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, 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. Altogether, our findings suggest that sex-biased expression changes are often facilitated by selection, including selection acting on the sex with lower expression, which is especially common for male expression of female-biased genes. 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

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

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

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

The characterization of expression variation both within and between species is important for inferring the type of selection that influences gene expression, as the 2 levels of variation are thought to represent, respectively, the short-term and long-term outcomes of the same underlying evolutionary process (Kimura 1983; Hudson et al. 1987; McDonald

86 and Kreitman 1991). For this reason, we measured gene expression in 2 body parts (head
87 and body) of adult males and females of 6 *Drosophila* species spanning divergence times of
88 up to 50 million years (Tamura et al. 2004). Within each species, we included 5–8 strains
89 derived from diverse geographic locations. These data allowed us to quantify the
90 conservation and turnover of SB gene expression across the genus in samples that included
91 or excluded the gonad, which is the tissue with the highest proportion of SB genes (Parisi et
92 al. 2004), and address 3 major questions: *i*) How much turnover is there in SB gene
93 expression across the *Drosophila* phylogeny? *ii*) How do genes gain SB expression? For
94 example, does expression increase/decrease in only 1 sex, or does it change in both sexes? If
95 it is the latter, does expression change in opposite directions in the 2 sexes, which is
96 expected if SB expression resolves sexual conflict, or are there concordant expression
97 changes in the 2 sexes, which would be indicative of shared regulatory constraints? *iii*) Do
98 lineage-specific expression changes show molecular signatures of positive selection? If so,
99 does selection occur predominantly in the sex that expression is biased towards? Answering
100 these questions will shed light on the evolutionary mechanisms responsible for the
101 maintenance and turnover of SB gene expression in the *Drosophila* genus.
102

103 **Results**

104 In order to characterize SB expression over a range of timescales, we performed
105 RNA-seq of male and female heads and bodies of 6 *Drosophila* species with divergence times
106 ranging between 2–50 million years (Fig 1), including *D. melanogaster* (*Dmel*), *D. simulans*
107 (*Dsim*), *D. suzukii* (*Dsuz*), *D. ananassae* (*Dana*), *D. subobscura* (*Dsub*), and *D. immigrans*
108 (*Dimm*). To get a comprehensive view of expression variation within each species, we
109 sampled 5–8 strains from a broad geographic distribution, encompassing strains from
110 multiple populations across 3 continents, including strains from the putative ancestral range
111 for *Dmel*, *Dsim*, *Dana*, and *Dsuz* (Table S1). We analyzed expression in 9,016 1:1 orthologs
112 present in all species (Thiébaut et al. 2024), 7,570–8,161 of which were expressed in the
113 head and 8,936–9,006 of which were expressed in the body of each species. To get an
114 overview of expression variation, we performed principal component analyses (PCAs) for all
115 samples, within each body part, or within each species. When considering the full dataset,
116 samples clustered strongly by body part (Fig 2A). In the head, the sexes showed only slight
117 separation (Fig 2B), while in the body, males and females were clearly differentiated (Fig 2A,
118 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 (Fig S1).

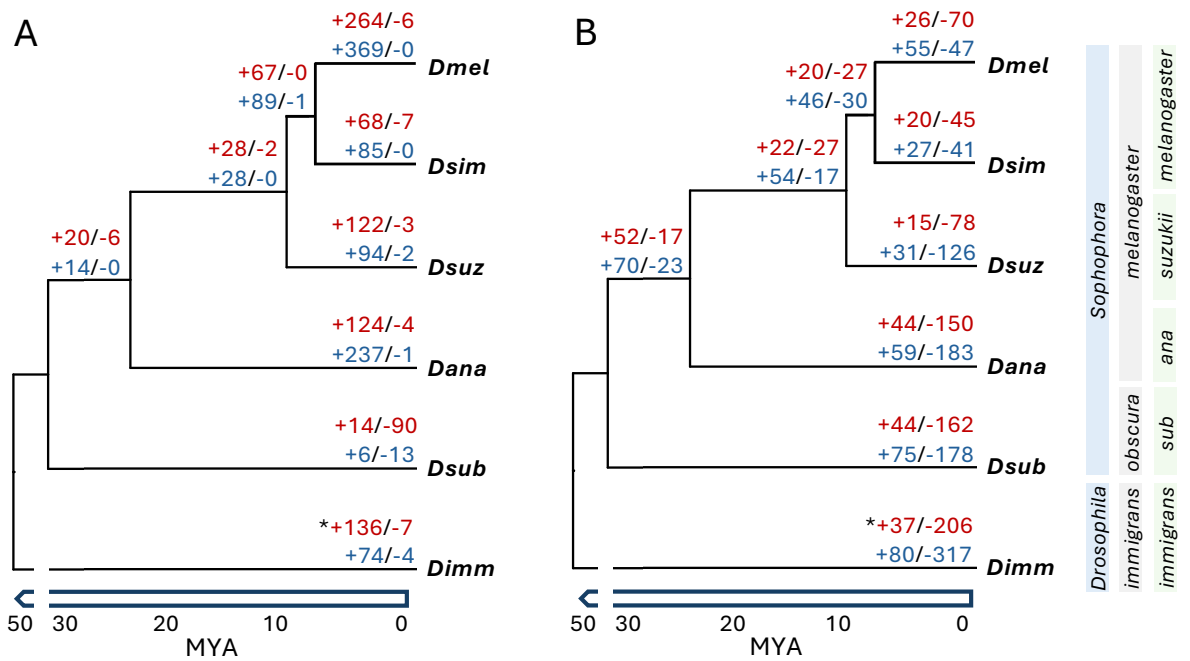


Fig 1: Phylogeny and SB expression turnover in the examined *Drosophila* species in the A) head and B) body. Branch lengths are shown only roughly to scale in millions of years ago (MYA). Numbers at each tip or node indicate sex bias gains (+) and losses (-) for female- (red) and male-biased (blue) genes. 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 is shown.

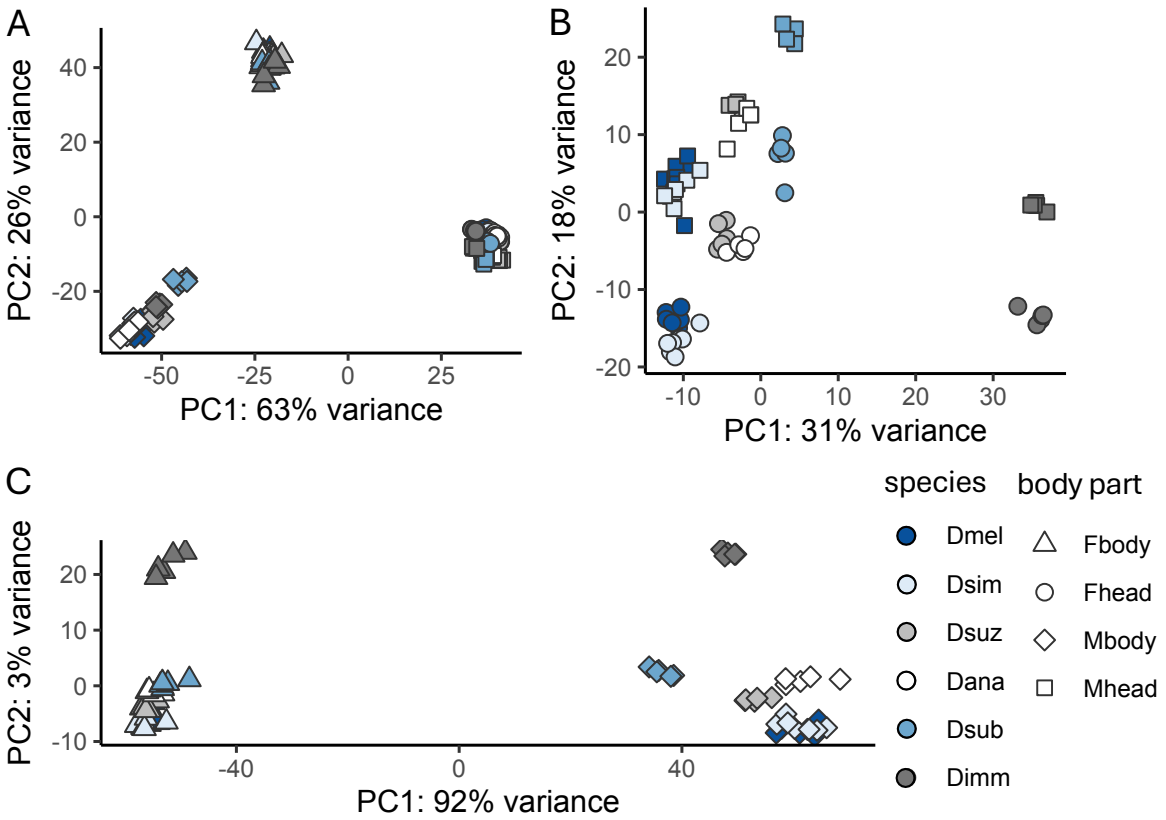


Fig 2: PCA of gene expression profiles in A) all samples, B) the head, and C) the body. The legend at the bottom right indicates the color associated with each species and the shape indicates the sex (male: M; female: F) and body part.

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

In order to compare overall prevalence, patterns, and degree of sex bias within and among the examined species, we used DESeq2 (Love et al. 2014; see Materials and Methods) to identify male-biased (MB), female-biased (FB), and unbiased (UB) genes within each species. SB gene expression was extensive in the body, with 71.9–81.6% of genes showing sex bias, but less common in the head, with 2.0–21.7% of genes showing sex bias (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 rather than any biological differences between species, as down-sampling resulted in similar numbers of SB genes as detected in the other species (Table S2). Because SB gene expression is often tissue-specific (Oliva et al. 2020), we compared sex bias between body parts. Due to the large amount of sex bias in the body, the majority of genes that could be examined in both

153 body parts (64.2–73.9%) were SB in the body but not the head (Fig 3C, Fig S2). The next
154 largest category of overlap was genes UB in both the body and head (14.1–25.2%), with
155 genes showing the same sex bias in both body parts being the third largest category (0.9–
156 13.0%). Interestingly, genes with opposite sex bias in the head and body were more common
157 than genes SB in the head but not the body (0.7–5.6% versus 0.4–3.1%, respectively). Thus,
158 genes SB in the head were 3–6 times more likely to also be SB in the body, and this sex bias
159 was often, but not always, in the same direction (Fig 3C, Fig S2).
160
161 **Table 1:** Sex bias in the head and body of 6 *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)

162 The number of analyzed genes after removal of outliers and genes with low counts (see
163 Materials and Methods), MB, FB, and UB genes in the head and body of each species are
164 shown. Percentages of analyzed genes rounded to the nearest decimal are shown in
165 parentheses.
166

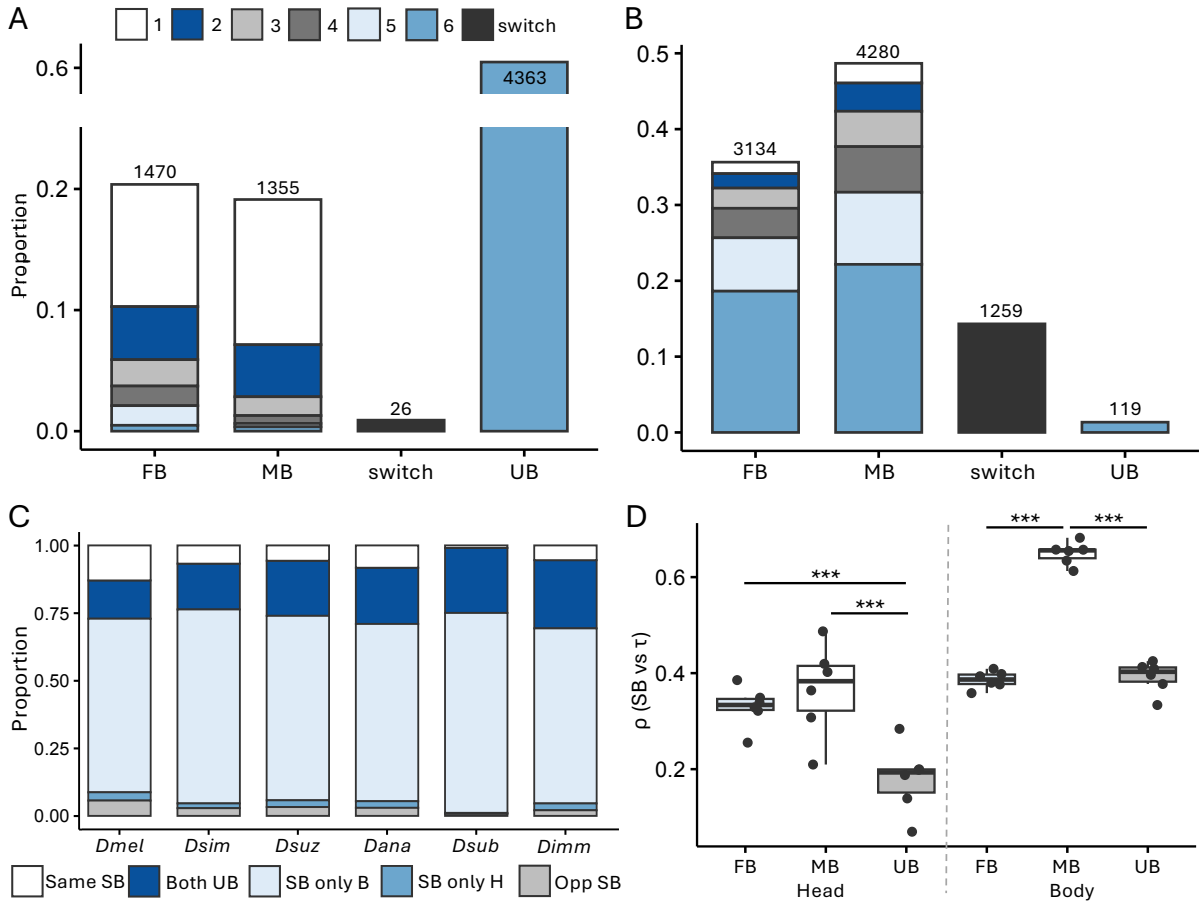


Fig 3: Sex bias within and among species. A,B) Proportion of genes with overlapping SB or UB expression among species in the A) head and B) body. Shown are the proportion of overlapping genes in the same SB category in 1 (white), 2 (dark blue), 3 (light grey), 4 (dark grey), 5 (light blue), and all 6 (medium blue) examined species. Proportion of analyzed genes that switched SB (FB to MB or vice versa) among any of the examined species is shown in black. The number of total genes in each category are shown above the bar. C) Proportion of overlapping genes that had shared SB (same SB; white) or UB (both UB; dark blue) expression, were SB in only the body (SB only B; light blue) or head (SB only H; medium blue), or opposite sex bias between the head and body (opp SB; light grey) are shown. D) Spearman's ρ correlations between τ and magnitude of sex bias in the head and body for MB, FB, and UB genes. Significance was assessed with a Mann-Whitney *U* test. ***$P < 0.005$. Non-significant comparisons not shown.

To determine if the magnitude of sex bias differed among body parts, the sex in which sex bias was detected, or species, we compared the degree of sex bias (i.e. the absolute value of our sex bias estimates) among categories. The magnitude of sex bias was significantly higher in the body than the head for all species for both MB and FB genes (*t*-

185 test; BH corrected $P < 1.5 \times 10^{-15}$ for all; Fig S3). In the head, the degree of sex bias was
186 higher for FB than for MB genes, while in the body, the opposite pattern was detected,
187 which was significant for all comparisons in all species (*t*-test; BH corrected $P < 4.5 \times 10^{-7}$ for
188 all; Fig S3), except *Dana* and *Dsub* heads (BH corrected $P = 1$). We also detected significant
189 differences in the degree of sex bias within the same body part and sex bias category (MB or
190 FB) among species, especially in the head, for many but not all comparisons (Fig S3). To
191 determine how these differences in the magnitude of sex bias relate to differences in overall
192 expression, we calculated the overall expression divergence (as measured by $1 - \rho$) between
193 body parts and species. Divergence between sexes was lowest in the head within species
194 (0.8–1.3%; Table S3), while divergence in the body was fairly similar to divergence between
195 body parts regardless of sex (27.7–36.5% versus 25.2–43.6%%; Tables S3–S4). Divergence
196 between species within the same body part and sex was higher than in the head but lower
197 than in the body between sexes within species (1.8–12.0%; Tables S3–S4). Thus, we detected
198 differences in the magnitude of expression bias based on the type of sex bias, the body part,
199 and the species, but overall expression levels showed the strongest correlation (between
200 sexes in the head) and weakest correlation (between the examined body parts in either sex)
201 within species.
202
203 **Tissue specificity and expression level are associated with differences in sex bias**
204 Previous studies have shown that SB genes often show a high degree of tissue
205 specificity (Meisel 2011; Dean et al. 2016; Khodursky et al. 2020). We therefore used the
206 adult *Dmel* tissue expression data in FlyAtlas2 (Krause et al. 2022) to examine expression of
207 our SB genes in individual tissues (Table S5). As might be expected for SB genes, MB genes in
208 the head or body were often most highly expressed in testes or a somatic tissue, while FB
209 genes were most highly expressed in the ovary or a somatic tissue. To better understand the
210 relationship between expression level, breadth, and sex bias in our dataset, we first
211 examined the overall expression level of SB and UB genes in the head and body of each
212 species. We detected significant effects of both species and sex bias category on the
213 expression level in both body parts (ANOVA; $P < 1.5 \times 10^{-6}$ for all; Fig S4). In both the head
214 and body, FB genes were the most highly expressed, followed by MB genes, and then UB
215 genes, except in *Dsub* heads, where MB genes were the most lowly expressed (Fig S4). Thus,
216 differences in sex bias were associated with differences in overall gene expression levels.

217 Next, to better understand the association between sex bias in our dataset and
218 tissue-specificity, we used the FlyAtlas2 adult tissue expression data (Krause et al. 2022) to
219 calculate the tissue specificity index, τ, which ranges from 0 to 1 with values closer to 1
220 indicating higher tissue specificity, for each gene. To determine if genes we identified as SB
221 were more tissue-specific than UB genes as previous studies have found (Meisel 2011; Dean
222 et al. 2016; Khodursky et al. 2020), we compared tissue specificity among SB and UB genes in
223 each species and body part. We detected a significant effect of sex bias category in both
224 body parts (ANOVA; $P < 1.6 \times 10^{-10}$ for all; Fig S5), suggesting that genes in different sex bias
225 categories display different levels of tissue specificity. Tissue specificity was highest for MB
226 genes in both the head and body for all species (Fig S5). In the head, τ was mostly similar
227 between FB and UB genes, while in the body, τ was lower in FB than in UB genes (Fig S5).
228 Next, we examined if the magnitude of sex bias was associated with the degree of tissue
229 specificity as well as the sex bias category. The magnitude of sex bias was significantly
230 positively correlated with τ in both body parts in all sex bias categories and all examined
231 species (Spearman's ρ; $P < 0.003$ for all) except MB genes in the head of *Dsub* ($P = 0.2483$).
232 Across all species, this correlation between the degree of sex bias and τ was highest for MB
233 genes in both body parts (Fig 3D), which was significantly higher than UB genes in both body
234 parts and FB genes in the body (Mann-Whitney *U* test; $P < 0.005$ for all), while the
235 correlation for FB genes was only significantly higher than UB genes in the head ($P < 0.005$;
236 Fig 3). Thus, tissue specificity was positively associated with sex bias, with the strongest
237 correlation in MB genes, which were also the most tissue-specific. This correlation was
238 particularly strong in the body, which might be driven at least in part by the presence of the
239 testes, which is the tissue with the highest proportion of MB genes (Parisi et al. 2004).
240

241 **Patterns of sex bias turnover among species differ between head and body**

242 To better understand how sex bias is conserved among species, we identified genes that
243 were SB or UB in all 6 species. In the head, we identified 4,363 (53.5–57.6%) genes with
244 conserved UB expression in all species, but only 1 (< 0.02%) and 35 (< 0.5%) genes as having
245 conserved MB or FB expression, respectively. On the other hand, we identified only 119
246 (1.3%) genes with UB expression in all species in the body, but 1,638 (18.2–18.3%) and 1,949
247 (21.6–21.8%) genes with conserved FB and MB expression, respectively. Next, to understand
248 how turnover in sex bias occurs among species, we employed a conservative approach (see
249 Materials and Methods) to identify lineage-specific gains, losses, or switching of FB or MB

250 expression that occurred on only 1 branch of the phylogeny (i.e. changes that occurred only
251 at 1 internal or external node of the phylogeny; Fig 1, Table S6). In the head, we detected
252 many more sex bias gains than losses for all species except *Dsub* (Fig 1A; 1,629 total lineage-
253 specific gains versus 135 losses, 103 of which were in *Dsub*); while, in the body we detected
254 more sex bias losses than gains (1,227 losses versus 663 gains; Fig 1B), although the pattern
255 was not as extreme as in the head. Across the entire phylogeny, only 171 genes switched sex
256 bias on a specific lineage, all of which occurred in the body and the majority of which (64.3%)
257 switched from FB to MB (Table S6).
258 To better understand how the sex bias of a gene differs among species across the entire
259 phylogeny, we examined the of genes analyzed in all species that were SB or UB in 1–6 of the
260 examined species. In the head, genes that were SB in 1 species were often UB in the other
261 species (10.1–12.0% of all analyzed genes; Fig 3A), but if a gene was SB in more than 1
262 species, it almost always shared the same sex bias between species (0.01–12.0% of genes
263 depending on the number of species in which a gene was detected as SB, 6.8–10.3% in total;
264 Fig 3A). On the other hand, only 26 genes switched their sex bias (i.e. from MB to FB or vice
265 versa) between species (0.4% of all analyzed genes; Fig 3A). In the body, SB genes were often
266 shared between species, with the majority showing sex bias towards the same sex in at least
267 2 species (1.5–22.2% of genes depending on the number of species in which a gene was
268 detected as SB, 34.1–46.1% in total; Fig 3B). A larger proportion of genes switched sex bias in
269 the body (14.3% of all analyzed genes; Fig 3B) compared to the head. To determine how
270 widespread this switching was among individual species, we performed pairwise
271 comparisons of sex bias in all examined genes between species for each body part (Figs S7,
272 S8). A small proportion (1.4–4.5%; Fig S8) of analyzed genes switched SB, suggesting that
273 relatively few genes switch sex bias among the individual species, but this number adds up
274 across the phylogeny. However, we should note that because the body is a composite of
275 multiple tissues, it is possible that some of these switches represent opposing changes in
276 different tissues rather than a switch in the same tissue. Overall, we detected a relatively
277 large amount of sex bias turnover in both body parts among species, which occurred in a
278 mostly species-specific manner in the head (Figs 1, 3). Moreover, when turnover occurred in
279 the head, a gene's expression most often switched between being UB and biased towards a
280 single sex, but rarely between the 2 sexes.
281

Sex bias is most often gained or lost via correlated expression changes in both sexes, especially in the head

In order to better understand how expression changes during the gain or loss of sex bias as well as how the magnitude of an expression change affects these patterns, we examined how expression in a focal species changed relative to the inferred ancestral expression within each sex (see Materials and Methods) for species-specific gains or losses of SB expression (i.e. how do male and female expression levels change when sex bias is gained or lost). In the head, the majority of expression changes occurred in the same direction (either up-regulation or down-regulation) in both sexes (75.6% of all sex bias gains and losses; Fig 4), and this pattern was even stronger for genes with large expression changes ($\log_2$ fold-change ≥ 1), 95.2% of which had concordant expression changes between the sexes (versus 74.3% of genes with small expression changes; Fig S8). Although concordant expression changes were also more frequent than opposing changes in the body (54.3% versus 45.7%), the direction of expression changes in males versus females was more evenly distributed compared to the head ($P < 2.2 \times 10^{-16}$; χ^2 test; Fig 4). Genes with large expression changes ($\log_2$ fold-change ≥ 2) were more similar to patterns in the head, with 66.7% of genes showing concordant expression changes between sexes (versus 53.3% of genes with small expression changes; Fig S8). If gene expression changes are concordant, we would expect there to be a positive correlation between the sexes. Consistant with this expectation, expression changes associated with SB gains were significantly positively correlated in the head and body in all species (Spearman's ρ; $P < 0.05$ for all) except for MB gains in the *Dsub* head and FB gains in the *Dmel* and *Dsim* body (Figs S9–S10). This correlation in gene expression changes between the sexes was significantly higher in the head than in the body (paired Mann-Whitney *U* test; $P = 0.0003$). However, this pattern was less apparent for SB losses, for which this correlation was sometimes negative and only significant in half of the comparisons (Figs S9–S10), although in the head this might be at least partially explained by the low number of SB losses in each species. Indeed, correlations in gene expression changes between the sexes were significantly higher for SB gains than for losses in both the head and the body ($P < 0.05$ for all; Fig 4).

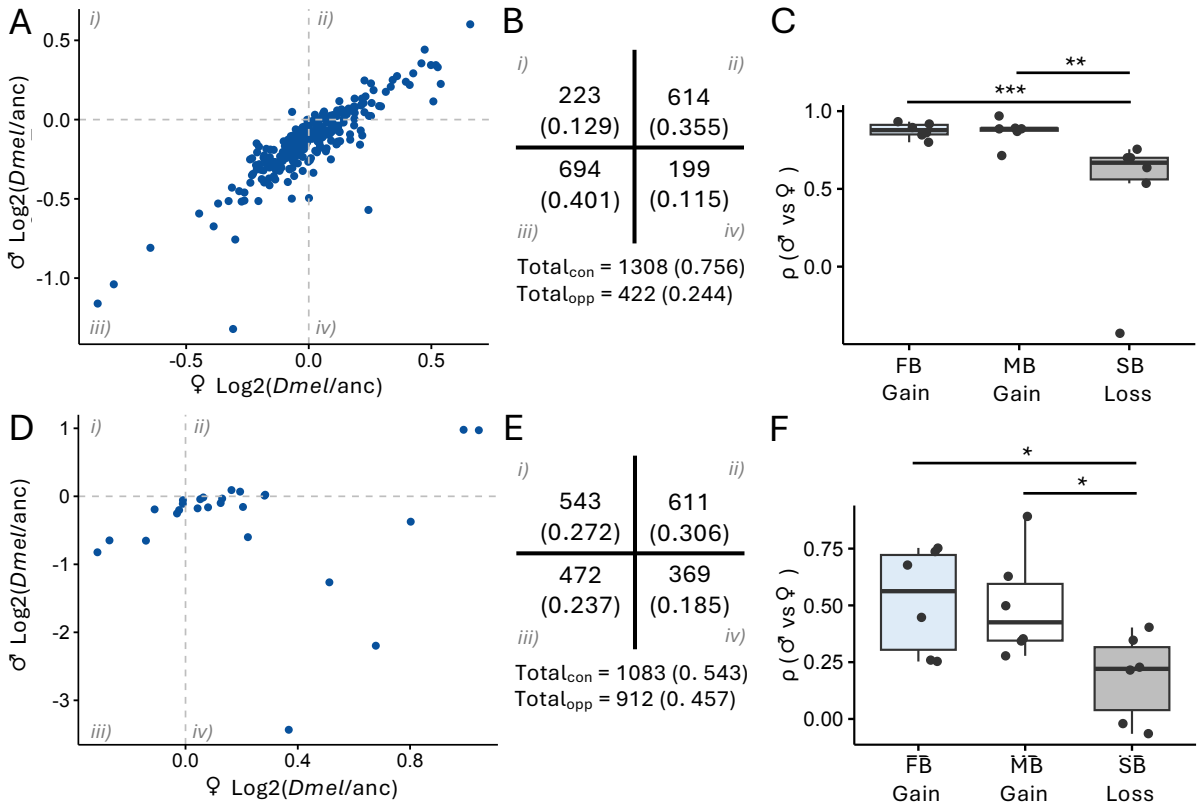
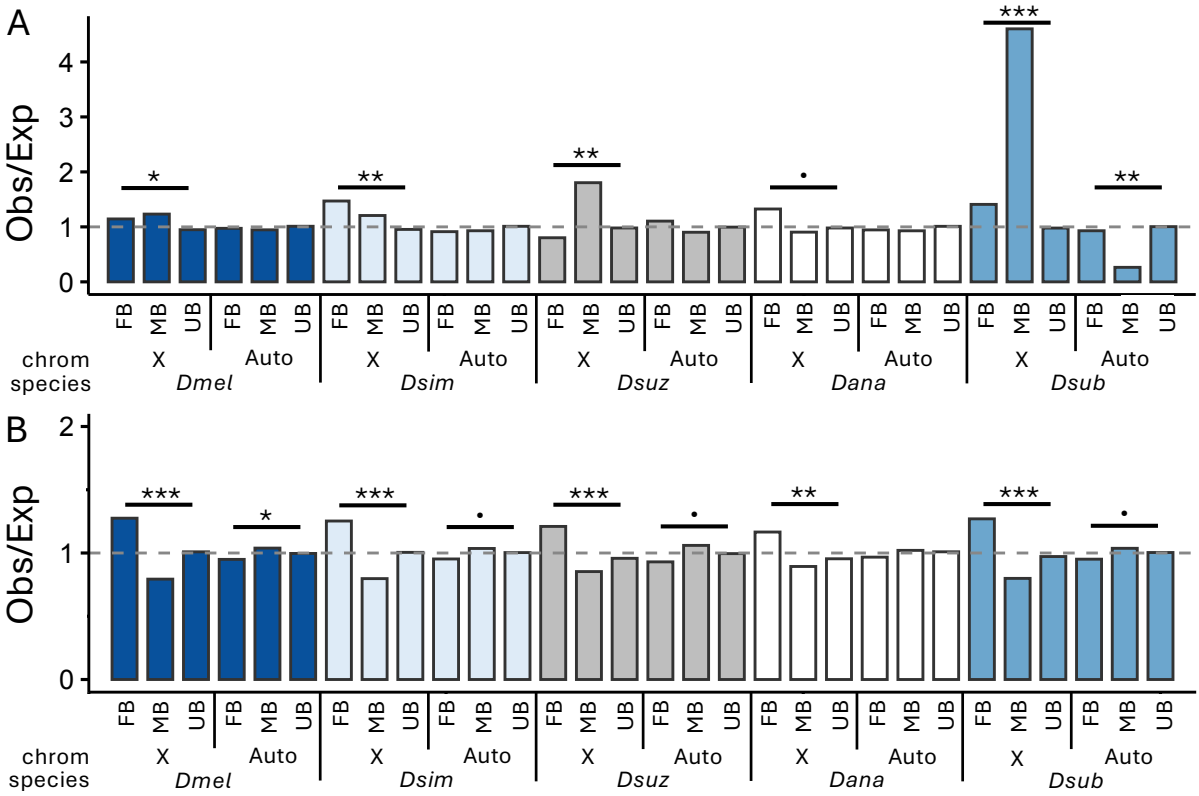


Fig 4: Expression changes underlying species-specific gains and losses of SB gene expression. As an example, scatterplots depicting the change between *Dmel* and ancestral expression in males versus females for genes with FB gains in the A) head and D) body are shown. All other scatterplots can be found in 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 affect the distribution of expression changes during SB loss or gain. In other words, are large expression changes more likely to be driven by up- or down-regulation of gene expression and how does ancestral sex bias affect these patterns? 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 (Fig S8). In contrast, up- or down-regulation in both sexes was

332 more evenly distributed for genes with small expression changes (32.3–36.5% and 21.0–
333 37.8% of genes for head and body, respectively; Fig S8). Due to the lack of SB reversals and
334 the low number of SB losses for most species in the head, it was difficult to determine if the
335 ancestral sex bias of a gene affected the distribution of expression changes, although MB
336 and FB losses tended to overlap with no clear separation based on the ancestral sex bias (Fig
337 S9). We observed a similar pattern for MB and FB gains in the body, with no clear clustering
338 based on whether sex bias was gained from an UB or SB gene (Fig S10). On the other hand,
339 FB and MB losses in the body were clearly separated (Fig S10), with genes that lost female
340 bias tending to show down-regulation in females and/or up-regulation in males and the
341 opposite for MB losses. Thus, a gain or loss of sex bias most often involved an expression
342 change in the same direction (up-regulation or down-regulation) in both sexes, but of
343 greater magnitude in 1 sex than the other, with larger expression changes tending to be
344 associated with down-regulated expression in both sexes. Overall, reversal-of-expression
345 changes were less common but were almost as frequent as concordant expression changes
346 in the body.
347
348 **Contrasting distributions of X versus autosomal SB genes in the head and body**
349 In male heterogametic species, such as *Drosophila*, females have 2 copies of the X
350 chromosome while males have only 1, which can lead to distinctive patterns of gene
351 expression and content on the X chromosome versus the autosomes. Indeed, previous
352 studies found that, in whole flies and gonads, FB genes were overrepresented on the X
353 chromosome, while MB genes were underrepresented (Parisi et al. 2003; Ranz et al. 2003;
354 Sturgill et al. 2007). In contrast, in the head and brain, genes with SB expression, and
355 especially MB expression, were enriched on the X chromosome (Catalán et al. 2012;
356 Huylmans and Parsch 2015; Khodursky et al. 2020). We detected similar patterns across the
357 species included in our study, with the proportion of X-linked genes depending upon the
358 body part and, in some cases, the species in which SB gene expression was detected (Fig 5).
359 In the head, SB genes were generally over-represented on the X chromosome, but whether
360 this enrichment occurred for MB or FB genes depended upon the species, and in some cases
361 (e.g. FB genes in *Dsuz* or MB genes in *Dana*) there was a slight underrepresentation of SB
362 genes on the X (Fig 5A). Interestingly, in *Dsub* MB genes were highly enriched on the X
363 chromosome but highly underrepresented on the autosomes (Fig 5A). In the body, FB genes
364 were enriched, while MB genes were underrepresented on the X chromosome for all species

365 (Fig 5B). Reciprocally, MB genes were enriched while FB genes were underrepresented on
366 the autosomes for all species except *Dana* (Fig 5B). Thus, SB genes displayed unique
367 chromosomal distributions depending on the examined body part.



368

369 **Fig 5:** Chromosomal distribution of SB genes in the A) head and B) body. Shown are the
370 ratios of observed (obs) to expected (exp) number of genes in each SB category on the X
371 versus the autosomes based on sex bias category and the number of analyzed on the X
372 versus the autosomes. Significance was assessed with a χ^2 test. ***$P < 0.005$, **$P < 0.01$, *P
373 < 0.05, marginally non-significant: .$P < 0.1$. Non-significant comparisons not shown.

374

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

376 A previous study in the brain of 3 *melanogaster* subgroup species found that SB
377 genes, and MB genes in particular, are enriched for signatures of directional selection
378 (Khodursky et al. 2020). In order to test for signs of putative positive selection at the gene
379 expression level in our dataset, we calculated Δx (see Materials and Methods) in both sexes
380 for all species relative to the outgroup species *Dimm*. Briefly, Δx is a measure of the ratio of
381 expression divergence to polymorphism within a species. An absolute Δx value greater than
382 1 indicates that divergence of expression in the focal species from *Dimm* exceeds expression
383 polymorphism within that species, indicating that positive selection may have occurred, with
384 Δx < -1 representing a down-regulation in the focal species and Δx > 1 indicating an up-

385 regulation. This measure has previously been applied to detect directional selection at the
386 gene expression level in multiple diverse taxa (e.g. Moghadam et al. 2012; Zemp et al. 2016;
387 Lichilín et al. 2021; Cossard et al. 2022).
388 We detected putative signs of positive selection (Δx > 1 or < -1) in 6.3–76.5% of genes
389 depending upon the sex, species, and sex bias category (Table S7; Fig S11), with the
390 proportion of putatively positively selected genes higher for both FB and MB than for UB
391 genes for all comparisons. Indeed, we detected significantly more FB and/or MB genes as
392 putatively positively selected than expected by chance in both sexes in the head for all
393 species (Fig 6A; χ^2 test; $P < 0.05$), except in *Dana* females and *Dsub* males, which were
394 marginally non-significant ($P < 0.09$). Putatively positively selected MB or FB genes were
395 similarly enriched to a lesser extent in 1 or both sexes in all species in the body, although we
396 detected fewer FB genes as putatively positively selected than expected in *Dsuz* females (Fig
397 6B). This enrichment of putatively positively selected MB and/or FB genes occurred on both
398 the X and the autosomes, although in some cases we also detected fewer SB genes than
399 expected both on the X and the autosomes, especially in the body (Fig S12). Overall, this
400 slightly higher enrichment of selected SB genes in the head suggests that although we
401 detected less SB expression in head, the genes that did show SB expression were more likely
402 to experience positive selection. Interestingly, SB genes were often enriched for putative
403 positive selection in the sex in which they were detected as SB (Fig 6A, B), but not always.
404 Unexpectedly, SB genes with signs of positive selection were often also (and sometimes
405 only) enriched in the opposite sex (i.e. the sex with lower expression; Table S8). Indeed, they
406 were enriched in at least 1 sex in all species in both the head and body, although this
407 enrichment was marginally non-significant in females in the *Dsub* body (Fig 6A, B, Table S8).
408 To assess differences in selection between the sexes, we compared Δx between males and
409 females. We detected significant differences in the magnitude of Δx for the majority of sex
410 bias categories and species in both head and body (80% for both; Fig 6C, D), with the
411 absolute value of Δx most often shifted higher in males, especially in the body (75 and 92%
412 of significant shifts in the head and body, respectively), and the mean absolute value in the
413 significantly higher sex usually close to or above 1 (88% of significant shifts; Fig 6C, D; Fig
414 S11). Despite this shift in magnitude, Δx was significantly positively correlated between the
415 sexes in all sex bias categories and species (Spearman's ρ; $P < 2.2 \times 10^{-16}$ for all; Table S8),
416 except *Dsub* MB genes in the head ($P = 0.153$), suggesting selection in the 2 sexes is
417 positively associated and, in general, selection drives gene expression in the same direction

in both sexes. Moreover, these positive correlations were significantly stronger in the body than in the head (mean of 0.827 versus 0.585 in the body and head, respectively; paired Mann-Whitney U test; $P = 5.37 \times 10^{-3}$). Thus, we detected an enrichment of putatively positively selected genes among SB genes in both the head and body, and this enrichment often occurred in either both sexes or only the sex with lower expression, mainly involving the male expression of FB genes. Moreover, the overall magnitude of Δx was significantly higher in males in comparison to females, especially in the body.

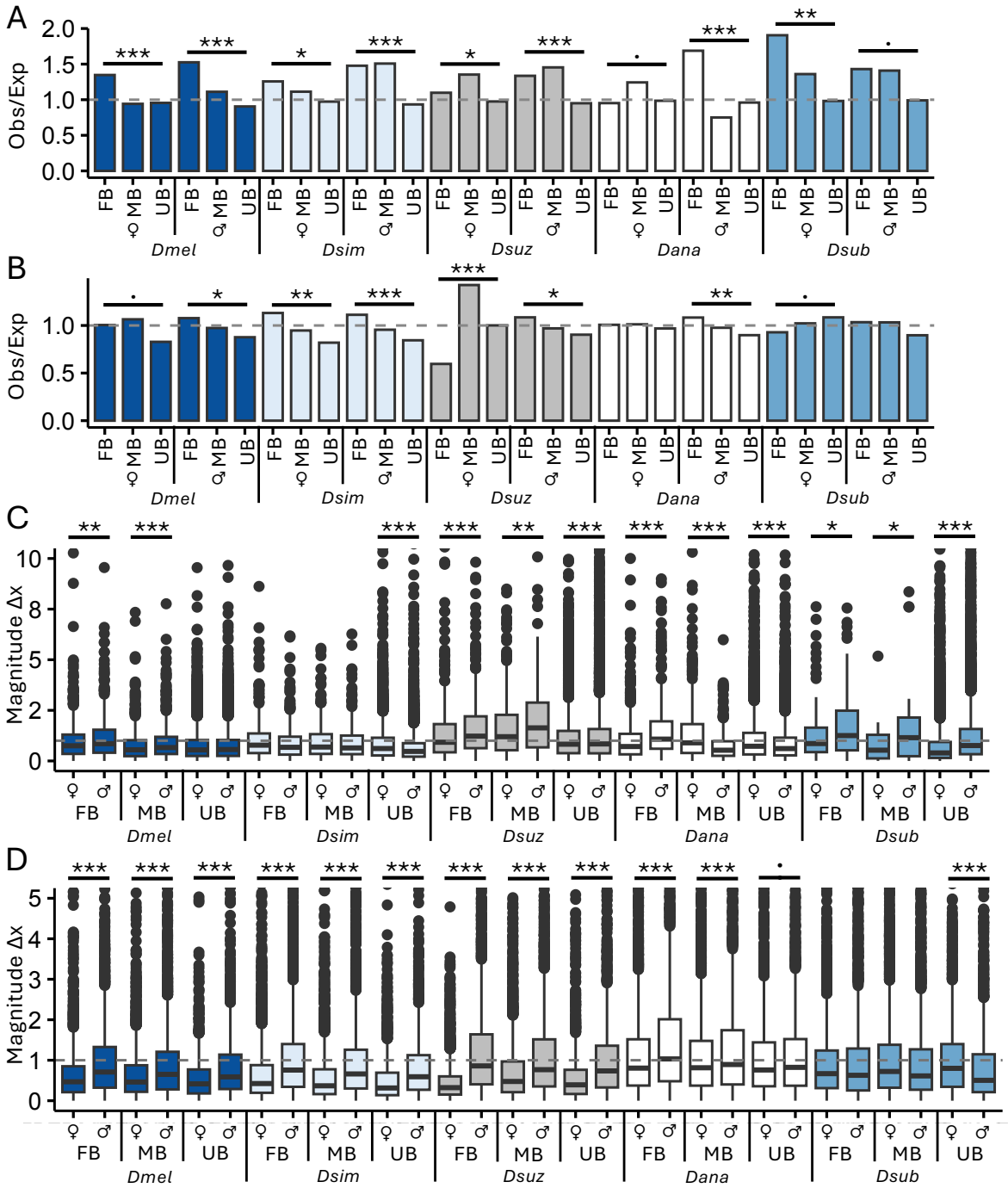


Fig 6: Signs of putative positive selection at the gene expression level. Shown are the ratios of observed (obs) to expected (exp) number of genes under putative positive selection (Δx >

428 1 or < -1) in each SB category and sex in the A) head and B) body. C, D) Shown is the
429 magnitude of Δx values for FB, MB and UB genes in females and males in the C) head and D)
430 body. The dashed gray line represents the cut-off above which a gene shows signs of
431 putative positive selection (absolute value of Δx > 1). For better visualization, absolute Δx
432 values above 5 are not shown. A, B) Significance was assessed with a χ^2 test. C, D) Significant
433 differences between sexes were assessed with a *t*-test. ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$,
434 marginally non-significant: .$P < 0.1$. Non-significant comparisons not shown.
435
436 Next, we examined if signs of putative positive selection could be associated with
437 differences in tissue specificity. We found that increased tissue specificity was associated
438 with an increased magnitude of sex bias, especially in MB genes (Fig 3D). However, a
439 previous study in the brains of 3 *melanogaster* group species found that expression breadth
440 did not selectively constrain the evolution of SB gene expression (Khodursky et al. 2020). To
441 determine if we could detect an association between tissue specificity and evidence of
442 putative positive selection, we compared τ between selected (Δx > 1 or < -1) and non-
443 selected genes in each sex among SB and UB genes in each species and body part. We
444 detected a significant effect of selection regime on tissue specificity (ANOVA, $P < 0.01$ for all;
445 Fig S13). However, in the body, there were no clear overall patterns in the association of
446 tissue specificity and selection regime, except that tissue specificity tended to differ between
447 selected and non-selected genes. In the head, SB genes with signs of putative positive
448 selection tended to show broader expression (i.e. lower τ values), suggesting that the action
449 of selection on more tissue-specific genes may be more constrained than in the body. Thus,
450 although increased tissue specificity was associated with a higher degree of sex bias (Fig 3D),
451 broader expression did not generally preclude the action of positive selection on a SB gene,
452 and was even associated with positive selection in the head (Fig S13).
453 In order to better understand how turnover in SB expression may be influenced by
454 selection, we examined Δx in genes with lineage-specific gains or losses of sex bias. We
455 detected many of the genes that gained or lost sex bias as under selection for up- or down-
456 regulation, with the exception of SB losses in the head, of which there were few (Table S9).
457 Similar to the results of the full dataset (Fig 6), we often detected similar or higher
458 proportions of genes as under selection in the more lowly expressed sex as in the more
459 highly expressed sex or both sexes (Table S9). However, when we examined if we detected
460 more (or fewer) genes under selection than expected by chance, we only sometimes

461 detected a significant excess of selected genes, which was especially common for male
462 expression during FB gains in the head (χ^2 test; Table S9). Because we could only include a
463 limited number of genes in this analysis (0–368, mean: 36.9), this general lack of significance
464 is likely at least partially due to a lack of power. Indeed, we could not assess significance for
465 25–36 of 56 lineage-specific gains/losses due to a lack of power (Tables S9). Because we
466 found that SB expression was often gained via concordant down-regulation in both sexes
467 (Figs 4 and S8), we next examined how often the detected positive selection occurred for a
468 down-regulation of expression in the focal species. We often detected a higher proportion of
469 genes with negative Δx values in 1 or both sexes, but especially in the more lowly expressed
470 sex and especially for male expression of FB genes (Table S9). This finding suggests that
471 selection for sex bias changes may occur fairly frequently for down-regulation, especially in
472 the sex with lower expression. In general, we detected genes under selection for up- or
473 down-regulation in both sexes, suggesting that at least some of the lineage-specific gains
474 and losses that we detected were the result of selection.
475

476 **Discussion**

477 We characterized SB gene expression in the head and body of 6 *Drosophila* species,
478 encompassing a broad geographic distribution within species and 2–50 million years of
479 evolution (Fig 1, Table S1). Overall, we detected contrasting patterns of SB gene expression
480 and turnover between the head and body (Table 1, Figs 1–3, S3, S6–S8), as well as
481 differences in expression level, tissue-specificity, and magnitude of sex bias depending on
482 the species, sex bias category, body part and/or sex (Figs S3–S5). Our findings that *i*) sex bias
483 was less extensive (Table 1), *ii*) conserved sex bias was rare while conserved UB expression
484 was extensive (Fig 3A), and *iii*) the least amount of expression divergence occurred in the
485 head (Tables S3–S4) suggest that gene expression is more constrained by shared regulatory
486 mechanisms in the head than in the body. Indeed, sex bias in the head was largely gained in
487 a species-specific manner (Fig 1). Unlike in the body, where sex bias sometimes switched
488 between species, these gains almost exclusively occurred in genes that were either UB or
489 biased towards the same sex in the head of other species (39.2% of examined genes; Fig
490 3A,B, Figs S6–S7), suggesting that there is greater genetic constraint against sex bias
491 reversals in the head throughout the *Drosophila* genus. Moreover, SB genes in the head
492 were usually also SB in the body irrespective of sex (Fig 3C, Fig S2), suggesting that sex bias is
493 more likely to arise in the head when the underlying regulatory mechanisms for sex bias

494 already exist for that gene. Taken together, these findings suggest that the gain of sex bias is
495 much more genetically constrained in the head than in the body. This pattern is likely driven,
496 at least in part, by expression in the brain, where expression levels are known to be more
497 selectively constrained than in other tissues (Khaitovich et al. 2005, 2006). In keeping with
498 our findings, Khodursky et al. (2020) also found that expression was genetically constrained
499 by shared regulatory mechanisms in the brain with SB expression being largely species-
500 specific; however, they also detected a much larger proportion of switching in sex bias in
501 their dataset. This discrepancy between studies is likely due to their more limited sampling
502 within species, which included either a limited number of isofemale lines (*N* = 2) or lines
503 derived from a single population (*N* = 6). On the other hand, our dataset included more lines
504 (*N* = 5–8) per species, which were derived from multiple, geographically diverse populations.
505 Thus, our samples represented a broader overview of within species variation and likely
506 "averaged out" potential population- or line-specific sex bias. Consistent with this
507 interpretation, a recent study found that somatic SB genes are more variable than UB genes
508 among individuals within sub-species of mice (Xie et al. 2025). This study also detected a
509 larger proportion of sex bias reversals among subspecies, but this discrepancy with our study
510 might be due to their shorter evolutionary timescale (Xi et al. 2025).
511 Interestingly, lineage-specific gains or losses of sex bias most often occurred via
512 concordant expression changes in both sexes, but with a greater change in 1 sex than the
513 other (75.6% in the head and 54.3% in the body; Fig 4, Figs S9–S10), and this pattern was
514 even stronger for sex bias gains (Fig 4C,F) as well as genes with large expression changes
515 (95.2% in the head and 66.7% in the body; Fig S8), the majority of which occurred via a
516 down-regulation of expression in both sexes. This concordance suggests that these lineage-
517 specific sex bias gains are not a way to resolve sexual antagonism, as in such a case we
518 would expect up-regulated expression in 1 sex and down-regulated expression in the other
519 (quadrants i and iv in Fig 4A,B,D,E). Such changes represented the minority of SB gains in our
520 data (24.4% in the head and 45.7% in the body), especially in the head and among genes
521 with large expression changes (4.8% in the head and 33.3% in the body). Thus, in general,
522 the presence of SB expression is likely to be a poor proxy for ongoing or resolved sexual
523 antagonism in *Drosophila*. In keeping with our findings, a recent study in *Dmel* found that
524 sexually antagonistic effects on starvation resistance could not be explained by SB gene
525 expression (Chen et al. 2025). Moreover, another study of 5 mammalian species found that
526 only 27% of lineage-specific sex bias changes could be associated with turnover in SB

527 transcription factor motifs (Naqvi et al. 2019), suggesting that in such cases the gain of sex-
528 specific regulation may be driving the gain or loss of sex bias. Thus, while opposing sex-
529 specific regulatory changes contribute to the gain or loss of lineage-specific SB gene
530 expression, our results suggest that the majority of lineage-specific SB gains occur via
531 concerted changes in expression with a larger magnitude of change in 1 sex than the other,
532 suggesting that the underlying regulatory architecture is largely shared between the sexes.
533 Indeed, a previous study in humans found that sex differences in the genetic regulation of
534 gene expression were not very common (Oliva et al. 2020), while another study of the brain
535 of 3 *melanogaster* group species found that male and female expression were positively
536 correlated relative to ancestral expression (Khodursky et al. 2020). We similarly detected a
537 positive correlation in lineage-specific SB gains and losses, but the strength of this
538 correlation was stronger for SB gains than losses (Fig 4), underscoring that many species-
539 specific SB gains are likely the result of differences in regulation that is shared between the
540 sexes.

541 We also detected contrasting patterns in the chromosomal distribution of SB genes
542 between the head and body (Fig 5). In the body, we detected a consistent enrichment of FB
543 and a paucity of MB genes on the X chromosome (Fig 5B), which is consistent with the
544 "feminization" and concurrent "de-masculinization" of the X that has been reported in
545 previous studies (e.g. Parisi et al. 2003; Ranz et al. 2003; Sturgill et al. 2007). On the other
546 hand, in the head, we often detected an enrichment of SB genes (MB, FB, or both) on the X
547 chromosome (Fig 5A). Previous work in *melanogaster* group species found that genes with
548 SB expression, and particularly MB expression, in the brain were enriched on the X
549 chromosome (Catalán et al. 2012; Khodursky et al. 2020) and that this signal from the brain
550 may contribute to a detectable enrichment of X-linked SB genes in studies of whole heads
551 (Huylmans and Parsch 2015). Thus, expression in the brain likely contributed to the
552 enrichment of X-linked SB genes that we detected in the head (Fig 5A).

553 SB genes, especially MB genes, are often more tissue-specific than UB genes (Meisel
554 2011; Dean et al. 2016; Khodursky et al. 2020). In keeping with previous findings, we found
555 that tissue specificity was highest in MB genes (Fig S5). Moreover, tissue specificity was
556 positively associated with the magnitude of sex bias, and this pattern was strongest in MB
557 genes, especially in the body (Fig 3D). However, this association did not limit the evolution of
558 sex bias in more broadly expressed genes (Fig S13). Together, these findings suggest that
559 increased tissue specificity may facilitate larger changes in SB expression, especially for MB

genes, which is in keeping with the idea that increased tissue specificity may be a driving force in the evolution of SB gene expression, and MB gene expression in particular (Miesel 2011; Dean et al. 2016). However, we should note that in this study we focused on expression in body parts, which are a composite of multiple tissues. While the head is composed of relatively few tissues, the body is composed of many, including reproductive tissues. Moreover, cell composition is more heterogeneous in the body, which together with the tissues it encompasses is composed of more body part-specific cell-type clusters than the head (Li et al. 2022b). Thus, it is possible we may have missed some smaller, tissue-, or cell-type specific changes in SB gene expression. Moreover, this higher heterogeneity may at least in part account for the higher amount of sex bias switching that we detected in the body (14.3% of examined genes versus 0.4% in the head). However, differences in cell-type heterogeneity between the sexes is unlikely to affect our detection of SB genes as a recent study found that cell-type composition differences between the sexes are not a major source of sex bias (Barata and Vicoso 2026).

When we examined Δx as a measure of putative positive selection at the gene expression level (where Δx > 1 or <-1 is indicative of positive selection), we found that *i*) the absolute value of Δx was most often higher in males compared to females, with the mean magnitude of male Δx usually close to or above 1 (Fig 6, Table S8), *ii*) SB genes were often also and sometimes only enriched for signs of positive selection in the opposite sex (i.e. the more lowly expressed sex), especially for male expression of FB genes (Fig 6), and *iii*) Δx tended to shift in the same direction in both sexes (Table S8). At the same time, changes in gene expression relative to ancestral expression during sex bias gains tended to be positively correlated between the sexes (Figs S9–S10), as did gene expression between the sexes within body parts (Tables S3–S4). Taken together, these findings suggest that lineage-specific sex bias changes may often be facilitated by selection acting on expression in both sexes or the sex with lower expression (i.e. selection may be occurring for lower expression, often resulting in a net gain of sex bias in the opposite sex), especially for male expression of FB genes. Indeed, we found that lineage-specific sex bias changes were most often concordant between the sexes, especially in the head, for SB gains, and for genes with large expression changes (Figs 4 and S8), the majority of which occurred via a down-regulation of expression in both sexes. We also detected signs of positive selection in these lineage-specific sex bias changes, especially for male expression of FB genes (Table S9). Thus, our results suggest that most lineage-specific sex bias gains may be the result of selection acting on the asymmetrical

593 down- (or up-) regulation of expression in both sexes via shared regulatory architecture, and
594 this mechanism may be particularly important when expression is highly genetically or
595 selectively constrained, such as in the head or brain, or when there are large overall
596 expression changes. This finding is rather counterintuitive, as SB gene expression is known to
597 evolve rapidly (Assis et al. 2012; Whittle and Johannesson 2013; Harrison et al. 2015; Yang et
598 al. 2016; Tosto et al. 2023; Xie et al. 2025), and theory predicts that shared genetic
599 architecture should slow down the evolution of sexual dimorphism (Lande 1980) given the
600 constraints that the shared genome imposes on gene expression (Griffin et al. 2013;
601 Khodursky et al. 2020). However, a recent study using mouse and human data detected
602 overlapping variances of SB gene expression between the sexes in somatic tissues (Xie et al.
603 2025), suggesting that patterns and evolutionary dynamics of SB genes are likely more
604 nuanced than previously thought. Therefore, in cases where sex bias is beneficial, but
605 genetic constraint on gene regulation is relatively high, working within the constraint of a
606 shared genome, rather than around it, may provide an alternative, more common route to
607 the establishment of SB gene expression.
608

609 ## Materials and Methods

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

611 All *Drosophila* species were maintained at 21°C with a 14 h light:10 h dark cycle on
612 standard cornmeal–yeast–molasses medium. For all species, strains were established as
613 isofemale lines and maintained by transferring 20–50 flies per generation for at least 10
614 generations. For all of the *Dmel* and some of the *Dsim* strains, additional inbreeding was
615 done by performing single sib-pair crosses for 5–10 generations. Expression was examined in
616 5–8 strains of 6 *Drosophila* species (Fig 1). To provide a broad geographical overview of
617 expression within each species, the *Drosophila* strains were sampled from locations across
618 Europe, Asia, and Africa and were either collected by ourselves or kindly provided by others
619 (see Table S1 for details on sampling locations and provenance). Briefly, we included strains
620 from at least 2 populations per species and included strains from the inferred ancestral
621 region for *Dmel*, *Dsim*, *Dana*, and *Dsuz*. For each strain, groups of 30–120 heads and 8–80
622 bodies of 3–6-day-old flies were dissected in cold 1X PBS and frozen in RNA-DNA shield
623 (Zymo research; Freiburg, Germany) at -70°C until RNA extraction. RNA was extracted using
624 a Qiagen RNeasy Mini or Plus Universal Kit (Qiagen; Hilden, Germany). Poly-A selection,
625 fragmentation, reverse transcription, library construction, and high- throughput RNA-seq

626 was performed by Novogene (Martinsried, Germany) using the Illumina NextSeq 550
627 platform (Illumina; San Diego, CA) with 150-bp paired-end reads. We sequenced 1 biological
628 replicate per strain, sex, and body part, resulting in 35 libraries for each sample type (140
629 libraries in total). Library size ranged from 22,874,617 to 34,733,318 million paired-end
630 reads, 43.29–78.74% of which could be mapped, which is relatively low for well-annotated
631 *Drosophila* species and likely due to only mapping to protein coding sequences (CDS) rather
632 than full-length transcripts and ncRNAs (Table S10; see description of read mapping below).
633
634 **Read mapping and estimation of gene expression levels**
635 In order to prevent potential bias due to lower quality annotations in some species
636 (for example, no full transcript or ncRNA information was available for *Dimm*; whereas, in
637 *Dmel* UTRs and ncRNAs are better annotated than in the other, less studied species), we
638 mapped RNA-seq reads to CDS sequences (excluding UTRs and ncRNAs) for each species.
639 Reference CDS sequences were downloaded (April 25, 2024) from the following sources:
640 NCBI (*Dsub* genome assembly UCBerk_Dsub_1.0, *Dana* genome assembly ASM1763931v2,
641 D*suz* Genome assembly LBDM_Dsuz_2.1.pri, and *Dsim* genome assembly Prin_Dsim_3.1),
642 FlyBase (version 6.57; Öztürk-Çolak et al. 2024; *Dmel*), and Li et al. (2022a; *Dimm*). Links to
643 the downloaded references are provided in Table S11.
644 RNA-seq reads were mapped to the respective species reference using NextGenMap
645 (Sedlazeck et al. 2013) in paired-end mode. Read pairs mapping to different genes were
646 discarded, while read pairs matching more than 1 isoform of a gene were randomly assigned
647 to 1 of the isoforms of that gene. For downstream analyses, we analyzed the sum of read
648 counts across all of a gene's isoforms (across all annotated exons), i.e. on the individual
649 gene-level. In order to make direct comparisons of gene expression among species and
650 sample types, we used our gene counts (Data S1) to calculate levels of gene expression as
651 transcripts per million (TPM; Data S2; Wagner et al. 2013) for all genes in each library, which
652 normalizes for both sequencing depth and potential gene length variation among samples
653 and species. For TPM calculations, CDS lengths were extracted from the *Dmel* reference files
654 or calculated from the reference files for all other species using the faidx function in
655 *samtools* (Danecek et al. 2021). In cases of multiple isoforms, the longest CDS length was
656 used.
657
658 **Identification of 1:1 orthologs**

659 In order to keep the datasets for each species comparable, we restricted our analyses
660 to 1:1 orthologs present in all species. To identify 1:1 orthologs, we downloaded (April 29,
661 2024) pairwise lists of orthologs between *Dmel* and all other species from Li et al. (2022a; for
662 *Dimm*) or DrosOMA (Thiébaut et al. 2024; for all other species; Table S11), as well as the full
663 pairwise orthologs list from DrosOMA (Thiébaut et al. 2024; Table S11). First, all non-1:1
664 orthologs were removed from each *Dmel* ortholog list and the FlyBase batch download
665 and/or ID validator tools (Öztürk-Çolak et al. 2024) were used to obtain FBgn numbers for
666 *Dmel* IDs from each list. Non-gene or non-coding matches were removed and in cases for
667 which submitted IDs yielded multiple matches, only the FBgn number that exactly
668 corresponded the original ID was retained. For *Dmel* IDs with multiple matches but no exact
669 match or duplicate FBgn numbers due to ID changes/gene re-naming, we used a
670 combination of FlyBase (Öztürk-Çolak et al. 2024), DrosOMA (Thiébaut et al. 2024), and
671 UniProt (Ahmad et al. 2025) to validate the IDs. IDs that did not match between our
672 reference files and our ortholog lists were validated in the same manner. The orthologous
673 *Dmel* sequences corresponding to IDs from the *Dimm* list (Li et al. 2022a) that could not be
674 validated using this method were blasted (blastn; Altschul et al. 1990) to identify the current
675 *Dmel* gene, and their isoform was verified in DrosOMA. All IDs that could not be verified
676 were removed from our 1:1 ortholog list as were IDs that were no longer classified as genes
677 by NCBI, not included in our reference files, and IDs that had been incorporated into a gene
678 model already represented in our list (i.e. duplicate IDs). Next, we used the full pairwise
679 orthologs list from DrosOMA (Thiébaut et al. 2024; Table S11) to remove genes that were
680 not 1:1 orthologs between the non-*melanogaster* species. Orthologs that could not be
681 confirmed as belonging to the same, known OMA group were also removed as were
682 orthologs belonging to the same OMA group (i.e. OMA groups that appeared more than
683 once in our list). After these filtering steps, we obtained a list of 9,016 1:1 orthologs for use
684 in our expression analyses.
685
686 **Expression analyses**
687 We identified sex-biased genes using gene counts (Data S1, S3) in the 9,016 1:1
688 orthologs and a negative binomial test as implemented in DESeq2 (Love et al. 2014) in R (R
689 core team 2022) with a 5% false discovery rate (FDR). In order to exclude lowly expressed
690 genes while retaining as many genes as possible for comparative analyses, genes with low
691 counts or identified as outliers by DESeq2 were removed using the independent filtering

692 function in DESeq2. Because this method retained some lowly expression genes within our
693 analyses (Table S12), we therefore filtered our dataset using a TPM cut-off of ≥ 0.5 for a gene
694 to be considered as expressed and repeated our expression analyses (outlined below).
695 Although applying a TPM cut-off resulted in fewer genes that could be included in the
696 analysis, our results remained qualitatively extremely similar (Tables S12–13 and Fig S14).
697 We therefore focused on our full dataset in the main text and the downstream analyses. To
698 estimate $\log_2$ fold-changes in gene expression between males and females, head and body
699 samples for each species were analyzed separately using a 1-factor model design with 2
700 levels corresponding to each sex. For PCAs within each species, we implemented a 2-factor
701 (sex and body part) model design with 2 levels for each factor (male versus female and head
702 versus body). For the PCA including all samples, we similarly implemented a 3-factor design
703 (sex, body part, and species), with the additional third factor consisting of 6 levels
704 corresponding to the 6 examined species. To examine similarities in overall expression
705 between samples (i.e. among body parts, species, and/or sexes), we calculated gene
706 expression divergence between 2 sample types as 1 – Spearman's ρ between the mean TPM
707 of the 2 sample types across all expressed genes. Significant differences in expression
708 divergence or the absolute value (i.e. magnitude) of sex bias between sample types or sex
709 bias categories were assessed with a *t*-test with a Benjamini-Hochberg correction (Benjamini
710 and Hochberg 1995) for the number of comparisons made per sample or category for each
711 type of data. For each body part, we tested for any effects of sex bias category and/or
712 species on overall expression levels using the *lme4* R package (Bates et al. 2015) and a type II
713 ANOVA as implemented in the Anova function of the *car* (Fox and Weisberg 2019) R package
714 and the $\log_2$ mean TPM of each gene in each species ($\log_2$ TPM ~ species + sex bias category
715 + 1 | gene; Table S13), which provides a more conservative, balanced approach than using
716 TPM values for each individual strain and sex.
717 To identify lineage-specific gains or losses of sex bias in each body part, we employed
718 a conservative, phylogeny-based approach in which a terminal species (or all species of a
719 terminal clade) was only considered to have a change in sex bias if all species on that lineage
720 shared the same sex bias, and this sex bias differed from that of all other outgroup species,
721 which were also all required to share the same sex bias. Due to the lack of an outgroup, we
722 could not polarize sex bias changes as gains or losses on the lineage leading to *Dimm*;
723 therefore, we categorized the gain or loss of expression relative to the other analyzed
724 species. For example, a gain of SB expression was considered to have occurred in *Dimm* if a

725 gene was SB in *Dimm*, but not in any of the other examined species. For each body part, in
726 order to determine how sex bias changes for individual genes across the entire phylogeny,
727 we calculated the proportion of genes UB, that switched SB anywhere in the phylogeny (i.e
728 were FB and MB in at least 1 species each), and SB in 1-6 of the examined species. Only
729 genes that could be categorized as FB, MB, or UB in all species were included in this analysis,
730 resulting in a total of 7,214 and 8,792 genes in the head and body respectively. To better
731 understand how sex bias changes individually among species, we similarly performed
732 pairwise comparisons between each species using all genes that could be assigned to a sex
733 bias category in both species.
734 In order to compare how expression changed in a particular species relative to the
735 inferred ancestral expression, we calculated the ancestral expression for each gene, sex and
736 body part for all internal nodes of our phylogenetic tree using the mean TPM values in each
737 species and a Brownian motion model fitted by maximum likelihood as implemented in the
738 ace function in the R package *ape* (Paradis and Schliep 2019). Changes in expression within
739 each sex, species, and body part in comparison to the inferred ancestral expression were
740 calculated as $\log_2(TPM_{Focal}/TPM_{Anc})$, where TPM_{Focal} was the mean TPM in the focal species
741 for a given body part and sex and TPM_{Anc} was the inferred ancestral TPM at the nearest
742 internal node. To test for differences in the distribution of expression changes (up- or down-
743 regulation) in males versus females between body parts, we employed a χ^2 test comparing
744 the number of genes for the entire phylogeny in each quadrant (i–iv, see Fig 4) between the
745 head and body. We further categorized genes as having a large or small expression change
746 by employing a $\log_2$ fold-change threshold of ≥ 2 in the body or ≥ 1 in the head to categorize
747 a gene as having a large expression change, with values below these thresholds categorized
748 as a small expression change. A lower threshold was applied in the head due to its lower
749 extent (Table 1) and magnitude (Fig S3) of sex bias. To test for significant correlations
750 between male and female expression changes during the gain or loss of sex bias, which
751 would indicate that expression changes are concordant between the sexes, in each body
752 part we calculated Spearman's ρ between expression changes in males versus changes in
753 females for all genes that gained or lost sex bias within each species. To detect differences in
754 the strength of the association between male and female expression changes when sex bias
755 is gained versus when it is lost, we then tested for differences in strength of these
756 correlations between MB or FB gains and SB losses using a Mann-Whitney *U* test or a paired
757 Mann-Whitney *U* test to test for differences between body parts.

758

759 **Chromosomal location and calculation of τ and Δx**

760 Chromosomal locations of all protein-coding genes for *Dsim*, *Dsuz*, *Dana*, and *Dsub*
761 were downloaded (July 11, 2025) from NCBI. For *Dsuz* the CBGP_Dsuzu_IsoJpt1.0 assembly,
762 which is chromosome-level, was used to determine the chromosomal location of analyzed
763 genes. *Dmel* chromosomal locations were extracted from the reference CDS file. Because a
764 chromosome-level assembly was not available for *Dimm*, this species was excluded from all
765 chromosomal location analyses. For all comparisons between X and autosomal genes, we
766 compared genes that could be assigned to the X chromosome (also known as Muller
767 element "A") to all genes that could be assigned to any autosome, excluding the "dot"
768 chromosome (also known as Muller element "F"), which is thought to have been an
769 ancestral X chromosome (Vicoso and Bachtrog 2013). To detect potential differences in the
770 distribution of SB genes on the X and autosomes for each species, we used a χ^2 test to test
771 for differences in the number of observed genes in each sex bias category versus the
772 expected number of genes in each category based on the overall proportion of genes in each
773 category for that species separately for the X and the autosomes.

774 In order to examine tissue specificity, we downloaded the FlyAtlas2 FPKM data
775 (Krause et al. 2022) for all adult *Dmel* tissues, excluding fat body, spermatheca, and heart,
776 which were sequenced using a different chemistry from the other tissues, from
777 https://motif.mvls.gla.ac.uk/downloads/FlyAtlas2_gene_data_2025.xlsx (April 24, 2025). We
778 then calculated the tissue-specificity index τ (Yanai et al. 2005) for each gene (Data S3) and
779 identified the tissue with the highest expression. For each body part, we tested for any effect
780 of sex bias category on τ using a type II ANOVA as implemented in the Anova function of the
781 *car* (Fox and Weisberg 2019) R package (τ ~ species + sex bias category; Table S13). Because
782 our estimates of τ are the same for every gene regardless of species, we could not include
783 gene or species as a random factor, which would result in overfitting. Moreover, any
784 significant effect of species is a reflection of a different composition of genes in each sex bias
785 category for each species, rather than a direct effect of species on levels of tissue specificity.
786 In order to test for any associations between sex bias and tissue specificity, we calculated
787 Spearman's ρ between τ and the magnitude of sex bias for each sex bias category in each
788 body part and species. To detect if the sex bias category affects the strength of the
789 association between tissue specificity and the degree of sex bias, we tested for differences in
790 the strength of these correlations among sex bias categories using a Mann-Whitney *U* test.

791 In order to identify putative signs of positive selection at the gene expression level,
792 for each species and body part we calculated Δx (Data S4) similar to Moghadam et al. (2012)
793 within each sex as

794 $$\Delta x = \frac{d}{fr}, (1)$$

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

800 $$d = \frac{mean(X_{Focal}) - mean(X_{Imm})}{mean(X_{Focal})}, (2)$$

801 the expression range (*r*) as

802 $$r = \frac{max(X_{Focal}) - min(X_{Focal})}{mean(X_{Focal})}, (3)$$

803 and the correction factor (*f*) as

804 $$f = \sqrt{\frac{mN-1}{mN-m}}, (4)$$

805 where *m* is the number of strains for the focal species and *N* is the most common number of
806 strains (for our data, *N* = 5). An absolute Δx value above 1 indicates that divergence of
807 expression in the focal species from *Dimm* exceeds expression polymorphism within that
808 species. Thus, a threshold of an absolute Δx value of 1 has previously been taken to indicate
809 that positive selection may have occurred (Moghadam et al. 2012, Zemp et al. 2016, Lichilín
810 et al. 2021, Cossard et al. 2022), with Δx < -1 representing a down-regulation in the focal
811 species and Δx > 1 indicating an up-regulation. To determine if we could detect more genes
812 under putative positive selection than expected by chance, for all genes and for the X
813 chromosome and autosomes separately, we used a χ^2 test to test for differences in the
814 number of observed putatively positively selected genes in each sex bias category versus the
815 number of expected putatively selected genes based on the proportion of genes in each sex
816 bias category for each sex, species, and body part. To detect potential differences in the
817 overall action of selection in males versus females, for each body part, species, and sex bias
818 category, we used a *t*-test to test for differences in the magnitude of Δx between males and
819 females. To detect potential associations between selection on male versus female
820 expression, we also tested for correlations in Δx between the sexes for each body part,
821 species, and sex bias category using Spearman's ρ. To determine if body part affects the
822 strength of the association between Δx in each sex, we tested for differences in the strength

823 of these correlations between the body and the head using a paired Mann-Whitney *U* test.
824 To test if putatively selected genes were enriched or depleted among lineage-specific sex
825 bias gains and losses, for expression in each sex we employed a χ^2 test comparing the
826 observed number of selected and non-selected genes versus the expected number based on
827 the proportion of selected genes in that sample type. Due to lack of power, we excluded
828 cases where the expected number of selected or non-selected genes was below 5 from
829 these analyses. To test if tissue specificity differed between genes identified as under
830 selection versus those identified as not under selection, for each body part and sex, we
831 tested for any effect of selection (i.e. identified as under putative positive selection or not)
832 on τ using a type II ANOVA as implemented in the Anova function of the *car* (Fox and
833 Weisberg 2019) R package (τ ~ species + sex bias category + selection; Table S13). Because
834 our estimates of τ are the same for every gene regardless of species or sex, we could not
835 include gene or species as a random factor, which would result in overfitting.
836
837 **Data Availability**
838 The RNA-seq data that support the findings of this study are publicly available from the
839 European Nucleotide Archive (ENA) at EMBL-EBI under Project accession number XXXXXXXX.
840 All other relevant data are within the manuscript and its Supplementary Material files.
841

842 ## Acknowledgements

843 We thank Hilde Lainer for excellent technical assistance in the laboratory. We are grateful to
844 members of the European *Drosophila* Populations Genomics Consortium (DrosEU) and the
845 following people for providing the fly lines used in this study: Jessica Abbott, Nicolas Gompel,
846 Josefa González, Sonja Grath, Katja Hoedjes, Mihailo Jelić, Maaria Kankare, Viola Nolte,
847 Darren Obbard, Marta Pascual, John Pool, Christian Schlötterer, Mads Fristrup Schou, Marina
848 Stamenković, Fabian Staubach, Cristina Vieira, Jorge Vieira, and Ricardo Wilches. This study
849 was supported by the Deutsche Forschungsgemeinschaft priority program "The genomic
850 basis of evolutionary innovations" (SPP2349; Project No. 503272152 awarded to JP).
851

852 ## References

853 Ahmad S, Jose da Costa Gonzales L, Bowler-Barnett EH, Rice DL, Kim M, Wijerathne S, et al.
854 2025.The UniProt website API: facilitating programmatic access to protein knowledge.
855 *Nucleic Acids Res.* 53(W1):W547-W53.

856 Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search
857 tool. *J Mol Biol.* 215(3):403-10.

858 Arbeitman MN, Furlong EE, Imam F, Johnson E, Null BH, Baker BS, et al. 2002. Gene
859 expression during the life cycle of *Drosophila melanogaster*. Science. 297(5590):2270-5.

860 Assis R, Zhou Q, Bachtrog D. 2012. Sex-biased transcriptome evolution in *Drosophila*.
861 Genome Biol Evol. 4(11):1189-200.

862 Barata C, Vicoso B. 2026. Single-nucleus resolution of sex-biased expression and dosage
863 compensation in *Drosophila melanogaster*. *Proc Biol Sci*. 293(2063).

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

866 Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate: a practical and
867 powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B*.
868 57(1):289-300.

869 Buchberger E, Reis M, Lu TH, Posnien N. 2019. Cloudy with a Chance of Insights: Context
870 Dependent Gene Regulation and Implications for Evolutionary Studies. *Genes* (Basel).
871 10(7).

872 Catalán A, Hutter S, Parsch J. 2012. Population and sex differences in *Drosophila*
873 *melanogaster* brain gene expression. *BMC Genomics*. 13:654.

874 Chen J, Liu C, Li W, Clark AG, Wang Z, Lu J. 2025. Pervasive sex-dependent effects in the
875 genetic architecture of starvation resistance in. *Sci Adv*. 11(39):eadt3560.

876 Cheng C, Kirkpatrick M. 2016. Sex-Specific Selection and Sex-Biased Gene Expression in
877 Humans and Flies. *PLoS Genet*. 12(9):e1006170.

878 Connallon T, Knowles LL. 2005. Intergenomic conflict revealed by patterns of sex-biased
879 gene expression. *Trends Genet*. 21(9):495-9.

880 Cossard GG, Godfroy O, Nehr Z, Cruaud C, Cock JM, Lipinska AP, et al. 2022. Selection
881 drives convergent gene expression changes during transitions to co-sexuality in haploid
882 sexual systems. *Nat Ecol Evol*. 6(5):579-89.

883 Danecek P, Bonfield JK, Liddle J, Marshall J, Ohan V, Pollard MO, et al. 2021. Twelve years
884 of SAMtools and BCFtools. *Gigascience*. 10(2).

885 Dean R, Mank JE. 2016. Tissue Specificity and Sex-Specific Regulatory Variation Permit the
886 Evolution of Sex-Biased Gene Expression. *Am Nat.* 188(3):E74-84.

887 Ellegren H, Parsch J. 2007. The evolution of sex-biased genes and sex-biased gene
888 expression. *Nat Rev Genet*. 8(9):689-98.

Fass SB, Mulvey B, Chase R, Yang W, Selmanovic D, Chaturvedi SM, et al. 2024. Relationship between sex biases in gene expression and sex biases in autism and Alzheimer's disease. *Biology of Sex Differences*. 15(1):47.

Gibson G, Riley-Berger R, Harshman L, Kopp A, Vacha S, Nuzhdin S, et al. 2004. Extensive sex-specific nonadditivity of gene expression in *Drosophila melanogaster*. *Genetics*. 167(4):1791-9.

Grath S, Parsch J. 2012. Rate of amino acid substitution is influenced by the degree and conservation of male-biased transcription over 50 myr of *Drosophila* evolution. *Genome Biol Evol*. 4(3):346-59.

Grath S, Parsch J. 2016. Sex-Biased Gene Expression. *Annu Rev Genet*. 50:29-44.

Griffin RM, Dean R, Grace JL, Rydén P, Friberg U. 2013. The shared genome is a pervasive constraint on the evolution of sex-biased gene expression. *Mol Biol Evol*. 30(9):2168-76.

Harrison PW, Wright AE, Zimmer F, Dean R, Montgomery SH, Pointer MA, et al. 2015. Sexual selection drives evolution and rapid turnover of male gene expression. *Proc Natl Acad Sci U S A*. 112(14):4393-8.

Hudson RR, Kreitman M, Aguadé M. 1987. A test of neutral molecular evolution based on nucleotide data. *Genetics*. 116(1):153-9.

24. Huylmans AK, Parsch J. Population- and sex-biased gene expression in the excretion organs of *Drosophila melanogaster*. *G3* (Bethesda). 2014;4(12):2307-15.

Huylmans AK, Parsch J. 2015. Variation in the X:Autosome Distribution of Male-Biased Genes among *Drosophila melanogaster* Tissues and Its Relationship with Dosage Compensation. *Genome Biol Evol*. 7(7):1960-71.

Innocenti P, Morrow EH. 2010. The sexually antagonistic genes of *Drosophila melanogaster*. *PLoS Biol*. 8(3):e1000335.

Jin W, Riley RM, Wolfinger RD, White KP, Passador-Gurgel G, Gibson G. 2001. The contributions of sex, genotype and age to transcriptional variance in *Drosophila melanogaster*. *Nat Genet*. 29(4):389-95.

Khaitovich P, Enard W, Lachmann M, Pääbo S. 2006. Evolution of primate gene expression. *Nat Rev Genet*. 7(9):693-702.

Khaitovich P, Hellmann I, Enard W, Nowick K, Leinweber M, Franz H, et al. 2005. Parallel patterns of evolution in the genomes and transcriptomes of humans and chimpanzees. *Science*. 309(5742):1850-4.

Khodursky S, Svetec N, Durkin SM, Zhao L. 2020. The evolution of sex-biased gene expression in the *Drosophila* brain. *Genome Res*. 30(6):874-84.

Kimura M. 1983. The neutral theory of molecular evolution. Cambridge: Cambridge University Press.

King MC, Wilson AC.1975. Evolution at two levels in humans and chimpanzees. *Science*. 188(4184):107-16.

Krause SA, Overend G, Dow JAT, Leader DP. 2022. FlyAtlas 2 in 2022: enhancements to the *Drosophila melanogaster* expression atlas. *Nucleic Acids Research*. 50(D1):D1010-D5.

Lande R. 1980. Sexual dimorphism, sexual selection, and adaptation in polygenic characters. *Evolution*. 34(2):292-305.

Li F, Rane RV, Luria V, Xiong Z, Chen J, Li Z, et al. 2022. Phylogenomic analyses of the genus *Drosophila* reveals genomic signals of climate adaptation. *Mol Ecol Resour*. 22(4):1559-81.

Li H, Janssens J, De Waegeneer M, Kolluru SS, Davie K, Gardeux V, et al. 2022. Fly Cell Atlas: A single-nucleus transcriptomic atlas of the adult fruit fly. *Science*. 375(6584):eabk2432.

Lichilín N, El Taher A, Böhne A. 2021. Sex-biased gene expression and recent sex chromosome turnover. *Philos Trans R Soc Lond B Biol Sci*. 376(1833):20200107.

Llopart A. 2012. The rapid evolution of X-linked male-biased gene expression and the large-X effect in *Drosophila yakuba*, *D. santomea*, and their hybrids. *Mol Biol Evol*. 29(12):3873-86.

Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15(12):550.

Ma WJ, Veltsos P, Toups MA, Rodrigues N, Sermier R, Jeffries DL, et al. 2018. Tissue Specificity and Dynamics of Sex-Biased Gene Expression in a Common Frog Population with Differentiated, Yet Homomorphic, Sex Chromosomes. *Genes* (Basel). 9(6).

McDonald JH, Kreitman M. 1991. Adaptive protein evolution at the Adh locus in *Drosophila*. *Nature*. 351(6328):652-4.

Meiklejohn CD, Coolon JD, Hartl DL, Wittkopp PJ. 2014. The roles of *cis*- and *trans*-regulation in the evolution of regulatory incompatibilities and sexually dimorphic gene expression. *Genome Res*. 24(1):84-95.

Meiklejohn CD, Parsch J, Ranz JM, Hartl DL. 2003. Rapid evolution of male-biased gene expression in *Drosophila*. *Proc Natl Acad Sci U S A*. 100(17):9894-9.

Meisel RP. 2011. Towards a more nuanced understanding of the relationship between sex-biased gene expression and rates of protein-coding sequence evolution. *Mol Biol Evol*. 28(6):1893-900.

Moghadam HK, Pointer MA, Wright AE, Berlin S, Mank JE. 2012. W chromosome expression responds to female-specific selection. *Proc Natl Acad Sci U S A*. 109(21):8207-11.

Müller L, Grath S, von Heckel K, Parsch J. 2012. Inter- and intraspecific variation in *Drosophila* genes with sex-biased expression. *Int J Evol Biol*. 2012:963976.

959 Naqvi S, Godfrey AK, Hughes JF, Goodheart ML, Mitchell RN, Page DC. 2019. Conservation,
960 acquisition, and functional impact of sex-biased gene expression in mammals. *Science*.
961 365(6450).

962 Oliva M, Muñoz-Aguirre M, Kim-Hellmuth S, Wucher V, Gewirtz ADH, Cotter DJ, et al. 2020.
963 The impact of sex on gene expression across human tissues. *Science*. 369(6509).

964 Öztürk-Çolak A, Marygold SJ, Antonazzo G, Attrill H, Goutte-Gattat D, Jenkins VK, et al.
965 2024. FlyBase: updates to the *Drosophila* genes and genomes database. *Genetics*. 227(1).

966 Pal S, Oliver B, Przytycka TM. 2023. Stochastic Modeling of Gene Expression Evolution
967 Uncovers Tissue- and Sex-Specific Properties of Expression Evolution in the. *J Comput Biol.*
968 30(1):21-40.

969 Paradis E, Schliep K. 2019. ape 5.0: an environment for modern phylogenetics and
970 evolutionary analyses in R. *Bioinformatics*. 35(3):526-8.

971 Parisi M, Nuttall R, Edwards P, Minor J, Naiman D, Lü J, et al. 2004. A survey of ovary-,
972 testis-, and soma-biased gene expression in *Drosophila melanogaster* adults. *Genome Biol.*
973 5(6):R40.

974 Parsch J, Ellegren H. 2013. The evolutionary causes and consequences of sex-biased gene
975 expression. *Nat Rev Genet*. 14(2):83-7.

976 R Core Team. 2022. R: A language and environment for statistical computing. Vienna,
977 Austria: R Foundation for Statistical Computing.

978 Ranz JM, Castillo-Davis CI, Meiklejohn CD, Hartl DL. 2003. Sex-dependent gene expression
979 and evolution of the *Drosophila* transcriptome. *Science*. 300(5626):1742-5.

980 Ranz JM, Namgyal K, Gibson G, Hartl DL. 2004. Anomalies in the expression profile of
981 interspecific hybrids of *Drosophila melanogaster and Drosophila simulans*. *Genome Res*.
982 14(3):373-9.

983 Rodríguez-Montes L, Ovchinnikova S, Yuan X, Studer T, Sarropoulos I, Anders S, et al. 2023.
984 Sex-biased gene expression across mammalian organ development and evolution. *Science*.
985 382(6670):eadf1046.

986 Sedlazeck FJ, Rescheneder P, von Haeseler A. 2013. NextGenMap: fast and accurate read
987 mapping in highly polymorphic genomes. *Bioinformatics*. 29(21):2790-1.

988 Shi L, Zhang Z, Su B. 2016. Sex Biased Gene Expression Profiling of Human Brains at Major
989 Developmental Stages. *Sci Rep*. 6:21181.

990 Sturgill D, Zhang Y, Parisi M, Oliver B. 2007. Demasculinization of X chromosomes in the
991 *Drosophila* genus. *Nature*. 450(7167):238-41.

992 Tamura K, Subramanian S, Kumar S. 2004. Temporal patterns of fruit fly (*Drosophila*)
993 evolution revealed by mutation clocks. *Mol Biol Evol*. 21(1):36-44.

994 Thiébaut A, Altenhoff AM, Campli G, Glover N, Dessimoz C, Waterhouse RM. 2024.
995 DrosOMA: the *Drosophila* Orthologous Matrix browser. *F1000Res*. 12:936.

996 Tosto NM, Beasley ER, Wong BBM, Mank JE, Flanagan SP. 2023. The roles of sexual
997 selection and sexual conflict in shaping patterns of genome and transcriptome variation.
998 *Nat Ecol Evol.* 7(7):981-93.

999 Vicoso B, Bachtrog D. 2013. Reversal of an ancient sex chromosome to an autosome in
1000 Drosophila. *Nature*. 499(7458):332-5.

1001 Wagner GP, Kin K, Lynch VJ. A 2013. model based criterion for gene expression calls using
1002 RNA-seq data. *Theory in Biosciences*. 132(3):159-64.

1003 Whittle CA, Johannesson H. 2013. Evolutionary dynamics of sex-biased genes in a
1004 hermaphrodite fungus. *Mol Biol Evol*. 30(11):2435-46.

1005 Wray GA, Hahn MW, Abouheif E, Balhoff JP, Pizer M, Rockman MV, et al. 2003. The
1006 Evolution of Transcriptional Regulation in Eukaryotes. *Molecular Biology and Evolution*.
1007 20(9):1377-419.

1008 Wright AE, Fumagalli M, Cooney CR, Bloch NI, Vieira FG, Buechel SD, et al. 2018. Male-
1009 biased gene expression resolves sexual conflict through the evolution of sex-specific
1010 genetic architecture. *Evol Lett*. 2(2):52-61.

1011 Xie C, Künzel S, Tautz D. 2025. Fast evolutionary turnover and overlapping variances of sex-
1012 biased gene expression patterns defy a simple binary sex classification of somatic tissues.
1013 *Elife*. 13.

1014 Yanai I, Benjamin H, Shmoish M, Chalifa-Caspi V, Shklar M, Ophir R, et al. 2005. Genome-
1015 wide midrange transcription profiles reveal expression level relationships in human tissue
1016 specification. *Bioinformatics*. 21(5):650-9.

1017 Yang L, Zhang Z, He S. 2016. Both Male-Biased and Female-Biased Genes Evolve Faster in
1018 Fish Genomes. *Genome Biol Evol*. 8(11):3433-45.

1019 Yang X, Schadt EE, Wang S, Wang H, Arnold AP, Ingram-Drake L, et al. 2006. Tissue-specific
1020 expression and regulation of sexually dimorphic genes in mice. *Genome Res*. 16(8):995-
1021 1004.

1022 Zemp N, Tavares R, Muyle A, Charlesworth D, Marais GA, Widmer A. 2016. Evolution of sex-
1023 biased gene expression in a dioecious plant. Nat Plants. 2(11):16168.

1024 Zhang Y, Sturgill D, Parisi M, Kumar S, Oliver B. 2007. Constraint and turnover in sex-biased
1025 gene expression in the genus *Drosophila*. *Nature*. 450(7167):233-7.
1026

1027 **Supplementary Material Captions**

1028 **Table S1:** Overview of *Drosophila* lines assayed in this study

1029 **Table S2:** Sex biased genes in *D. melanogaster* in a random sub-sampling of 5 lines

1030 **Table S3:** Expression divergence within species

1031 **Table S4:** Expression divergence among species

1032 **Table S5:** Number of SB genes with highest expression in testis, ovary, or a somatic tissue

1033 **Table S6:** Lineage-specific gains and losses of sex biased gene expression

1034 **Table S7:** Sex-biased genes on the X versus autosomes

1035 **Table S8:** Genes showing signs of putative positive selection at the expression level

1036 **Table S9:** Putative positive selection in genes with lineage-specific SB gains and losses

1037 **Table S10:** Library size and mapping efficiency

1038 **Table S11:** Download location of files used for analysis

1039 **Table S12:** Sex bias in the examined species using TPM cut-off

1040 **Table S13:** Results of Type II ANOVAs to test the effect of sex bias category and species on
1041 gene expression levels and τ

1042 **Fig S1:** Principal component analysis of gene expression profiles in A) *Dmel*, B) *Dsim*, C) *Dsuz*,
1043 D) *Dana*, E) *Dsub*, and F) *Dimm*. Triangles and diamonds indicate female and male body,
1044 respectively, while circles and squares indicate female and male head, respectively.

1045 **Fig S2:** Overlap in SB genes between head (H) and body (B). Shown are upset plots
1046 demonstrating the overlap in UB, FB, or MB genes in A) *Dmel* (mel), B) *Dsim* (sim), C) *Dsuz*
1047 (suz), D) *Dana* (ana), E) *Dsub* (sub), and F) *Dimm* (imm). Horizontal bars represent the total
1048 number of genes in a body part and sex bias category combination. Vertical bars represent
1049 the number of genes in an intersection class. Connected, filled circles underneath a vertical
1050 bar indicate that a body part and sex bias category combination is included in an intersection
1051 class. Blue represents genes UB in one body part and SB in the other, dark gray represents
1052 gene in the same bias category, and light blue indicates genes with the opposite sex bias
1053 between head and body.

1054 **Fig S3:** Magnitude of sex bias in the A) head and B) body of *D. melanogaster* (mel), *Dsim*
1055 (sim), *Dsuz* (suz), *Dana* (ana), *Dsub* (sub), and *Dimm* (im/imm). Significant differences
1056 between species for MB and FB genes were assessed with a *t*-test. Shown are the BH-
1057 corrected *P*-values in the C) head and D) body. Non-significant *P*-values are shown in bold.

1058 **Fig S4:** Overall expression levels in the A) head and B) body for MB (M), FB (F), and UB (U)
1059 genes in *Dmel*, *Dsim*, *Dsuz*, *Dana*, *Dsub*, and *Dimm*. Significance was assessed for each body
1060 part with a type II ANOVA with sex bias (SB) category and species as fixed factors and gene
1061 as a random factor.

1062 **Fig S5:** Tissue specificity τ in the A) head and B) body for MB (M), FB (F), and UB (U) genes in
1063 *Dmel*, *Dsim*, *Dsuz*, *Dana*, *Dsub*, and *Dimm*. Significance was assessed for each body part with
1064 a type II ANOVA with sex bias (SB) category and species as factors.
1065 **Fig S6:** Overlap in SB genes within the head (H) between species. Shown are upset plots
1066 demonstrating the overlap in UB, FB, or MB genes in pairwise comparisons between A–E)
1067 *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*
1068 (sub), and E,I,L,N,O) *Dimm* (imm). Horizontal bars represent the total number of genes in a
1069 species and sex bias category combination. Vertical bars represent the number of genes in
1070 an intersection class. Connected, filled circles underneath a vertical bar indicate that a
1071 species and sex bias category combination is included in an intersection class. Blue
1072 represents genes UB in one species and SB in the other, dark gray represents genes in the
1073 same sex bias category, and light blue indicates genes that switch sex bias between species.
1074 P) Summary of pairwise overlap in sex bias categories among species. Shown are the
1075 proportion of overlapping genes that had the same SB (white) or were both UB (dark blue),
1076 were SB in only one of the examined species (SB 1 species; light blue), or switched sex bias
1077 (Switch SB; gray) between *Dmel* (*m*), *Dsim* (*si*), *Dsuz* (*su*), *Dana* (*a*), *Dsub* (*sb*), and *Dimm* (*im*).
1078 **Fig S7:** Overlap in sex biased genes within the body (B) between species. Shown are upset
1079 plots demonstrating the overlap in UB, FB, or MB genes in pairwise comparisons between A–
1080 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)
1081 *Dsub* (sub), and E,I,L,N,O) *Dimm* (imm). Horizontal bars represent the total number of genes
1082 in a species and sex bias category combination. Vertical bars represent the number of genes
1083 in an intersection class. Connected, filled circles underneath a vertical bar indicate that a
1084 species and sex bias category combination is included in an intersection class. Blue
1085 represents genes UB in one species and SB in the other, dark gray represents genes in the
1086 same sex bias category, and light blue indicates genes that switch sex bias between species.
1087 P) Summary of pairwise overlap in sex bias categories among species. Shown are the
1088 proportion of overlapping genes that had the same SB (white) or were both UB (dark blue),
1089 were SB in only one of the examined species (SB 1 species; light blue), or switched sex bias
1090 (Switch SB; gray) between *Dmel* (*m*), *Dsim* (*si*), *Dsuz* (*su*), *Dana* (*a*), *Dsub* (*sb*), and *Dimm* (*im*).
1091 **Fig S8:** All SB gains and losses for genes with A,B) large and C,D) small expression changes
1092 between the focal and ancestral species in the A,C) head and B,D) body. The cut-off for a
1093 large expression change was a $\log_2$ fold-change of magnitude 1 or higher in the head and 2
1094 or higher in the body. Genes with concordant (con) changes in expression between the sexes

1095 are located in quadrants ii and iii; while genes with opposing (opp) expression changes
1096 between the sexes are located in quadrants i and iv.
1097 **Fig S9**: Expression changes during sex bias turnover in the head. Shown are scatterplots of
1098 expression in A–C) *Dmel*, D–F) *Dsim*, G–I) *Dsuz*, J–L) *Dana*, M–O) *Dsub*, and P–R) *Dimm* in
1099 each sex relative to ancestral (anc) expression for A,D,G,J,M,P) FB or B,E,H,K,N,O) MB gains
1100 or C,F,I, L,O,R) losses. Spearman's ρ and the associated *P* value for each category are shown.
1101 **Fig S10:** Expression changes during sex bias turnover in the body. Shown are scatterplots of
1102 expression in A–C) *Dmel*, D–F) *Dsim*, G–I) *Dsuz*, J–L) *Dana*, M–O) *Dsub*, and P–R) *Dimm* in
1103 each sex relative to ancestral (anc) expression for A,D,G,J,M,P) FB or B,E,H,K,N,O) MB gains
1104 or C,F,I, L,O,R) losses. Spearman's ρ and the associated *P* value for each category are shown.
1105 **Fig S11**: Δx in the A) head and B) body of 5 *Drosophila* species. Shown are Δx values for FB,
1106 MB and UB genes in females (F) and males (M) of *Dmel*, *Dsim*, *Dsuz*, *Dana*, and *Dsub*. The
1107 dashed gray line represents the cut-off above or below which a gene shows signs of putative
1108 positive selection. For better visualization, Δx values above 5 or below -5 are not shown.
1109 **Fig S12:** Chromosomal distribution of signs of putative positive selection at the gene
1110 expression level. Shown are the ratios of observed (obs) to expected (exp) number of genes
1111 under putative positive selection (Δx > 1 or < -1) in each sex bias category and sex on the X
1112 chromosome and autosomes (auto) in the A) head and B) body. Significance was assessed
1113 with a χ^2 test. ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$, marginally non-significant: .$P < 0.1$. Non-
1114 significant comparisons not shown.
1115 **Fig S13:** Tissue specificity, τ, in genes identified as putatively under positive selection (S) or
1116 not under selection (N) in A,C) females (F) and B,D) males (M) the A,B) head and C,D) body
1117 for MB (M), FB (F), and UB (U) genes in *Dmel*, *Dsim*, *Dsuz*, *Dana*, *Dsub*, and *Dimm*.
1118 Significance was assessed for each body part with a type II ANOVA with sex bias (SB)
1119 category, species, and if under putative positive selection as factors.
1120 **Fig S14:** Expression analyses using a TPM cut-off of 0.5. A–D) Shown are A,B) overall
1121 expression levels and C,D) tissue specificity, τ, in the A,C) head and B,D) body for MB (M), FB
1122 (F), and UB (U) genes in *Dmel* (*mel*), *Dsim* (*sim*), *Dsuz* (*suz*), *Dana* (*ana*), *Dsub* (*sub*), and
1123 *Dimm* (*imm*). A,B) Significance was assessed for each body part with a type II ANOVA with
1124 sex bias (SB) category and species as fixed factors and gene as a random factor. C,D)
1125 Significance was assessed for each body part with a type II ANOVA with SB category and
1126 species as factors. E,F) Shown are Spearman's ρ correlations between male and female
1127 expression changes within each species for SB gains and losses in the E) head (H) and F) body

1128 (B). Significance was assessed with a Mann-Whitney U test. ***P < 0.005, **P < 0.01, *P <
1129 0.05. Non-significant comparisons not shown. G–L) Genes with concordant (con) changes in
1130 expression between the sexes are located in quadrants ii and iii; while genes with opposing
1131 (opp) expression changes between the sexes are located in quadrants i and iv. Total genes in
1132 each quadrant for the entire phylogeny in the G) head and H) body are shown. In
1133 parentheses, the proportion of total gene expression changes are shown. G–J) Shown are all
1134 SB gains and losses for genes with I,J) large and K,L) small expression changes between the
1135 focal and ancestral species in the I,K) head and J,L) body. The cut-off for a large expression
1136 change was a $\log_2$ fold-change of magnitude 1 or higher in the head and 2 or higher in the
1137 body.
1138 **Data S1:** Data underlying gene expression analyses: gene counts.
1139 **Data S2:** Data underlying gene expression analyses: TPM.
1140 **Data S3:** Data underlying gene expression analyses: SB gene expression, τ, and expression
1141 changes.
1142 **Data S4:** Data underlying selection analyses.