Conservation and divergence of sex-biased gene expression across 50 million years of *Drosophila* evolution

Amanda Glaser-Schmitt* and John Parsch*

Division of Evolutionary Biology, Faculty of Biology, Ludwig-Maximilians-Universität München, Munich, Germany

Running title: Sex-biased gene expression in *Drosophila*

*Corresponding authors:
Email: glaser@bio.lmu.de (AGS); parsch@bio.lmu.de (JP)

Abstract

In sexually dimorphic species, sex-biased gene expression plays an important role in driving morphological, physiological, and behavioral differences between males and females. Here, we examined patterns of sex-biased gene expression within and among 6 *Drosophila* species with divergence times ranging from 2–50 million years. We detected contrasting patterns of sex bias conservation and turnover between heads and bodies, with more extensive sex-biased expression and greater conservation of sex-biased expression in the body, but more species-specific turnover of sex-biased expression in the head, where conserved, unbiased expression was common. Interestingly, in both body parts lineage-specific gains of sex-biased expression occurred most often via concordant expression changes in both sexes that differed only in their magnitude, with this pattern being particularly strong in the head, suggesting that the majority of lineage-specific sex bias gains do not represent a resolution of sexual antagonism, but instead reflect regulatory changes shared between the sexes. We detected an enrichment of putatively positively selected expression changes among sex-biased genes in both body parts. Taken together, our findings suggest sex-biased changes may often be facilitated by selection acting on the sex with lower expression, especially for male expression of female-biased genes in the body. We also detected differences in the proportion of sex-biased genes located on the X chromosome depending on sex bias and body part. Overall, our results provide novel insights into the dynamics of sex-biased gene expression, as well as the molecular mechanisms and selective forces underlying its turnover, across short and long evolutionary timescales.

Introduction

Gene expression variation underlies much of the phenotypic variation that we observe among species, populations, and individuals of the same species (King and Wilson 1975; Wray et al. 2003; Buchberger et al. 2019). In species with sexual dimorphism, this gene expression variation is particularly important as the vast majority or, in some cases, all of the genome is shared by both sexes (Ellegren and Parsch 2007). Therefore, sex-biased (SB) gene expression, i.e. the differential expression of a shared genome between males and females, plays an important role in driving morphological, physiological, and behavioral differences between the sexes (reviewed in Parsch and Ellegren 2013; Grath and Parsch 2016; Tosto et al. 2023). Indeed, quantifying and understanding patterns of sex-biased gene expression over both short and longer evolutionary timescales can help us better understand sexual

53 conflict and the evolutionary forces that shape and maintain phenotypic variation, especially
54 between the sexes, which in turn can shed light on biodiversity and the evolution of complex
55 phenotypic traits (reviewed in Tosto et al. 2023). For example, sex-specific gene expression
56 and regulation have been implicated in the susceptibility and presentation of several human
57 diseases (e.g. Shi et al. 2016; Khodursky et al. 2022; Fass et al. 2024), as well as variation in
58 human height (Naqvi et al. 2019), all of which represent complex, highly polygenic
59 phenotypes.

60 Studies have shown that sex-biased gene expression is extensive (Meiklejohn et al.
61 2003; Yang et al. 2006; Oliva et al. 2020), can be tissue-, developmental stage-, and cell type-
62 specific (Yang et al. 2006; Ma et al. 2018; Naqvi et al. 2019; Oliva et al. 2020; Khodursky et al.
63 2022; Rodríguez-Montes et al. 2023), and evolves rapidly (Assis et al. 2012; Whittle and
64 Johannesson, 2013; Harrison et al. 2015; Yang et al. 2016; Xie et al. 2025). The shared
65 genome has long been thought to impose genetic constraints on the sexes (Lande 1980;
66 Griffen et al. 2013), and SB gene expression is thought to be an indication of this ongoing
67 sexual conflict (i.e. sexual antagonism) or its resolution (Connallon and Knowles 2005;
68 Innocenti and Morrow 2010). Indeed, previous studies of SB gene expression in diverse taxa
69 have found that sexual conflict is often resolved via SB gene expression and/or sex-specific
70 regulation (e.g. Cheng and Kirkpatrick 2016; Dean et al. 2016; Wright et al. 2018).

71 The model insect, *Drosophila melanogaster*, was utilized in some of the earliest
72 studies of SB gene expression (Jin et al. 2001; Arbeitman et al. 2002; Ranz et al. 2003; Parisi
73 et al. 2003) and has continued to be an important model system for studying its evolution at
74 both the interspecific and intraspecific levels (e.g. Meiklejohn et al. 2003; Ranz et al 2004;
75 Gibson et al. 2004; Catalán et al. 2012; Llopart et al. 2012; Meiklejohn et al. 2014; Huylmans
76 and Parsch 2014; Khodursky et al. 2020). Despite this progress, transcriptomic studies that
77 systematically compare SB gene expression among species while also assessing the full range
78 of within-species expression diversity are currently lacking. Previous studies in the genus
79 *Drosophila* were restricted in their phylogenetic scale (e.g. Ranz et al. 2003; Grath and
80 Parsch 2012; Müller et al. 2012; Khodursky et al. 2020) or had limited samples for
81 determining within-species expression variation (e.g. Zhang et al. 2007; Pal et al. 2023).

82 The characterization of expression variation both within and between species is
83 important for inferring the type of selection that influences gene expression, as the two
84 levels of variation are thought to represent, respectively, the short-term and long-term
85 outcomes of the same underlying evolutionary process (Kimura 1983; Hudson et al. 1987;

86 McDonald and Kreitman 1991). To address this issue, we measured gene expression in two
87 body parts (head and body) of adult males and females of 6 *Drosophila* species spanning
88 divergence times of up to 50 million years (Tamura et al. 2004). Within each species, we
89 included 5–8 strains derived from diverse geographic locations. These data allowed us to
90 detect the conservation and turnover of SB gene expression across the genus in samples that
91 include or exclude the gonad, which is the tissue with the highest proportion of SB genes
92 (Parisi et al. 2004). Furthermore, we were able to compare levels of expression
93 polymorphism and divergence to identify genes showing evidence for adaptive regulatory
94 evolution. We found that there is a much greater extent and conservation of SB expression
95 in the body than in the head. Most genes in the head showed conserved unbiased (UB)
96 expression, with reversals in the sex bias of a gene, i.e. from male-biased (MB) to female-
97 biased (FB) or vice versa, occurring only very rarely. Interestingly, in both body parts,
98 lineage-specific gains of SB expression typically involved concordant expression changes in
99 both sexes that differed only in their magnitude, with this pattern being particularly strong in
100 the head. We detected an enrichment of putatively positively selected gene expression
101 changes among SB genes in both body parts, and found evidence suggesting that these
102 changes may often be facilitated by selection acting on expression in the sex with lower
103 expression, especially for male expression of FB genes in the body. Together, these results
104 suggest that, intriguingly, the most common route to a gain in sex bias may be through
105 shared regulatory mechanisms between the sexes, rather than sex-specific regulation.
106 Overall, our results shed light on the long-term evolutionary dynamics of sex-biased gene
107 expression in the *Drosophila* genus.
108

109 **Results**

110 In order to study the evolution of sex-biased gene expression over a range of
111 timescales, we performed RNA-seq of male and female heads and bodies of 6 *Drosophila*
112 species with divergence times ranging between 2–50 million years (Fig 1), including *D.*
113 *melanogaster* (*Dmel*), *D. simulans* (*Dsim*), *D. suzukii* (*Dsuz*), *D. ananassae* (*Dana*), *D.*
114 *subobscura* (*Dsub*), and *D. immigrans* (*Dimm*). To get a comprehensive view of expression
115 variation within each species, we sampled 5–8 strains from a broad geographic distribution,
116 encompassing strains from multiple populations across three continents, including strains
117 from the putative ancestral range for *Dmel*, *Dsim*, *Dana*, and *Dsuz* (Supplementary Table S1).
118 We analyzed expression in 9,016 1:1 orthologs present in all species (Thiébaut et al. 2024),

7,570–8,161 of which were expressed in the head and 8,936–9,006 of which were expressed in the body of each species. When we examined gene expression variation across all samples in all species and sexes, the strains clustered strongly by body part (Fig 2A). In the head, the sexes showed only slight separation (Fig 2B), while in the body, males and females were clearly differentiated (Fig 2A, C). Within each sex and body part, strains of each species clustered together, although these clusters often overlapped among species. *Dimm*, which is the most distantly related to the other species (Fig 1), also showed the greatest interspecific differentiation (Fig 2B, C). Within each species, patterns were similar, with body parts and sexes clustering by sample type but differentiation between the sexes much stronger in the body than the head (Supplementary Fig S1).

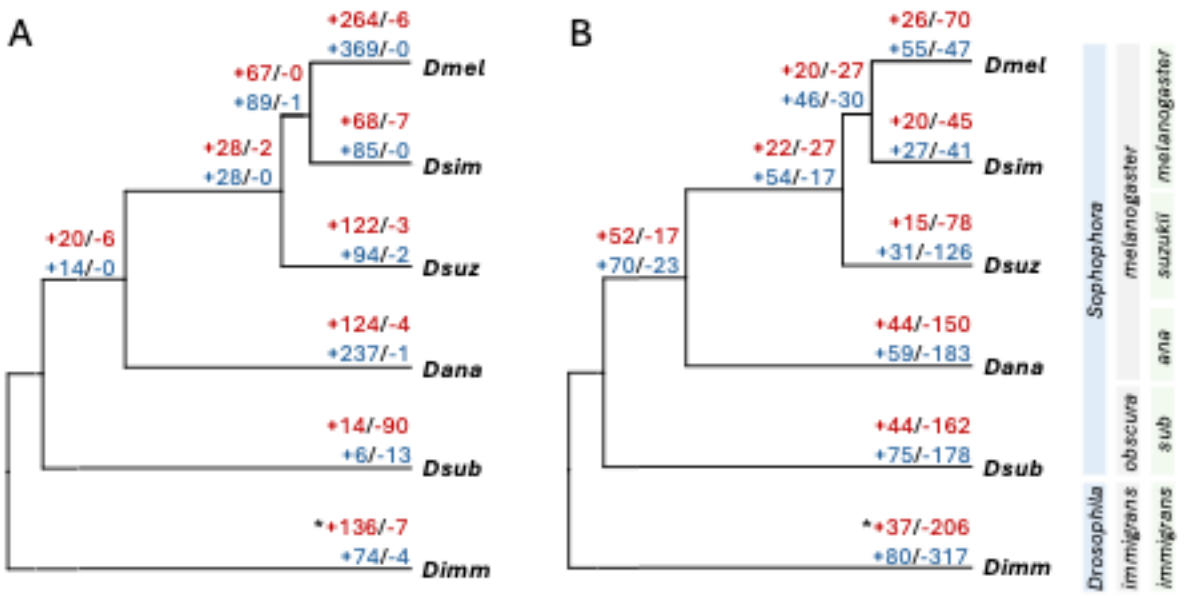


Fig 1: Phylogeny and sex-biased expression turnover in the examined *Drosophila* species in the A) head and B) body. Branch lengths are shown only roughly to scale. Numbers at each tip or node indicate sex bias gains (+) and losses (-) for female- (red) and male-biased (blue) genes. The subgenera are shown in the light blue boxes, the species groups in the gray boxes, and the subgroups in the light green boxes (*subobscura*: *sub*; *ananassae*: *ana*). *Gains and losses could not be polarized for *Dimm* due to the lack of an outgroup; therefore, the number of genes with different sex bias relative to the other species are shown.

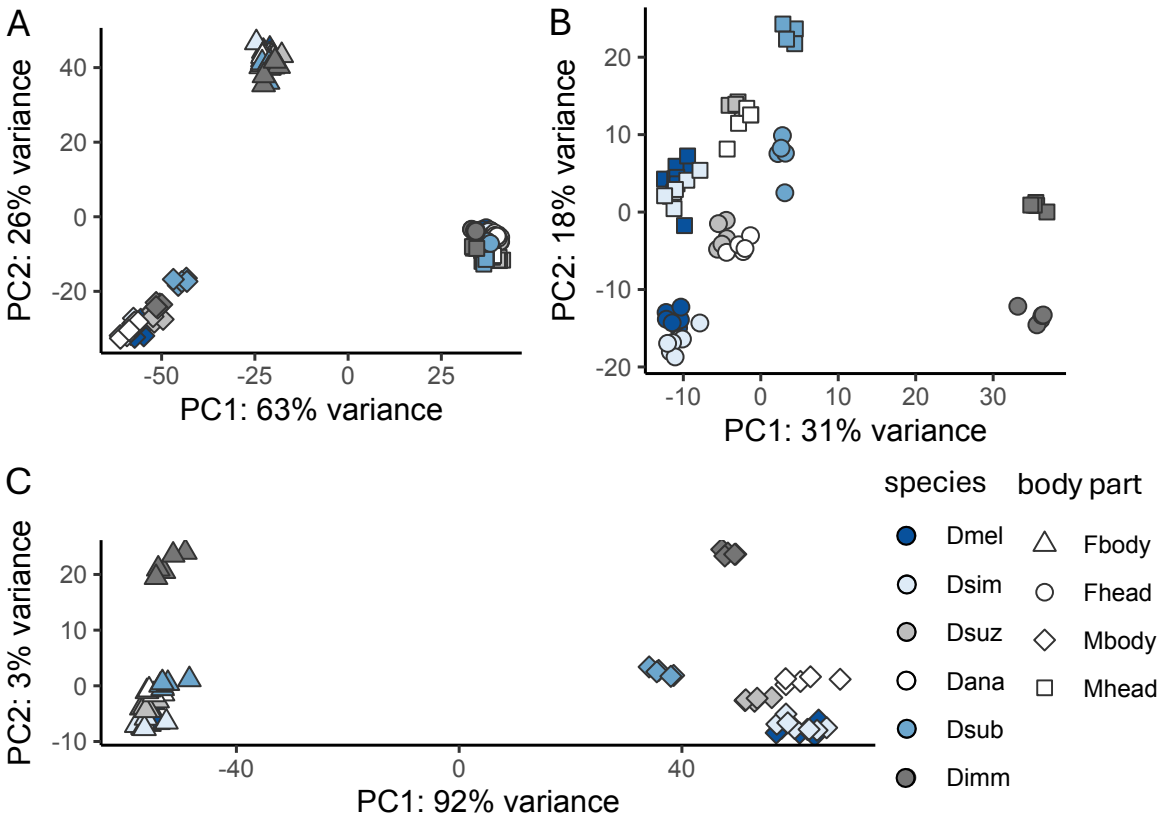


Fig 2: Principal component analysis of gene expression profiles in A) all samples, B) the head, and C) the body. The legend at the bottom right indicates the color associated with each species and the shape indicates the sex (male: M; female: F) and body part.

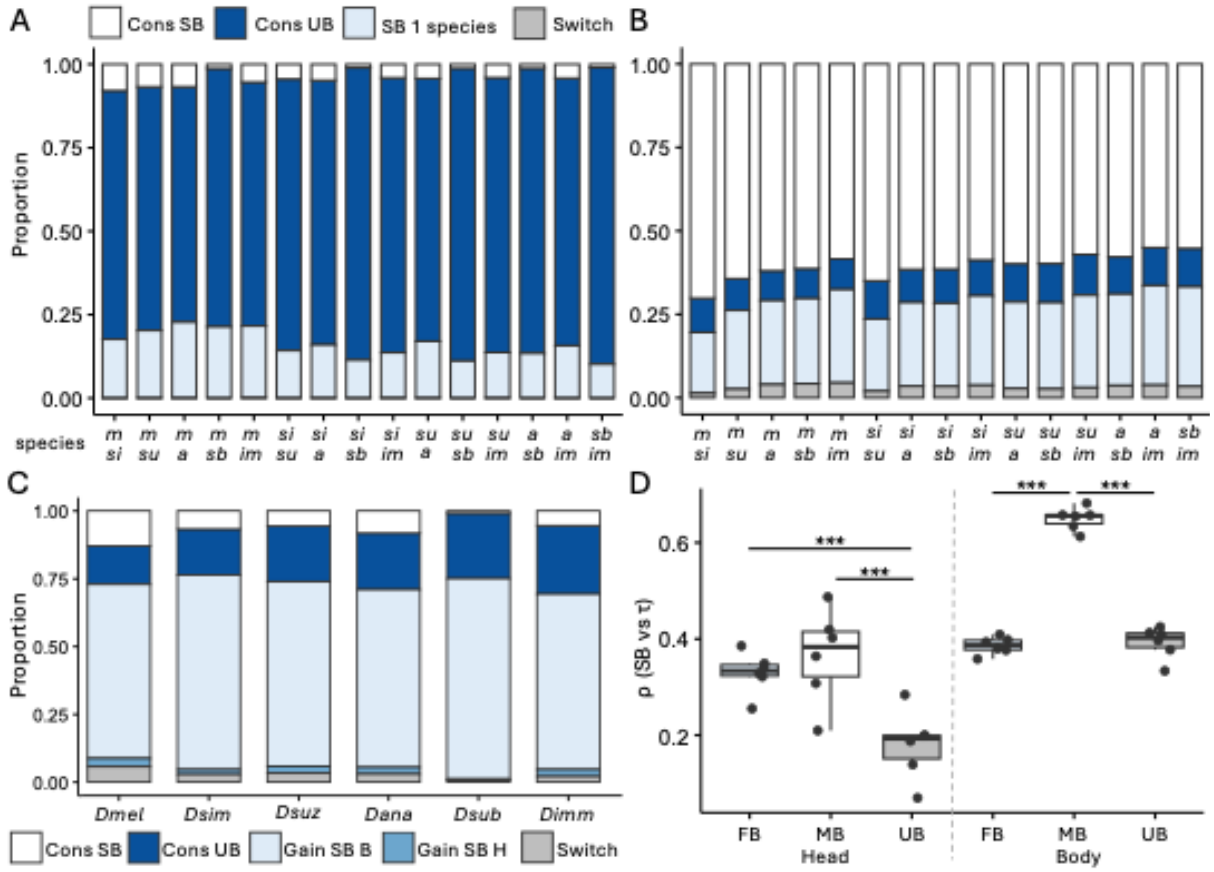
Patterns and magnitude of sex-biased expression differ among body parts and species

Within each species, SB gene expression was extensive in the body, with 71.9–81.6% of genes showing sex bias versus 2.0–21.7% of genes in the head (Table 1). In comparison to the other species, sex bias in *Dsub* heads was very low (2.0% versus 10.1–21.7%; Table 1), especially when considering MB genes (32 versus 212–814 genes; Table 1). We detected more genes as SB in the *Dmel* head in comparison to the other species, but this excess was most likely driven by the sampling of additional strains in this species, as down-sampling resulted in the detection of similar numbers of SB genes as in the other species (Supplementary Table S2). Due to the large amount of sex bias in the body, the majority of genes that could be examined in both body parts (64.2–73.9%) were SB in the body but not the head (Fig 3C, Supplementary Fig S2). The next largest category of overlap was genes UB in both the body and head (14.1–25.2%), with genes showing the same sex bias in both body parts being the third largest category (0.9–13.0%). Interestingly, genes that switched sex bias between the head and body were more common than genes SB in the head but not the body

159 (0.7–5.6% versus 0.4–3.1%, respectively). Thus, genes SB in the head were 3–6 times more
160 likely to also be SB in the body, and this sex bias was often, but not always, in the same
161 direction (Fig 3C, Supplementary Fig S2).
162
163 **Table 1:** Sex bias in the head and body of six *Drosophila* species

	Head				Body			
Species	**Genes**	**MB**	**FB**	**UB**	**Genes**	**MB**	**FB**	**UB**
Dmel	7899	814 (10.3)	898 (11.4)	6187 (78.3)	9006	4229 (47.0)	3121 (34.7)	1656 (18.4)
Dsim	7828	370 (4.7)	520 (6.6)	6940 (88.7)	9002	3998 (44.4)	3166 (35.2)	1838 (20.4)
Dsuz	7591	304 (4.0)	562 (7.4)	6725 (88.6)	8936	3715 (41.6)	3035 (34.0)	2186 (24.6)
Dana	7570	490 (6.5)	548 (7.2)	6532 (86.3)	8955	3727 (41.6)	3007 (33.6)	2221 (24.8)
Dsub	8161	32 (0.4)	135 (1.7)	7994 (98.0)	8981	3727 (41.5)	3004 (33.4)	2250 (25.1)
Dimm	7715	212 (2.7)	565 (7.3)	6938 (89.9)	8938	3527 (39.5)	2896 (32.4)	2515 (28.1)

164 The number of analyzed genes, MB, FB, and UB genes in the head and body of each species
165 are shown. Percentages of analyzed genes rounded to the nearest decimal are shown in
166 parentheses.
167



168
169 **Fig 3:** Sex bias within and among species. A,B) Pairwise overlap in sex bias categories among
170 species in the A) head and B) body. Shown are the proportion of overlapping genes that had
171 conserved SB (Cons SB; white) or conserved UB expression (Cons UB; dark blue), were SB in

172 only one of the examined species (SB 1 species; light blue), or switched sex bias (Switch;
173 gray) between *Dmel* (*m*), *Dsim* (*si*), *Dsuz* (*su*), *Dana* (*a*), *Dsub* (*sb*), and *Dimm* (*im*). C)
174 Proportion of overlapping genes that had conserved SB or conserved UB expression, were SB
175 in only the body (Gain SB B; light blue) or head (Gain SB H; medium blue), or switched sex
176 bias between the head and body are shown. D) Spearman's ρ correlations between τ and
177 magnitude of sex bias in the head and body for MB, FB, and UB genes. Significance was
178 assessed with a Mann-Whitney *U* test. ***$P < 0.005$. Non-significant comparisons not
179 shown.
180
181 The magnitude of sex bias was significantly higher in the body than the head for all
182 species for both MB and FB genes (*t*-test; BH corrected $P < 1.5 \times 10^{-15}$ for all; Supplementary
183 Fig S3). Within the head, the degree of sex bias was higher for FB than for MB genes, while in
184 the body, the opposite pattern was detected, which was significant for all comparisons in all
185 species (*t*-test; BH corrected $P < 4.5 \times 10^{-7}$ for all; Supplementary Fig S3), except *Dana* and
186 *Dsub* heads (BH corrected $P = 1$). We also detected significant differences in the degree of
187 sex bias within the same body part and sex bias category (MB or FB) among species,
188 especially in the head, for many but not all comparisons (Supplementary Fig S3). Despite
189 these differences in the magnitude of sex bias, overall expression divergence (as measured
190 by 1 - ρ) between sexes was lowest in the head within species (0.8–1.3%; Supplementary
191 Table 3), while divergence in the body was fairly similar to divergence between body parts
192 regardless of sex (27.7–36.5% versus 25.2–43.6%%; Supplementary Tables 3–4). Divergence
193 between species within the same body part and sex was higher than in the head but lower
194 than in the body between sexes within species (1.8–12.0%; Supplementary Tables 3–4).
195 Thus, we detected differences in the magnitude of expression bias based on the type of sex
196 bias, the body part, and the species, but overall expression levels showed the strongest
197 correlation (between sexes in the head) and weakest correlation (between the examined
198 body parts in either sex) within species.
199
200 **Tissue specificity and expression level are associated with differences in sex bias**
201 According to FlyAtlas2 data (Krause et al. 2022; Supplementary Table S5), genes we
202 identified as MB in the head or body were often most highly expressed in testes or a somatic
203 tissue, while FB genes were most highly expressed in the ovary or a somatic tissue. To better
204 understand the relationship between expression level, breadth, and sex bias in our dataset,

205 we first examined the overall expression level of SB and UB genes in the head and body of
206 each species. We detected significant effects of both species and sex bias category on the
207 expression level in both body parts (ANOVA; $P < 1.5 \times 10^{-6}$ for all; Supplementary Fig S4). In
208 both the head and body, FB genes were the most highly expressed, followed by MB genes,
209 and then UB genes, except in *Dsub* heads, where MB genes were the most lowly expressed
210 (Supplementary Fig S4). Thus, differences in sex bias were associated with differences in
211 overall gene expression levels.
212 Next, we used the *Dmel* FlyAtlas2 adult tissue expression data (Krause et al. 2022) to
213 calculate the tissue specificity index, τ, which ranges from 0 to 1 with values closer to 1
214 indicating higher tissue specificity, for each gene. When we compared tissue specificity
215 among SB and UB genes in each species and body part, we detected a significant effect of
216 sex bias category in both body parts as well as an effect of species in the head (ANOVA; $P <$
217 1.6×10^{-10} for all; Supplementary Fig S5). Tissue specificity was highest for MB genes in both
218 the head and body for all species (Supplementary Fig S5). In the head, τ was mostly similar
219 between FB and UB genes, while in the body, τ was lower in FB than in UB genes
220 (Supplementary Fig S5). Tissue specificity was significantly positively correlated with the
221 magnitude of sex bias in both body parts in all sex bias categories and all examined species
222 (Spearman's ρ; $P < 0.003$ for all) except MB genes in the head of *Dsub* ($P = 0.2483$). Over all
223 species, this correlation between the degree of sex bias and τ was highest for MB genes in
224 both body parts (Fig 3D), which was significantly higher than UB genes in both body parts
225 and FB genes in the body (Mann-Whitney *U* test; $P < 0.005$ for all), while the correlation for
226 FB genes was only significantly higher than UB genes in the head ($P < 0.005$; Fig 3). Thus,
227 tissue specificity was positively associated with sex bias, with the strongest correlation in MB
228 genes, which were also the most tissue specific. This correlation was particularly strong in
229 the body, which might be driven at least in part by the presence of the testes, which is the
230 tissue with the highest proportion of MB genes (Parisi et al. 2004).
231
232 **Patterns of sex bias turnover among species differ between the heads and body**
233 In order to better understand how sex bias is conserved among species, we identified
234 genes that were SB or UB in all 6 species. In the head, we identified 4,363 (53.5–57.6%)
235 genes with conserved UB expression in all species, but only 1 (< 0.02%) and 35 (< 0.5%)
236 genes as having conserved MB or FB expression, respectively. On the other hand, we
237 identified only 119 (1.3%) genes with UB expression in all species in the body, but 1,638

238 (18.2–18.3%) and 1,949 (21.6–21.8%) genes with conserved FB and MB expression,
239 respectively. In order to understand how turnover in sex bias occurs among species, we
240 identified lineage-specific gains or losses of FB or MB that occurred on only one branch of
241 the phylogeny (Fig 1). In the head, we detected many more sex bias gains than losses for all
242 species except *Dsub* (Fig 1A; 1,629 total lineage-specific gains versus 135 losses, 103 of which
243 were in *Dsub*); while, in the body we detected more sex bias losses than gains (1,227 losses
244 versus 663 gains; Fig 1B), although the pattern was not as extreme as in the head. Across the
245 entire phylogeny, only 171 genes switched sex bias on a specific lineage, all of which
246 occurred in the body and the majority of which (64.3%) switched from FB to MB
247 (Supplementary Table S6).
248 To better understand how sex bias changed among species, we made pairwise
249 comparisons of sex bias in all examined genes between species for each body part (Fig 3A–B,
250 Supplementary Figs S6–S7). In the head, genes that were SB in one species were usually UB
251 in the other species (10.2–22.7% of genes), but if a gene was SB in both species, it almost
252 always shared the same sex bias between species (1.0–7.9% of genes), with only 0–9 genes
253 switching their sex bias (i.e. from MB to FB or vice versa) between species (0–0.12% of
254 genes; Fig 3A,B, Supplementary Fig S6). In the body, SB genes were often shared between
255 species, with the majority showing sex bias towards the same sex (55.0–70.2% of genes), but
256 there were also many SB genes that were UB in the other species (18.2–29.9% of genes;
257 Supplementary Figs S7, S8) as well as a small proportion (1.4–4.5%) that switched sex bias
258 between species. However, we should note that because the body is a composite of multiple
259 tissues, it is possible that some of these switches represent opposing changes in different
260 tissues rather than a switch in the same tissue. Thus, we detected a relatively large amount
261 of sex bias turnover in both body parts among species, which occurred in a mostly species-
262 specific manner in the head (Figs 1–2). Moreover, when turnover occurred in the head, a
263 gene's expression switched between being UB and biased towards a single sex, but never
264 between the two sexes.
265
266 **Sex bias is most often gained or lost via correlated expression changes in both sexes,**
267 **especially in the head**
268 In order to better understand how expression changes during the gain or loss of sex
269 bias, we examined how expression in a focal species changed relative to the inferred
270 ancestral expression within each sex (see Materials and Methods) for species-specific gains

271 or losses of SB expression. In the head, the majority of expression changes occurred in the
272 same direction (either up-regulation or down-regulation) in both sexes (75.6% of all sex bias
273 gains and losses; Fig 4), and this pattern was even stronger for genes with large expression
274 changes ($\log_2$ fold-change ≥ 1), 95.2% of which had concordant expression changes between
275 the sexes (versus 74.3% of genes with small expression changes; Supplementary Fig S8).
276 Although concordant expression changes were also more common than opposing changes in
277 the body (54.3% versus 45.7%), the direction of expression changes in males versus females
278 was more evenly distributed compared to the head ($P < 2.2 \times 10^{-16}$; χ^2 test; Fig 4). Genes with
279 large expression changes ($\log_2$ fold-change ≥ 2) were more similar to patterns in the head,
280 with 66.7% of genes showing concordant expression changes between sexes (versus 53.3%
281 of genes with small expression changes; Supplementary Fig S8). In keeping with the
282 observation that expression changes were often concordant between sexes, expression
283 changes associated with SB gains were significantly positively correlated in the head and
284 body in all species (Spearman's ρ; $P < 0.05$ for all) except for MB gains in the *Dsub* head and
285 FB gains in the *Dmel* and *Dsim* body (Supplementary Figs S9–S10). This correlation in gene
286 expression changes between the sexes was significantly higher in the head than in the body
287 (paired Mann-Whitney *U* test; $P = 0.0003$). However, this pattern was less apparent for SB
288 losses, for which this correlation was sometimes negative and only significant in half of the
289 comparisons (Supplementary Figs S9–S10), although in the head part of this observation
290 might be at least partially explained by the low number of SB losses in each species. Indeed,
291 correlations in gene expression changes between the sexes were significantly higher for SB
292 gains than for losses in both the head and the body ($P < 0.05$ for all; Fig 4).
293

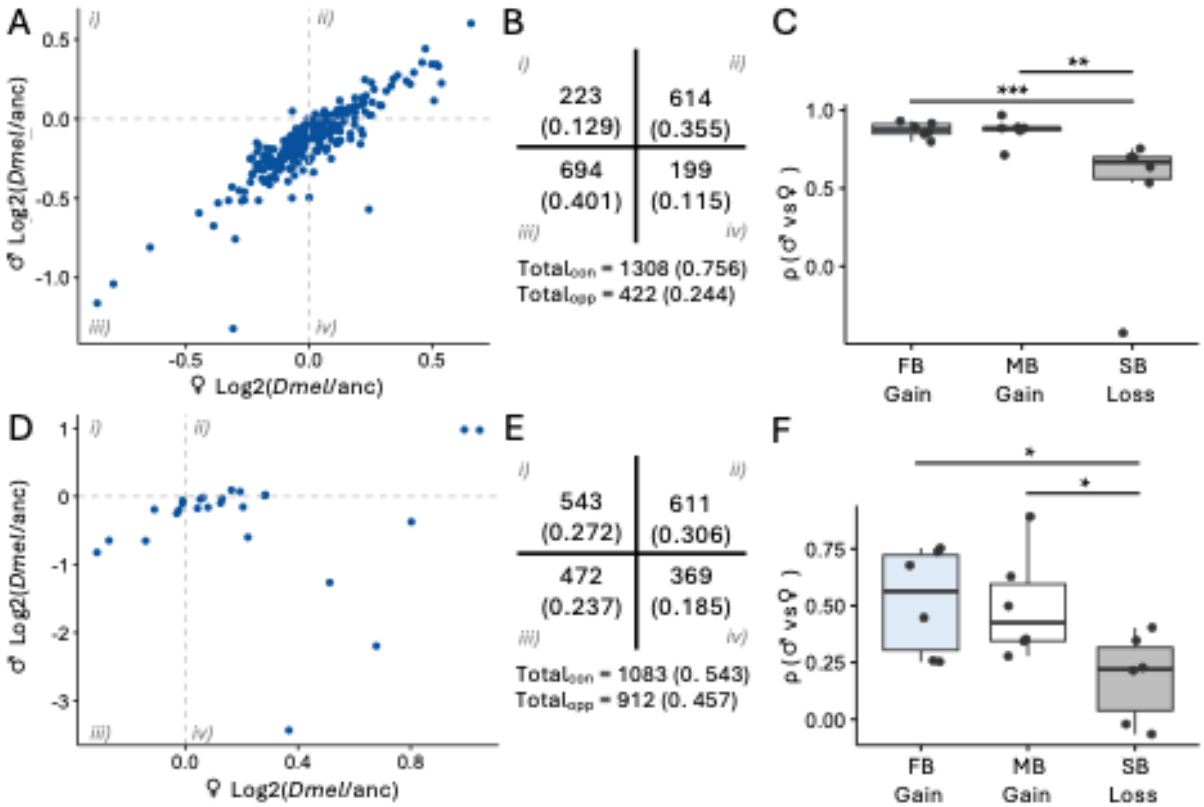


Fig 4: Expression changes underlying species-specific gains and losses of SB gene expression. As an example, scatterplots depicting the change between *Dmel* and ancestral expression in males versus females for genes with FB gains in the A) head and D) body are shown. All other scatterplots can be found in Supplementary Figs S9–S10. A,B,D,E) Genes with concordant (con) changes in expression between the sexes are located in quadrants ii and iii; while genes with opposing (opp) expression changes between the sexes are located in quadrants i and iv. B, E) Total genes in each quadrant for the entire phylogeny in the B) head and E) body are shown. In parentheses, the proportion of total gene expression changes are shown. C, F) Spearman's ρ correlations between male and female expression changes within each species for SB gains and losses in the C) head and F) body. Significance was assessed with a Mann-Whitney *U* test. ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$. Non-significant comparisons not shown.

Next, we examined how the size of expression changes and ancestral sex bias affected the distribution of expression changes during SB loss or gain. For the majority of large gene expression changes ($\log_2$ fold-change ≥ 1 in the head or ≥ 2 in the body; 56.7–75.2% of genes), gain or loss of sex bias occurred via a down-regulation in both sexes with only 10–20% of genes up-regulated (Supplementary Fig S8). In contrast, up- or down-regulation in both sexes was more evenly distributed for genes with small expression changes (32.3–36.5% and 21.0–37.8% of genes for head and body, respectively;

314 Supplementary Fig S8). Due to the lack of SB reversals and the low number of SB losses for
315 most species in the head, it is difficult to determine if the ancestral sex bias of a gene
316 affected the distribution of expression changes, although MB and FB losses tended to
317 overlap with no clear separation based on the ancestral sex bias (Supplementary Fig S9). We
318 observed a similar pattern for MB and FB gains in the body, with no clear clustering based on
319 whether sex bias was gained from an UB or SB gene (Supplementary Fig S10). On the other
320 hand, FB and MB losses in the body were clearly separated (Supplementary Fig S10), with
321 genes that lost female bias tending to show down-regulation in females and/or up-
322 regulation in males and the opposite for MB losses. Thus, a gain or loss of sex bias most
323 often involved an expression change in the same direction (up-regulation or down-
324 regulation) in both sexes, but of greater magnitude in one sex than the other, with larger
325 expression changes tending to be associated with down-regulated expression in both sexes.
326 Overall, reversal-of-expression changes were less common but were almost as frequent as
327 concordant expression changes in the body.
328
329 **Contrasting distributions of X versus autosomal SB genes in the head and body**
330 Previous studies found that, in whole flies and gonads, FB genes were
331 overrepresented on the X chromosome, while MB genes were underrepresented (Parisi et
332 al. 2003; Ranz et al. 2003; Sturgill et al. 2007). In contrast, in the head and brain, genes with
333 SB expression, and especially MB expression, were enriched on the X chromosome (Catalán
334 et al. 2012; Huylmans and Parsch 2015; Khodursky et al. 2020). We detected similar patterns
335 across the species included in our study, with the proportion of X-linked genes depending
336 upon the body part and, in some cases, the species in which SB gene expression was
337 detected (Fig 5; Supplementary SX). In the head, SB genes were generally over-represented
338 on the X chromosome, but whether this enrichment occurred for MB or FB genes depended
339 upon the examined species, and in some cases (e.g. FB genes in *Dsuz* or MB genes in *Dana*)
340 there was a slight underrepresentation of SB genes on the X (Fig 5A). Interestingly, in *Dsub*
341 MB genes were highly enriched on the X chromosome but highly underrepresented on the
342 autosomes (Fig 5A). In the body, FB genes were enriched, while MB genes were
343 underrepresented on the X chromosome for all species (Fig 5B). Reciprocally, MB genes were
344 enriched while FB genes were underrepresented on the autosomes for all species except
345 *Dana* (Fig 5B). Thus, SB genes displayed unique chromosomal distributions depending on the
346 examined body part.

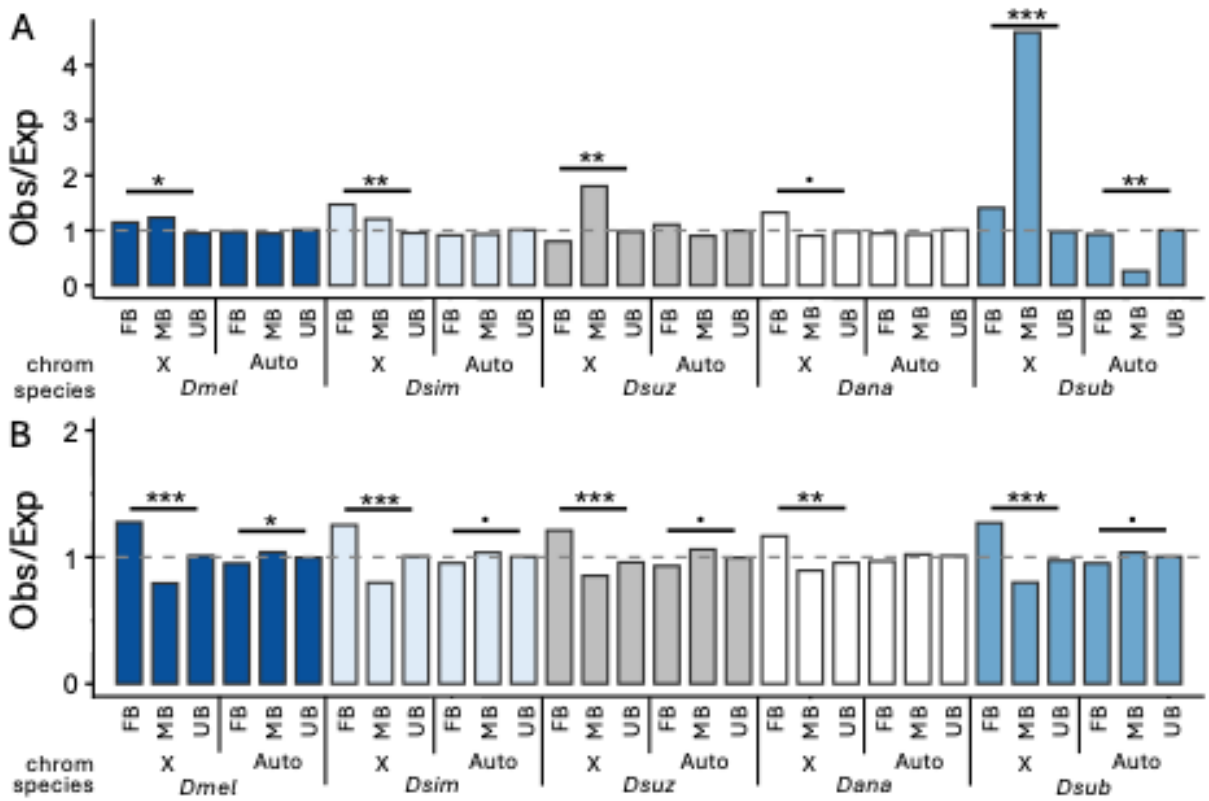


Fig 5: Chromosomal distribution of SB genes in the A) head and B) body. Shown are the ratios of observed (obs) to expected (exp) number of genes in each SB category on the X versus the autosomes based on sex bias category and the number of analyzed on the X versus the autosomes. Significances was assessed with a χ^2 test. ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$, marginally non-significant: .$P < 0.1$. Non-significant comparisons not shown.

Signs of positive selection at the gene expression level are pervasive in SB genes

A previous study in the brain of three *melanogaster* subgroup species found that SB genes, and MB genes in particular, are enriched for signatures of directional selection (Khodursky et al. 2020). In order to test for signs of putative positive selection at the gene expression level, we calculated Δx (see Materials and Methods), which acts similarly to a McDonald-Kreitman (MK) test (McDonald and Kreitman 1991), in both sexes for all species relative to the outgroup species *Dimm*. Briefly, in cases where $-1 < \Delta x > 1$, divergence of expression in the focal species from *Dimm* exceeds expression polymorphism within that species, indicating that positive selection may have occurred. We detected putative signs of positive selection in 6.3–76.5% of genes depending upon the sex, species, and sex bias category (Supplementary Table S7; Supplementary Fig S11), with the proportion of putatively positively selected genes higher for both FB and MB than for UB genes for all

367 comparisons. Indeed, we detected significantly more FB and/or MB genes as putatively
368 positively selected than expected in both sexes in the head for all species (Fig 6A; χ^2 test; $P <$
369 0.05), except in *Dana* females and *Dsub* males, which were marginally non-significant ($P <$
370 0.09). Putatively positively selected MB or FB genes were similarly enriched to a lesser
371 extent in one or both sexes in all species in the body, although we detected fewer FB genes
372 as putatively positively selected than expected in *Dsuz* females (Fig 6B). This enrichment of
373 putatively positively selected MB and/or FB genes occurred on both the X and the
374 autosomes, although in some cases we also detected fewer SB genes than expected both on
375 the X and the autosomes, especially in the body (Supplementary Fig S12). Interestingly, SB
376 genes were not always enriched for putative positive selection in the sex in which they were
377 detected as SB (Fig 6A, B). Indeed, SB genes with signs of positive selection were enriched in
378 the opposite sex (i.e. the sex with lower expression) in at least one sex in all species in both
379 the head and body, although this enrichment was marginally non-significant in females in
380 the *Dsub* body (Fig 6A, B). When we compared Δx between males and females, we detected
381 significant differences in the magnitude of Δx for the majority of sex bias categories and
382 species in both head and body (80% for both; Fig 6C, D), with the absolute value of Δx most
383 often shifted higher in males, especially in the body (75 and 92% of significant shifts in the
384 head and body, respectively), and the mean absolute value in the significantly higher sex
385 usually close to or above 1 (88% of significant shifts; Fig 6C, D; Supplementary Fig S11).
386 Despite this shift in magnitude, Δx was significantly positively correlated between the sexes
387 in all sex bias categories and species (Spearman's ρ; $P < 2.2 \times 10^{-16}$ for all; Supplementary
388 Table S8), except *Dsub* MB genes in the head ($P = 0.153$), and these positive correlations
389 were significantly stronger in the body than in the head (mean of 0.827 vs 0.585 in the body
390 and head, respectively; paired Mann-Whitney U test; $P = 5.37 \times 10^{-3}$). Thus, we detected an
391 enrichment of putatively positively selected genes among SB genes in both the head and
392 body, and this enrichment often occurred in the sex with lower expression, mainly involving
393 the male expression of FB genes. Moreover, the overall magnitude of Δx was significantly
394 higher in males in comparison to females, especially in the body.

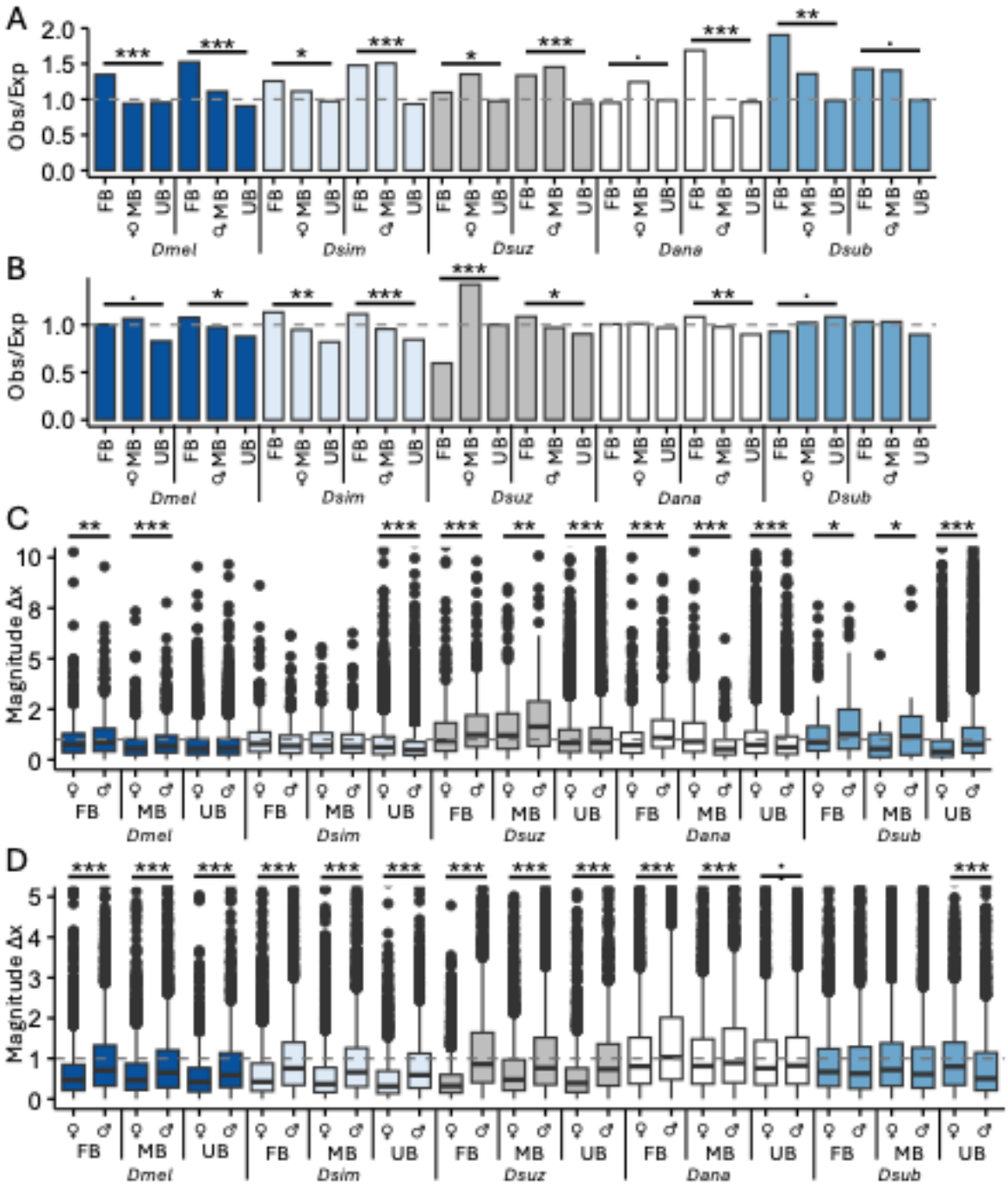


Fig 6: Signs of putative positive selection at the gene expression level. Shown are the ratios of observed (obs) to expected (exp) number of genes under putative positive selection (-1 < Δx > 1) in each SB category and sex in the A) head and B) body. C, D) Shown is the magnitude of Δx values for FB, MB and UB genes in females and males in the C) head and D) body. The dashed gray line represents the cut-off above which a gene shows signs of putative positive selection (absolute value of Δx > 1). For better visualization, absolute Δx values above 5 are not shown. A, B) Significance was assessed with a χ^2 test. C, D) Significant differences between sexes were assessed with a *t*-test. ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$, marginally non-significant: .$P < 0.1$. Non-significant comparisons not shown.

405

406 **Discussion**

407 We characterized SB gene expression in the head and body of 6 *Drosophila* species,
408 encompassing a broad geographic distribution within species and 2–50 million years of
409 evolution (Fig 1; Supplementary Table S1). Overall, we detected contrasting patterns of SB
410 gene expression and turnover between the head and body (Table 1; Figs 1–3; Supplementary
411 Figs S3, S6–S8), as well as differences in expression level, tissue-specificity, and magnitude of
412 sex bias depending on the species, sex bias category, body part and/or sex (Supplementary
413 Figs S3–S5). Our findings that *i*) sex bias was less extensive (Table 1), *ii*) conserved sex bias
414 was rare while conserved UB expression was extensive (Fig 3A), and *iii*) the least amount of
415 expression divergence occurred in the head (Supplementary Tables 3–4) suggest that gene
416 expression is more constrained in the head than in the body. Indeed, sex bias in the head
417 was largely gained in a species-specific manner (Fig 1). Unlike in the body, where sex bias
418 sometimes switched between species, these gains almost exclusively occurred in genes that
419 were either UB or biased towards the same sex in the head of other species (99.88–100% of
420 examined genes; Fig 3A,B, Supplementary Figs S6–S7), suggesting that there is greater
421 constraint against sex bias reversals in the head throughout the *Drosophila* genus.
422 Moreover, SB genes in the head were usually also SB in the body irrespective of sex (Fig 3;
423 Supplementary Fig S2), suggesting that sex bias is more likely to arise in the head when the
424 underlying regulatory mechanisms for sex bias already exist for that gene. Taken together,
425 these findings suggest that the gain of sex bias is much more constrained in the head than in
426 the body. This pattern is likely driven, at least in part, by expression in the brain, where
427 expression is known to be more constrained than in other tissues (Khaitovich et al. 2005,
428 2006). In keeping with our findings, Khodursky et al. (2020) also found that expression was
429 constrained in the brain with SB expression being largely species-specific; however, they also
430 detected a much larger proportion of switching in sex bias in their dataset. This discrepancy
431 between studies is likely due to their more limited sampling within species, which included
432 either a limited number of isofemale lines (N = 2) or lines derived from a single population (N
433 = 6). On the other hand, our dataset included more lines (N = 5–8) per species, which were
434 derived from multiple, geographically diverse populations. Thus, our samples represented a
435 broader overview of within species variation and likely "averaged out" potential population-
436 or line-specific sex bias. Consistent with this interpretation, a recent study found that
437 somatic SB genes are more variable than UB genes among individuals within sub-species of

438 mice (Xie et al. 2025). This study also detected a larger proportion of sex bias reversals
439 among subspecies, but this discrepancy with our study might be due to their shorter
440 evolutionary timescale (Xi et al. 2025).

441 Interestingly, lineage-specific gains or losses of sex bias most often occurred via
442 concordant expression changes in both sexes, but with a greater change in one sex than the
443 other (75.6% in the head and 54.3% in the body; Fig 4, Supplementary Figs S9–S10), and this
444 pattern was even stronger for sex bias gains (Fig 4C,F) as well as genes with large expression
445 changes (95.2% in the head and 66.7% in the body; Supplementary Fig S8), the majority of
446 which occurred via a down-regulation of expression in both sexes. This concordance
447 suggests that these lineage-specific sex bias gains are not a way to resolve sexual
448 antagonism, as in such a case we would expect up-regulated expression in one sex and
449 down-regulated expression in the other (quadrants i and iv in Fig 4A,B,D,E). Such changes
450 represented the minority of SB gains in our data (24.4% in the head and 45.7% in the body),
451 especially in the head and among genes with large expression changes (4.8% in the head and
452 33.3% in the body). Thus, in general, the presence of SB expression is likely to be a poor
453 proxy for ongoing or resolved sexual antagonism in *Drosophila*. In keeping with our findings,
454 a recent study in *Dmel* found that sexually antagonistic effects on starvation resistance could
455 not be explained by SB gene expression (Chen et al. 2025). Moreover, another study of 5
456 mammalian species found that only 27% of lineage-specific sex bias changes could be
457 associated with turnover in SB transcription factor motifs (Naqvi et al. 2019), suggesting that
458 in such cases the gain of sex-specific regulation may be driving the gain or loss of sex bias.
459 Thus, while opposing sex-specific regulatory changes contribute to the gain or loss of
460 lineage-specific SB gene expression, our results suggest that the majority of lineage-specific
461 SB gains occur via concerted changes in expression with a larger magnitude of change in one
462 sex than the other, suggesting that the underlying regulatory architecture is largely shared
463 between the sexes. Indeed, a previous study in humans found that sex differences in the
464 genetic regulation of gene expression are not very common (Oliva et al. 2020), while another
465 study of the brain of three *melanogaster* group species found that male and female
466 expression were positively correlated relative to ancestral expression (Khodursky et al.
467 2020). We similarly detected a positive correlation in lineage-specific SB gains and losses, but
468 the strength of this correlation was stronger for SB gains than losses (Fig 4), underscoring
469 that many species-specific SB gains are likely the result of differences in regulation that is
470 shared between the sexes.

471 We also detected contrasting patterns in the chromosomal distribution of SB genes
472 between the head and body (Fig 5). In the body, we detected a consistent enrichment of FB
473 and a paucity of MB genes on the X chromosome (Fig 5B), which is consistent with the
474 "feminization" and concurrent "de-masculinization" of the X that has been reported in
475 previous studies (e.g. Parisi et al. 2003; Ranz et al. 2003; Sturgill et al. 2007). On the other
476 hand, in the head, we often detected an enrichment of SB genes (MB, FB, or both) on the X
477 chromosome (Fig 5A). Previous work in *melanogaster* group species found that genes with
478 SB expression, and particularly MB expression, in the brain were enriched on the X
479 chromosome (Catalán et al. 2012; Khodursky et al. 2020) and that this signal from the brain
480 may contribute to a detectable enrichment of X-linked SB genes in studies of whole heads
481 (Huylmans and Parsch 2015). Thus, expression in the brain likely contributed to the
482 enrichment of X-linked SB genes that we detected in the head (Fig 5A).
483 SB genes, especially MB genes, are often more tissue-specific than UB genes (Meisel
484 2011; Dean et al. 2016; Khodursky et al. 2020). In keeping with previous findings, we found
485 that tissue specificity was highest in MB genes (Supplementary Fig S5). Moreover, tissue
486 specificity was positively associated with the magnitude of sex bias, and this pattern was
487 strongest in MB genes, especially in the body (Fig 3D). Together, these findings suggest that
488 increased tissue specificity may facilitate larger changes in SB expression, especially for MB
489 genes, which is in keeping with the idea that increased tissue specificity may be a driving
490 force in the evolution of SB gene expression, and MB gene expression in particular (Miesel
491 2011; Dean et al. 2016). However, we should note that in this study we focused on
492 expression in body parts, which are a composite of multiple tissues. While the head is
493 composed of relatively few tissues, the body is composed of many, including reproductive
494 tissues; therefore, it is possible we may have missed some smaller or tissue-specific changes
495 in SB gene expression.
496 When we examined Δx as a measure of putative positive selection at the gene
497 expression level (where $-1 < \Delta x > 1$ is indicative of positive selection), we found that *i*) the
498 absolute value of Δx was most often shifted higher in males compared to females, with the
499 magnitude of male Δx usually close to or above 1 (Fig 6; Supplementary Table S8), *ii*) SB
500 genes were often enriched for signs of positive selection in the opposite sex (i.e. the more
501 lowly expressed sex), especially FB genes for male expression (Fig 6), and *iii*) Δx tended to
502 shift in the same direction in both sexes (Supplementary Table S8). At the same time,
503 changes in gene expression relative to ancestral expression during sex bias gains tended to

504 be positively correlated between the sexes (Supplementary Figs S9–S10), as did gene
505 expression between the sexes within body parts (Supplementary Tables S3–S4). Taken
506 together, these findings suggest that lineage-specific sex bias changes may often be
507 facilitated by selection acting on expression in the sex with lower expression (i.e. selection
508 may be occurring for lower expression, often resulting in a net gain of sex bias in the
509 opposite sex), especially for male expression of FB genes in the body. Indeed, we found that
510 lineage-specific sex bias changes were most often concordant between the sexes, especially
511 in the head, for SB gains, and for genes with large expression changes (Fig 4; Supplementary
512 Fig 8), the majority of which occurred via a down-regulation of expression in both sexes.
513 Thus, our results suggest that most lineage-specific sex bias gains may be the result of
514 selection acting on the asymmetrical down- (or up-) regulation of expression in both sexes
515 via shared regulatory architecture, and this mechanism may be particularly important when
516 expression is highly constrained, such as in the head or brain, or when there are large overall
517 expression changes. This finding is rather counterintuitive, as SB gene expression is known to
518 evolve rapidly (Assis et al. 2012; Whittle and Johannesson 2013; Harrison et al. 2015; Yang et
519 al. 2016; Tosto et al. 2023; Xie et al. 2025), and theory predicts that shared genetic
520 architecture should slow down the evolution of sexual dimorphism (Lande 1980) given the
521 constraints that the shared genome imposes on gene expression (Griffin et al. 2013;
522 Khodursky et al. 2020). However, a recent study using mouse and human data detected
523 overlapping variances of SB gene expression between the sexes in somatic tissues,
524 suggesting that patterns and evolutionary dynamics of SB genes are likely more nuanced
525 than previously thought. Therefore, in cases where sex bias is beneficial, but constraint on
526 gene expression is relatively high, working within the constraint of a shared genome, rather
527 than around it, may provide an alternative, more common route to the establishment of SB
528 gene expression.
529

530 ## Materials and Methods

531 ### *Drosophila* strains and RNA-seq

532 All *Drosophila* species were maintained at 21°C with a 14 h light:10 h dark cycle on
533 standard cornmeal–yeast–molasses medium. For all species, strains were established as
534 isofemale lines and maintained by transferring 20–50 flies per generation for at least 10
535 generations. For all of the *Dmel* and some of the *Dsim* strains, additional inbreeding was
536 done by performing single sib-pair crosses for 5–10 generations. Expression was examined in

537 5–8 strains of 6 *Drosophila* species (Fig 1). To provide a broad geographical overview of
538 expression within each species, the *Drosophila* strains were sampled from locations across
539 Europe, Asia, and Africa and were either collected by ourselves or kindly provided by others
540 (see Supplementary Table S1 for details on sampling locations and provenance). Briefly, we
541 included strains from at least two populations per species and included strains from the
542 inferred ancestral region for *Dmel*, *Dsim*, *Dana*, and *Dsuz*. For each strain, groups of 30–120
543 heads and 8–80 bodies of 3–6-day-old flies were dissected in cold 1X PBS and frozen in RNA-
544 DNA shield (Zymo research; Freiburg, Germany) at -70°C until RNA extraction. RNA was
545 extracted using a Qiagen RNeasy Mini or Plus Universal Kit (Qiagen; Hilden, Germany). Poly-
546 A selection, fragmentation, reverse transcription, library construction, and high- throughput
547 RNA-seq was performed by Novogene (Martinsried, Germany) using the Illumina NextSeq
548 550 platform (Illumina; San Diego, CA) with 150-bp paired-end reads. We sequenced one
549 biological replicate per strain, sex, and body part, resulting in 35 libraries for each sample
550 type (140 libraries in total). Library size ranged from 22,874,617 to 34,733,318 million
551 paired-end reads, 43.29–78.74% of which could be mapped (Supplementary Table S9; see
552 description of read mapping below).
553
554 **Read mapping and estimation of gene expression levels**
555 In order to prevent potential bias due to lower quality annotations in some species
556 (for example, no full transcript information was available for *Dimm*; whereas, in *Dmel* UTRs
557 are better annotated than in the other, less studied species), we mapped RNA-seq reads to
558 CDS sequences (excluding UTRs) for each species. Reference CDS sequences were
559 downloaded (April 25, 2024) from the following sources: NCBI (*Dsub* genome assembly
560 UCBerk_Dsub_1.0, *Dana* genome assembly ASM1763931v2, D*suz* Genome assembly
561 LBDM_Dsuz_2.1.pri, and *D simulans* genome assembly Prin_Dsim_3.1), FlyBase (version
562 6.57; Öztürk-Çolak et al. 2024; *Dmel*), and Li et al. (2022; *Dimm*). Links to the downloaded
563 references are provided in Supplementary Table S10.
564 RNA-seq reads were mapped to the respective species reference using NextGenMap
565 (Sedlazeck et al. 2013) in paired-end mode. Read pairs mapping to different genes were
566 discarded, while read pairs matching more than one isoform of a gene were randomly
567 assigned to one of the isoforms of that gene. For downstream analyses, we analyzed the
568 sum of read counts across all of a gene's isoforms (across all annotated exons), i.e. on the
569 individual gene-level. In order to make direct comparisons of gene expression among species

570 and sample types, we used our gene counts to calculate levels of gene expression as
571 transcripts per million (TPM; Wagner et al. 2013) for all genes in each library. For TPM
572 calculations, CDS lengths were extracted from the *Dmel* reference files or calculated from
573 the reference files for all other species using the faidx function in *samtools* (Danecek et al.
574 2021). In cases of multiple isoforms, the longest CDS length was used.
575
576 **Identification of 1:1 orthologs**
577 In order to keep the datasets for each species comparable, we restricted our analyses
578 to 1:1 orthologs present in all species. To identify 1:1 orthologs, we downloaded (April 29,
579 2024) pairwise lists of orthologs between *Dmel* and all other species from Li et al. (2022; for
580 *Dimm*) or DrosOMA (Thiébaut et al. 2024; for all other species; Supplementary Table S10), as
581 well as the full pairwise orthologs list from DrosOMA (Thiébaut et al. 2024; Supplementary
582 Table S10). First, all non-1:1 orthologs were removed from each *Dmel* ortholog list and the
583 FlyBase batch download and/or ID validator tools (Öztürk-Çolak et al. 2024) were used to
584 obtain FBgn numbers for *Dmel* IDs from each list. Non-gene or non-coding matches were
585 removed and in cases for which submitted IDs yielded multiple matches, only the FBgn
586 number that exactly corresponded the original ID was retained. For *Dmel* IDs with multiple
587 matches but no exact match or duplicate FBgn numbers due to ID changes/gene re-naming,
588 we used a combination of FlyBase (Öztürk-Çolak et al. 2024), DrosOMA (Thiébaut et al.
589 2024), and UniProt (Ahmad et al. 2025) to validate the IDs. IDs that did not match between
590 our reference files and our ortholog lists were validated in the same manner. The
591 orthologous *Dmel* sequences corresponding to IDs from the *Dimm* list (Li et al. 2022) that
592 could not be validated using this method were blasted (blastn; Altschul et al. 1990) to
593 identify the current *Dmel* gene, and their isoform was verified in DrosOMA. All IDs that could
594 not be verified were removed from our 1:1 ortholog list as were IDs that were no longer
595 classified as genes by NCBI, not included in our reference files, and IDs that had been
596 incorporated into a gene model already represented in our list (i.e. duplicate IDs). Next, we
597 used the full pairwise orthologs list from DrosOMA (Thiébaut et al. 2024; Supplementary
598 Table S10) to remove genes that were not 1:1 orthologs between the non-*melanogaster*
599 species. Orthologs that could not be confirmed as belonging to the same, known OMA group
600 were also removed as were orthologs belonging to the same OMA group (i.e. OMA groups
601 that appeared more than once in our list). After these filtering steps, we obtained a list of
602 9,016 1:1 orthologs for use in our expression analyses.

603

604 **Expression analyses**

605 We identified sex-biased genes (Supplementary Data S1) using gene counts in the
606 9,016 1:1 orthologs and a negative binomial test as implemented in DESeq2 (Love et al.
607 2014) in R (R core team 2022) with a 5% false discovery rate (FDR). Head and body samples
608 for each species were analyzed separately using a one-factor model design with two levels
609 corresponding to each sex. For PCAs within each species, we implemented a 2-factor (sex
610 and body part) model design with two levels for each factor (male versus female and head
611 versus body). For the PCA including all samples, we similarly implemented a 3-factor design
612 (sex, body part, and species), with the additional third factor consisting of 6 levels
613 corresponding to the 6 examined species. Gene expression divergence between 2 sample
614 types was calculated as 1 – Spearman's ρ between the mean TPM of the 2. Significant
615 differences in divergence or magnitude of sex bias between samples were assessed with a *t*-
616 test with a Benjamini-Hochberg correction (Benjamini and Hochberg 1995) for the number
617 of comparisons made per sample. We tested for an effect of sex bias category and species
618 on overall expression levels using the mean of the $\log_2$ TPM of each gene in each species and
619 body part and a type II ANOVA.

620 To identify lineage-specific gains or losses of sex bias, we employed a conservative
621 approach in which a terminal species (or all species of a terminal clade) was only considered
622 to have a change in sex bias if all species on that lineage shared the same sex bias, and this
623 sex bias differed from that of all other outgroup species, which were also all required to
624 share the same sex bias. Due to the lack of an outgroup, we could not polarize sex bias
625 changes as gains or losses on the lineage leading to *Dimm*; therefore, we categorized the
626 gain or loss of expression relative to the other analyzed species. In order to compare how
627 expression changed in a particular species relative to the inferred ancestral expression, we
628 calculated the ancestral expression for each gene, sex and body part for all internal nodes of
629 our phylogenetic tree using the mean TPM values in each species and a Brownian motion
630 model fitted by maximum likelihood as implemented in the ace function in the R package
631 *ape* (Paradis and Schliep 2019). Changes in expression within each sex, species, and body
632 part in comparison to the inferred ancestral expression were calculated as
633 $\log_2(TPM_{Focal}/TPM_{Anc})$, where TPM_{Focal} was the mean TPM in the focal species for a given
634 body part and sex and TPM_{Anc} was the inferred ancestral TPM at the nearest internal node.
635 We tested for differences in the distribution of the direction (up- or down-regulation) of

636 expression changes in males versus females using a χ^2 test for the number of genes in
637 quadrants i–iv (see Fig 4). We further categorized genes as having a large or small expression
638 change based on a $\log_2$ fold-change threshold of ≥ 2 in the body or ≥ 1 in the head, where
639 the extent (Table 1) and magnitude (Supplementary Fig S3) of sex bias was lower. To test for
640 significant correlations between male and female expression changes during changes in sex
641 bias, we calculated Spearman's ρ between expression changes in males versus female for all
642 genes that gained or lost sex bias within each species. We then tested for differences in
643 these correlations between MB or FB gains and SB losses using a Mann-Whitney *U* test or a
644 paired Mann-Whitney *U* test to test for differences between body parts.
645
646 **Chromosomal location and calculation of τ and Δx**
647 Chromosomal locations of all protein-coding genes for *Dsim*, *Dsuz*, *Dana*, and *Dsub*
648 were downloaded (July 11, 2025) from NCBI. For *Dsuz* the CBGP_Dsuzu_IsoJpt1.0 assembly,
649 which is chromosome-level, was used to determine the chromosomal location of analyzed
650 genes. *Dmel* chromosomal locations were extracted from the reference CDS file. Because a
651 chromosome-level assembly was not available for *Dimm*, this species was excluded from all
652 chromosomal location analyses. For all comparisons between X and autosomal genes, we
653 compared genes that could be assigned to the X chromosome (also known as Muller
654 element "A") to all genes that could be assigned to any autosome within each species.
655 Separately for the X and the autosomes, we tested for differences in the number of observed
656 genes in each sex bias category versus the expected number of genes in each category based
657 on the overall proportion of genes in each category for that species using a χ^2 test.
658 In order to examine tissue specificity, we downloaded the FlyAtlas2 FPKM data
659 (Krause et al 2022) for all adult *Dmel* tissues, excluding fat body, spermatheca, and heart,
660 which were sequenced using a different chemistry from the other tissues, from
661 https://motif.mvls.gla.ac.uk/downloads/FlyAtlas2_gene_data_2025.xlsx (April 24, 2025). We
662 then calculated the tissue-specificity index τ (Yanai et al 2005) for each gene (Supplementary
663 Data S1) and identified the tissue with the highest expression. We tested for an effect of sex
664 bias category and species on τ in each body part using a type II ANOVA. In order to test for
665 correlations between sex bias and tissue specificity, we calculated Spearman's ρ between τ
666 and the magnitude of sex bias for each sex bias category in each body part and species. We
667 tested for differences in these correlations among sex bias categories using a Mann-Whitney
668 *U* test.

In order to identify putative signs of positive selection at the gene expression level, for each species and body part we calculated Δx (Supplementary Data S1) similar to Moghadam et al. (2012) within each sex as

$$x = \frac{d}{fr}, (1)$$

where, *d* is the divergence in gene expression between a focal species and *Dimm*, *r* is the range of expression levels for the focal species, and *f* is a modifier correcting for differences in sample size among species. To calculate Δx, we used the $\log_2$ TPM as a measure of expression for each gene in each fly strain and calculated the divergence (*d*) in expression between the focal species expression (X_{Focal}) and *Dimm* expression (X_{Imm}) as

$$d = \frac{mean(X_{Focal}) - mean(X_{Imm})}{mean(X_{Focal})}, (2)$$

the expression range (*r*) as

$$r = \frac{max(X_{Focal}) - min(X_{Imm})}{mean(X_{Focal})}, (3)$$

and the correction factor (*f*) as

$$f = \sqrt{\frac{mN-1}{mN-m}}, (4)$$

where *m* is the number of strains for the focal species and *N* is the most common number of strains (for our data, *N* = 5). For all genes, and for the X chromosome and autosomes separately, we tested for differences in the number of observed putatively positively selected genes in each sex bias category and the number of putatively selected genes expected based on the proportion of genes in each sex bias category for each sex, species and body part using a χ^2 test. For each body part, species, and sex bias category, we tested for differences in the magnitude of Δx between males and females using a *t*-test. We also tested for correlations in Δx between the sexes for each body part, species, and sex bias category using Spearman's ρ. We tested for differences in the strengths of these correlations between the body and the head using a paired Mann-Whitney *U* test

Data Availability

The RNA-seq data that support the findings of this study are publicly available from the European Nucleotide Archive (ENA) at EMBL-EBI under Project accession number XXXXXXXXXXX. All other relevant data are within the manuscript and its Supplementary Material files.

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872 ## Supplementary Material Captions

873 **Supplementary Table S1:** Overview of *Drosophila* lines assayed in this study

874 **Supplementary Table S2:** Sex biased genes in *D. melanogaster* in a random sub-sampling of
875 5 lines

876 **Supplementary Table S3:** Expression divergence within species

877 **Supplementary Table S4:** Expression divergence among species
878 **Supplementary Table S5:** Number of sex-biased genes expressed highest in testis, ovary, or a
879 somatic tissue
880 **Supplementary Table S6:** Lineage-specific gains and losses of sex biased gene expression
881 **Supplementary Table S7:** Sex-biased genes on the X versus autosomes
882 **Supplementary Table S8:** Genes showing signs of putative positive selection at the
883 expression level
884 **Supplementary Table S9:** Library size and mapping efficiency
885 **Supplementary Table S10:** Download location of files used for analysis
886 **Supplementary Fig S1:** Principal component analysis of gene expression profiles in A) *Dmel*,
887 B) *Dsim*, C) *Dsuz*, D) *Dana*, E) *Dsub*, and F) *Dimm*. Triangles and diamonds indicate female
888 and male body, respectively, while circles and squares indicate female and male head,
889 respectively.
890 **Supplementary Fig S2:** Overlap in SB genes between head (H) and body (B). Shown are upset
891 plots demonstrating the overlap in UB, FB, or MB genes in A) *Dmel* (mel), B) *Dsim* (sim), C)
892 *Dsuz* (suz), D) *Dana* (ana), E) *Dsub* (sub), and F) *Dimm* (imm). Horizontal bars represent the
893 total number of genes in a body part and sex bias category combination. Vertical bars
894 represent the number of genes in an intersection class. Connected, filled circles underneath
895 a vertical bar indicate that a body part and sex bias category combination is included in an
896 intersection class. Blue represents genes UB in one body part and SB in the other, dark gray
897 represents gene in the same bias category, and light blue indicates genes that switch sex bias
898 between head and body.
899 **Supplementary Fig S3:** Magnitude of sex bias in the A) head and B) body of *D. melanogaster*
900 (mel), *Dsim* (sim), *Dsuz* (suz), *Dana* (ana), *Dsub* (sub), and *Dimm* (im/imm). Significant
901 differences between species for MB and FB genes were assessed with a *t*-test. Shown are the
902 BH-corrected *P*-values in the C) head and D) body. Non-significant *P*-values are shown in
903 bold.
904 **Supplementary Fig S4:** Overall expression levels in the A) head and B) body for male (M) and
905 female (F) biased and unbiased (U) genes in *D. melanogaster* (mel), *D. simulans* (sim), *D.*
906 *suzukii* (suz), *D. ananassae* (ana), *D. subobscura* (sub), and *D. immigrans* (imm). Significance
907 was assessed for each body part with a type II ANOVA with sex bias (SB) category and
908 species as factors.

Supplementary Fig S5: Tissue specificity τ in the A) head and B) body for MB (M), FB (F), and UB (U) genes in *Dmel*, *Dsim*, *Dsuz*, *Dana*, *Dsub*, and *Dimm*. Significance was assessed for each body part with a type II ANOVA with sex bias (SB) category and species as factors.

Supplementary Fig S6: Overlap in SB genes within the head (H) between species. Shown are upset plots demonstrating the overlap in UB, FB, or MB genes in pairwise comparisons between A–E) *Dmel* (mel), A, F–I) *Dsim* (sim), B,F,J–L) *Dsuz* (suz), C,G,J,M,N) *Dana* (ana), D,H,K,M,O) *Dsub* (sub), and E,I,L,N,O) *Dimm* (imm). Horizontal bars represent the total number of genes in a species and sex bias category combination. Vertical bars represent the number of genes in an intersection class. Connected, filled circles underneath a vertical bar indicate that a species and sex bias category combination is included in an intersection class. Blue represents genes UB in one species and SB in the other, dark gray represents genes in the same sex bias category, and light blue indicates genes that switch sex bias between species.

Supplementary Fig S7: Overlap in sex biased genes within the body (B) between species. Shown are upset plots demonstrating the overlap in UB, FB, or MB genes in pairwise comparisons between A–E) *Dmel* (mel), A, F–I) *Dsim* (sim), B,F,J–L) *D. suz* (suz), C,G,J,M,N) *Dana* (ana), D,H,K,M,O) *Dsub* (sub), and E,I,L,N,O) *Dimm* (imm). Horizontal bars represent the total number of genes in a species and sex bias category combination. Vertical bars represent the number of genes in an intersection class. Connected, filled circles underneath a vertical bar indicate that a species and sex bias category combination is included in an intersection class. Blue represents genes UB in one species and SB in the other, dark gray represents genes in the same sex bias category, and light blue indicates genes that switch sex bias between species.

Supplementary Fig S8: All SB gains and losses for genes with A,B) large and C,) small expression changes between the focal and ancestral species in the A,C) head and B,D) body. The cut-off for a large expression change was a $\log_2$ fold-change of magnitude 1 or higher in the head and 2 or higher in the body. Genes with concordant (con) changes in expression between the sexes are located in quadrants ii and iii; while genes with opposing (opp) expression changes between the sexes are located in quadrants i and iv.

Supplementary Fig S9: Expression changes during sex bias turnover in the head. Shown are scatterplots of expression in A–C) *Dmel*, D–F) *Dsim*, G–I) *Dsuz*, J–L) *Dana*, M–O) *Dsub*, and P–R) *Dimm* in each sex relative to ancestral (anc) expression for A,D,G,J,M,P) FB or B,E,H,K,N,O)

941 MB gains or C,F,I, L,O,R) losses. Spearman's ρ and the associated *P* value for each category
942 are shown.
943 **Supplementary Fig S10:** Expression changes during sex bias turnover in the body. Shown are
944 scatterplots of expression in A–C) *Dmel*, D–F) *Dsim*, G–I) *Dsuz*, J–L) *Dana*, M–O) *Dsub*, and P–
945 R) *Dimm* in each sex relative to ancestral (anc) expression for A,D,G,J,M,P) FB or B,E,H,K,N,O)
946 MB gains or C,F,I, L,O,R) losses. Spearman's ρ and the associated *P* value for each category
947 are shown.
948 **Supplementary Fig S11**: Δx in the A) head and B) body of 5 *Drosophila* species. Shown are
949 Δx values for FB, MB and UB genes in females (F) and males (M) of *Dmel*, *Dsim*, *Dsuz*, *Dana*,
950 and *Dsub*. The dashed gray line represents the cut-off above or below which a gene shows
951 signs of putative positive selection. For better visualization, Δx values above 5 or below -5
952 are not shown.
953 **Supplementary Fig S12:** Chromosomal distribution of signs of putative positive selection at
954 the gene expression level. Shown are the ratios of observed (obs) to expected (exp) number
955 of genes under putative positive selection ($-1 < \Delta x > 1$) in each sex bias category and sex on
956 the X chromosome and autosomes (auto) in the A) head and B) body. Significance was
957 assessed with a χ^2 test. ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$, marginally non-significant: .$P <$
958 0.1. Non-significant comparisons not shown.
959 **Supplementary Data S1:** Data underlying analyses.