were significant based on
669 permutation *p*-values < 0.05, the bootstrap confidence intervals for the estimate did not overlap zero.

670

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