

1 **Sex-biased gene expression in *Drosophila melanogaster* is constrained by**
2 **ontogeny and genetic architecture**

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16 **Sexual dimorphism is predicted to be constrained by the underlying**
17 **genetic architecture shared between the sexes and through ontogeny, but**
18 **whole-transcriptome data for both sexes across genotypes and**
19 **developmental stages are lacking. Within a quantitative genetic**
20 **framework, we sequenced RNA from *Drosophila melanogaster* at different**
21 **developmental stages to examine sex-biased gene expression and how**
22 **selection acts upon it. We found evidence that gene expression is**
23 **constrained by both univariate and multivariate shared genetic variation**
24 **between genes, sexes and developmental stages, but may be resolved by**
25 **differential splicing. These results provide a comprehensive picture of how**
26 **conflict over sexual dimorphism varies through development and clarifies**
27 **the conditions under which it is predicted to evolve.**
28

29 Sexual dimorphism is widespread across animals and plants, and is likely to have
30 evolved in response to the different reproductive roles of the sexes within a
31 species ¹. However, in most species, the majority of genes are shared between
32 males and females, and sex-specific fitness optima for shared traits can create
33 conflict, called intralocus sexual conflict ². Intralocus sexual conflict is driven by
34 sexually antagonistic selection, which pushes the phenotypic value of the trait in
35 different directions. It is therefore thought that sexually antagonistic selection
36 leads to the evolution of sexual dimorphism and ultimately the resolution of
37 conflict, by allowing each sex to express the trait independently and to each
38 achieve their optimum phenotype ^{3,4}.

39

40 For sexually dimorphic phenotypes to develop from the same genes, we might
41 expect differences in gene expression (or in downstream modifications and
42 regulation) between the sexes at some stage of development ^{5,6}. Sexually
43 dimorphic gene expression, or sex-biased gene expression, has been examined in
44 detail in a wide-range of species ⁷. But, although it is known that there can be
45 substantial variation in gene expression between developmental stages, less is
46 understood about the sex-specific dynamics of gene expression throughout
47 development ^{6,8,9}. This is a potentially significant gap in our knowledge in the
48 face of evidence that gene expression during early development can strongly
49 impact on adult phenotype ^{10,11}, as can the early environment more generally ¹²⁻
50 ¹⁵.

51

52 In terms of understanding how sex-biased gene expression relates to sexual
53 conflict and conflict resolution, very few studies have directly associated sex
54 differences in gene expression with sex-specific fitness ^{16,17}. Furthermore, our
55 understanding of how the transcriptome is related to fitness is largely based on
56 adult gene expression, and little is known about how sex differences laid down
57 during development might influence overall fitness. Further research is
58 necessary to synthesise a comprehensive picture of how sex-biased gene
59 expression might mediate sexual conflict. We argue that a quantitative genetic
60 and developmental perspective will provide valuable insights into the genetics
61 and ontogeny of conflict and resolution, by enabling genetic covariance across

62 sexes and developmental stages to be quantified and by testing the association of
63 these patterns of gene expression with fitness.

64

65 Here, we present the first quantification of a transcriptome across sexes,
66 developmental stages and genotypes, and interpret the variation in gene
67 expression in terms of sex-specific fitness. We demonstrate widespread sex-
68 biased gene expression in *Drosophila melanogaster* larvae, pupae and adults, as
69 well as considerable differences in the fitness consequences of gene expression
70 throughout development. We combine this developmental perspective with a
71 quantitative genomic approach that has been used increasingly in recent
72 research¹⁶⁻¹⁹. By analysing gene expression in different genetic lines, we find
73 evidence for potential constraints on conflict resolution through genetically
74 correlated gene expression between genes, across sexes and across
75 development. Importantly, we use multivariate analyses alongside our univariate
76 analyses, to account for genetic covariance between different genes, an aspect
77 that is overlooked in more common univariate analyses of gene expression. In
78 addition, although our data does not allow full examination of all potential
79 downstream modifications to gene expression, we do explore a potential route
80 for conflict resolution via differential splicing between males and females. In
81 sum, our results offer new insight into the genetics of sex differences in gene
82 expression throughout development, and how these differences could mediate
83 sexual conflict.

84

85 **RESULTS**

86 RNA was extracted from male and female third-instar larvae, pupae and adults
87 from each of 10 hemiclinal lines, and gene expression was quantified by RNA-
88 sequencing. Each hemiclinal line is generated through a series of crosses¹⁷ that
89 result in each fly within a line (both males and females) sharing a haplotype that
90 is expressed alongside a random haplotype from the base population, allowing
91 additive genetic variation to be estimated for each trait. We examined the
92 expression of 14008 genes in total, of which 13501, 13602 and 13495 genes
93 were expressed in larvae, pupae and adults respectively. Sex-specific fitness for
94 each hemiclinal line (hereafter referred to as a 'line') was measured as
95 reproductive success under competitive conditions.

96

97 **Sex-biased and sexually antagonistic gene expression**

98 Initial analyses partitioned variance in the expression of each gene within each
99 stage between sex, line and sex-by-line effects. Table 1A summarises the
100 numbers of sex-biased genes at each stage (significant 'sex' term at FDR<0.001).
101 Sex-biased expression was generally high throughout development (83.4% and
102 78.7% of larval and pupal genes, respectively), and highest in adults, with 89.9%
103 of genes showing significant differences in expression between adult males and
104 females. In larvae and pupae, sex-biased gene expression is predominantly male-
105 biased, but in adults there are more equal numbers of female- and male-biased
106 genes (Table 1A). Imposing the additional criteria of a fold change >2 reduces the
107 numbers of genes called as sex-biased at each developmental stage, although this
108 reduction is most noticeable in larvae and pupae, indicating that the magnitude
109 of sex-biased expression is generally higher in adults (Table 1A).

110

111 From the same models, significant genetic (between-line) and sex-specific
112 genetic (sex*line) variation were identified at FDR<0.05 (Table 1B). The
113 numbers of genes with significant genetic variation increased throughout
114 development, from approximately a fifth of all genes tested in larvae, to just over
115 a third of all genes tested in adults. Notably, sex-by-line variation - which
116 indicates some extent of genetic variation for sex differences in gene expression -
117 was very low in larvae (1.4%), but higher in adults (15.1%) and considerably

118 higher in pupae (22.1%), suggesting a higher capacity for the evolution of
119 sexually dimorphic gene expression in pupae. In a species with a holometabolous
120 life cycle like *D. melanogaster*, the pupae represent the most dynamic phase of
121 development in terms of tissue differentiation and the development of sex-
122 specific phenotypes, whereas the larval stage mostly concerns growth rather
123 than differentiation, regardless of sex. Although the samples were carefully
124 staged, pupal metamorphosis is such a dynamic developmental stage that we
125 cannot rule out the possibility that genetic variation for gene expression is
126 confounded with genetic differences in the precise timing and process of
127 metamorphosis. Nonetheless, the particularly high level of sex-specific genetic
128 variation in pupae shows that we identified significant genetic variation for sex
129 differences in pupal metamorphosis, consistent with our other findings.

130

131 Candidate sexually antagonistic genes were identified by regressing fitness
132 against gene expression, where a significant sex*fitness interaction (FDR<0.05)
133 was used to call candidate sexually antagonistic (SA) genes, and a significant
134 fitness term in the model (FDR<0.05), with a non-significant sex*fitness
135 interaction, interpreted here as sexually concordant (SC) fitness consequences
136 (Table 1C). Overall numbers of fitness-associated genes (SA and SC combined)
137 were highest in pupae, which consisted predominantly of SC genes. Numbers of
138 SA candidate genes were highest in adults (9.6% of genes tested) and lowest in
139 pupae (4.5%). The overlap in SA and SC calls between stages was low (Figure 1),
140 suggesting that both forms of selection vary widely throughout development. For
141 all tests of sex-biased expression, overall genetic variation, and fitness
142 association, genes are identified in Supplementary File 1.

143

144 Across development, 2571 genes (18.4% of the total 14008 genes tested
145 throughout development) were identified as SA candidate genes in at least one
146 developmental stage, suggesting a higher overall level of conflict throughout the
147 genome than estimated from analyses of adult gene expression alone in the same
148 laboratory population over five years previously¹⁷. Notably, even within the
149 adult subset of our data, there was low and non-significant overlap (approx.
150 10%) between the genes that were called as SA candidates compared to the

151 genes called as SA candidates previously¹⁷. Studies like these capture a snapshot
152 of sexually antagonistic fitness variation at a given time point, but it appears that
153 conflict and resolution are dynamic^{17,20}.

154

155 **Modules of correlated gene expression**

156 In an attempt to simplify a complex, whole-transcriptome dataset and identify
157 patterns of potential functional interest, we identified modules of correlated
158 gene expression among the fitness-related genes (SA and SC combined) within
159 each developmental stage. First, we calculated genetic correlations for each
160 pairwise combination of fitness-related genes for both sexes within each stage,
161 and used the mean absolute value of the correlations for each gene with all other
162 genes as a measure of gene 'connectivity'¹⁶. Average connectivity was generally
163 high, and highest in adults (Figure 2). In larvae and adults, connectivity was
164 significantly higher for SA genes than for SC genes, but the opposite was true in
165 pupae, although the absolute difference was small (Figure 2).

166

167 Next, we inferred functionality by using the pairwise correlations calculated
168 above to cluster the fitness-associated genes into transcription modules within
169 each stage²¹. We tested these modules for enrichment with SA candidate genes
170 at each stage, and tested the largest modules (>100 genes) for enrichment with
171 tissue-specific genes identified from the FlyAtlas database²² of larval and adult
172 tissues (results in Supplementary File 2). There were 27 transcription modules
173 identified in larvae, 5 of which were significantly enriched with larval SA
174 candidate genes at FDR<0.05 (Figure 3A). A large larval transcription module
175 (#11 in Figure 3A) was enriched for both SA candidate genes as well as salivary
176 gland and malpighian tubule-specific genes, suggesting this module may be
177 associated with feeding, and that larval feeding behaviour might have SA fitness
178 consequences. In pupae, there were 56 transcription modules, of which only 4
179 were significantly enriched for pupal SA candidates (Figure 3B). The clustering
180 results for pupal fitness-associated genes were dominated by one large
181 transcription module (#36 in Figure 3B). This module, similarly to the largest
182 transcription module in larvae (#27 in Figure 3A), was enriched for a variety of
183 different tissue-specific genes, all from somatic rather than germ line tissues. In

184 adults, we found 36 transcription modules, of which 8 were enriched with adult
185 SA candidate genes (Figure 3C). Modules #5, 6 and 9 were heavily enriched with
186 testes-specific genes, whereas modules #11 and 19 were enriched for multiple
187 tissue-specific sets of genes, including a combination of head, brain and CNS
188 tissue-specific genes, suggesting a putative link between behaviour and sex-
189 specific fitness. These results are consistent with previous research that has
190 demonstrated sexual conflict over adult locomotory behaviour in *D.*
191 *melanogaster*²³. Module #19 was also significantly enriched with adult SA
192 candidate genes, and so these transcription modules may harbour interesting
193 candidate genes for further research (Figure 3C).

194

195 **Shared quantitative genetic variation**

196 To explore the potential for genetic constraints to hinder the resolution of sexual
197 conflict over gene expression, we measured quantitative genetic variation
198 underlying gene expression. In particular, we were interested in shared genetic
199 variation between sexes and between developmental stages, as this shared
200 genetic variation could prevent the independent evolution of sex- and stage-
201 specific gene expression.

202

203 Initially, we considered genetic variation from a univariate perspective. For each
204 gene with significant genetic variation in at least one developmental stage
205 (N=8761), we ran a model that partitioned variance in gene expression between
206 sex, stage and line, creating a 6x6 genetic variance-covariance matrix for each
207 gene individually (2 sexes x 3 stages), as shown inset in Figures 4A-D. This
208 matrix can be split into four sources of variance: (1) sex- and stage-specific
209 genetic variance (Figure 4A), (2) between-sex genetic covariance within each
210 stage (Figure 4B), (3) between-stage genetic covariance within each sex (Figure
211 4C), and (4) genetic covariance between both sexes and stages (Figure 4D).

212 Covariance estimates were scaled to the total amount of genetic variation in the
213 full matrix. Overall, genes that were identified as SA in at least one stage had
214 significantly more sex- and stage-specific genetic variation than genes that were
215 not SA in any stage of development (Figure 4A). This was expected, as theory
216 predicts that SA selection will help maintain genetic variation^{3,4}. There is also

217 substantial shared genetic variation both between sexes and between stages, and
218 interestingly this is significantly higher for SA genes than non-SA genes in all
219 instances (Figures 4B-D). Notably, there is even evidence of considerable shared
220 genetic variation between-sex and between-stage (Figure 4D), suggesting
221 complex genetic covariance of gene expression across males and females at
222 different developmental stages. The results of these models were also used to
223 calculate the intersexual genetic correlation, r_{mf} , for each gene at each stage. As
224 might be expected from the results of overall between-sex genetic covariance
225 shown in Figure 4B, r_{mf} is higher for SA genes than non-SA genes at each stage,
226 although this difference is small in pupae (Figure 5).

227

228 Next, we used multivariate analyses of quantitative genetic variation, combined
229 with a re-sampling technique, to examine shared genetic variation between-sex
230 and between-gene. This involved calculating the full genetic variance-covariance
231 matrix for sub-samples of genes expressed in males and females (\mathbf{G}_{mf} , or \mathbf{G}
232 matrix). The \mathbf{G} matrix includes the sub-matrix \mathbf{B} , which summarises between-sex
233 and between-gene genetic covariance²⁴ (see Supplementary File 3). We
234 estimated the average \mathbf{G} matrix for SA and SC genes for larvae, pupae and adults
235 independently. Overall, we found that \mathbf{B} tended to have a higher magnitude for
236 SA genes than for SC genes in larvae and adults, indicating that there is higher
237 between-sex and between-gene genetic covariance in SA genes. The opposite
238 was true for pupae (Figure 6A), consistent with the patterns of connectivity for
239 SA genes compared with SC genes in Figure 2. We also estimated the matrix
240 correlation between the upper and lower halves of the \mathbf{B} matrix, and found that
241 this was significantly higher for SA genes than for SC genes at each
242 developmental stage (Figure 6B), although this difference was small in pupae.
243 This shows that genetic covariances are more strongly correlated between sexes
244 for SA than for SC genes, implying more potential for male and female gene
245 expression to evolve independently in SC genes.

246

247 Finally, we used the multivariate breeder's equation²⁴ to estimate the vectors of
248 the predicted response to selection for males and females for SA and SC genes.
249 We then use the angle between these vectors as a measure of the predicted

250 divergence between male and female trait evolution. When the between-sex,
251 between-gene shared genetic variation in **B** is included in these calculations, the
252 divergence between the sexes tends to decrease, as shared genetic variation
253 forces the predicted response to selection between the sexes to realign with one
254 another to some extent. However, this realignment is stronger for SA than SC
255 genes in larvae (Figure 6C), pupae (Figure 6D) and especially in adults (Figure
256 6E), suggesting that the multivariate between-sex genetic covariance within **B**
257 presents a significant constraint on the independent evolution of the sexes,
258 particularly in adults.

259

260 **Evidence for differential splicing**

261 Differential splicing was examined by comparing splicing between male and
262 female samples within each developmental stage, for all genes with evidence of
263 alternative isoform expression. There was most evidence of differential splicing
264 between the sexes in adults, where 1089 genes out of 2822 genes tested (38.6%)
265 showed significant differential splicing between sexes (FDR<0.05). Evidence for
266 significant sex-specific splicing in larvae (0.9%, 14/1636) and pupae (3.8%,
267 77/2015) was low. However, the overall extent of differential splicing (measured
268 as the square root of the Jensen-Shannon distance between the male and female
269 splicing distributions, see methods) was higher in non-SA genes than for SA
270 genes (Figure 7A-C), although this difference was generally small and only
271 significant in larvae (Figure 7A). We also found that genes with a high
272 intersexual genetic correlation tended to exhibit lower levels of differential
273 splicing between the sexes (Figure 7D). The negative correlation between r_{mf} and
274 $\sqrt{J_{S(m,f)}}$ was significant for larvae ($r^2=-0.070$, $P=0.007$) and adults ($r^2=-0.081$,
275 $P<0.001$) but not for pupae ($r^2=-0.038$, $P=0.110$).

276

277 **DISCUSSION**

278 Our results clearly demonstrate that although the developmental transcriptome
279 of *D. melanogaster* exhibits high levels of sexual dimorphism, sexual conflict over
280 the expression of shared genes persists. In fact, 18.4% of the genes tested were
281 identified as sexually antagonistic candidates in at least one stage of
282 development, implying that this dimorphism is not a signature of fully resolved
283 conflict. Furthermore, our analyses provide a detailed account of how conflict
284 could be mediated at the level of the transcriptome, as we find evidence for a
285 number of different sources of constraint that could prevent conflict resolution,
286 as well as finding evidence to support one specific mechanism of resolution.

287
288 First, the overall patterns of sex-specific selection and sex-specific genetic
289 variation - the two main ingredients necessary for independent trait evolution
290 between the sexes²⁵ - vary considerably throughout development. Here, we used
291 lifetime reproductive success (LRS) to estimate selection on genes at each stage.
292 From an evolutionary perspective, LRS provides arguably the most relevant
293 approximation of fitness: selection will favour individuals who leave behind the
294 most offspring (i.e. have the highest LRS). If there is a significant correlation
295 between LRS and the pre-adult expression of a particular gene, then it suggests
296 that the gene might contribute to an aspect of development that affects LRS. Sex-
297 specific selection on gene expression appears inconsistent from one
298 developmental stage to the next, with very little overlap between genes that
299 were identified as either SA or SC across stages. This is perhaps unsurprising
300 given previous research that has demonstrated changes in sex-specific selection
301 through development using different experimental approaches^{9,26}. The numbers
302 of genes with significant sex-specific genetic variation also varied across
303 development, and were especially low in larvae and, to some extent, adults,
304 limiting the potential for independent evolution between the sexes at these
305 stages.

306
307 Second, the results revealed potential genetic constraints on conflict resolution
308 that stemmed from several sources of shared genetic variation: between-gene,
309 between-sex and between-stage, and sometimes a combination of these. Even if

310 there is considerable overall genetic variation for gene expression, if this genetic
311 variation is not independent between contexts where the gene is under different
312 selection, then adaptive evolution of gene expression can be constrained. It is
313 unlikely that any gene would be expressed completely independently^{19,27};
314 however, the extent of genetic covariance is almost always significantly higher
315 for SA genes than it is for SC genes, strongly supporting the idea that this shared
316 genetic variation could prevent conflict resolution. The exception to this was that
317 gene connectivity was significantly lower for pupal SA genes than for pupal SC
318 genes, and similarly, between-sex and between-gene multivariate shared genetic
319 variation in the **B** matrix was lower for pupal SA than SC genes. This may result
320 from the unusual modularity of the pupal transcriptome, where most fitness-
321 related genes clustered into a single large, highly correlated gene module that
322 consisted predominantly of SC fitness-related genes.

323

324 Consistent with previous research²⁸, univariate between-sex genetic covariance
325 and the intersexual genetic correlation is higher for SA genes than for non-SA
326 genes in our data, indicating a putative constraint of genetic variation shared
327 between the sexes within developmental stages. However, the multivariate
328 analyses in this study provide additional insight into between-sex and between-
329 gene genetic covariance that has previously been overlooked. Genetic covariance
330 within the **B** matrix seems particularly influential in adults, where the constraint
331 imposed by **B** on the independent evolution of SA genes between the sexes is
332 high. The results highlight the instability of the genetic covariance in **B** across
333 development, and emphasise the usefulness of a multivariate perspective to
334 examine trait evolution in a more realistic, multi-gene context.

335

336 Of particular interest was the amount of shared genetic variation between both
337 sex and stage. This genetic covariance indicates that the expression of a gene in,
338 for example, male larvae, is not independent of the expression of the same gene
339 in, for example, adult females. Since the fitness consequences for a particular
340 gene are unlikely to be aligned between two different sexes and developmental
341 stages, this could be a source of genetic constraint. This genetic covariance has
342 not, to our knowledge, previously been measured for gene expression, but it

343 might be expected that such covariance would be relatively low and unimportant
344 due to the intuitively weak link between different sexes and stages. In fact, this
345 covariance is of a similar magnitude to the other sources of genetic covariance
346 that were measured, and, also similarly to the other genetic covariance
347 components, it is significantly higher in SA than non-SA genes. This could have
348 implications for antagonistic pleiotropy between developmental stages ^{6,29,30},
349 suggesting that such relationships might be sex-specific. We also find higher
350 genetic covariance identified for SA genes between stages within each sex than
351 for non-SA genes, supporting a link between developmental antagonistic
352 pleiotropy and sexual conflict ⁶. Given the magnitude of between-stage, and
353 between-sex/between-stage, genetic covariance measured here, developmental
354 genetics could have important consequences for conflict resolution. Such
355 developmental covariance has been studied outside of the sexual conflict
356 literature ³¹, but this is clearly also relevant to sexual conflict and the evolution
357 of sexual dimorphism.

358

359 Finally, we present some limited evidence to support the idea that a lack of
360 differential splicing between sexes might also hinder conflict resolution.
361 Previous research has considered a role of sex-specific splicing ^{32,33} or
362 differential exon usage and duplication ³⁴ in allowing the sexes to achieve
363 differential expression from a shared gene. Consistent with this, we find firstly
364 that larval SA genes have significantly less evidence of sex-specific splicing
365 patterns than non-SA genes, and secondly that the intersexual genetic
366 correlation and the extent of sex-specific splicing are significantly negatively
367 associated in larvae and adults - i.e. genes with more shared quantitative genetic
368 variation between the sexes also exhibit less evidence of sex-specific splicing
369 patterns. This indicates another potential genetic constraint on the independent
370 expression of shared genes between the sexes.

371

372 We have therefore identified multiple routes through which conflict resolution
373 could be constrained or facilitated. The novel developmental perspective of this
374 quantitative genomic data is particularly interesting not only because it allowed
375 us to identify genetic covariance between stages, but also because it highlights

376 the different dynamics of sexual conflict at each stage, and the potential to
377 underestimate the extent of conflict or resolution by focussing on only one stage.
378 In short, the larval and adult stages appear to be characterised by conflict and
379 constraint on sex-specific phenotypes, whereas there is less evidence of this in
380 pupae. This is attributable to a complex combination of constraints on larvae and
381 adults that ultimately result in the transcriptome being less independent
382 between the sexes. Since the pupae undergo dramatic metamorphosis and
383 differentiation, compared to the relatively stable larvae and adults, it seems
384 likely that strong selection on the metamorphic process for the formation of
385 optimised sex-specific phenotypes via tissue differentiation may have lead to
386 more extensive conflict resolution in pupae. Indeed, these results are in line with
387 previous work on *D. melanogaster*, which demonstrated some of the most
388 dynamic patterns of gene expression at the start and end of the pupal stage ⁸,
389 indicating that this developmental stage may have evolved a more flexible
390 transcriptome to allow for sex-specific metamorphosis, in contrast to larval and
391 adult stages that are characterised by tissue growth and maintenance,
392 respectively. A holometabolous life cycle allows, to some extent, for a discrete
393 phase of concentrated differentiation between the sexes, with potential for some
394 aspects of development to become uncoupled across metamorphosis ³⁵. Since
395 this phase is absent in hemimetabolous insects, and in other animals more
396 generally, there is potential for conflict to be more prominent during
397 development in other species. It is clear that constraints on conflict resolution
398 are likely to result from a combination of different sources, making the evolution
399 of conflict resolution a complex problem, not least one that appears unlikely to
400 be consistent even throughout the life cycle.
401

402 **METHODS**

403 Hemiclonal haplotypes were sampled from a *D. melanogaster* base population
404 (LH_M) that had been maintained in the laboratory for more than 500 generations
405 as a large outbred population with overlapping generations. Haplotypes were
406 expressed as male or female hemiclonal individuals following a series of crosses
407 ^{17,26}. All flies were reared on a standard molasses diet at 25°C and 65% relative
408 humidity, with a 12:12h light:dark incubator cycle.

409
410 Male and female flies used for the parental cross were allowed to interact and
411 mate for 48h before the males were removed and females were flipped into
412 lightly yeasted laying vials. Females oviposited in these vials for 2h before being
413 flipped into a holding vial. Females were given further 2h laying periods in fresh
414 vials after 4 and 7 days, creating staged vials of developing offspring. For each set
415 of vials, larvae were visually inspected under a dissecting microscope after 4
416 days, when developing testes can be seen through the larval body wall. Larvae
417 were split into sex-specific vials to continue development, with 10 larvae per
418 vial. Eleven days after the initial laying vials were set up, third instar larvae,
419 pupae and 1-day old virgin adults (unable to mate as they eclosed in sex-specific
420 vials) were harvested. Individuals were frozen at -80°C and sample processing
421 took place over the course of 4 weeks.

422
423 RNA extractions were carried out on individual larvae/pupae/adults using
424 TRIzol (Invitrogen) according to the manufacturer's instructions (adjusted
425 protocol for a small amount of starting material). Our aim was to sequence RNA
426 from 180 samples in total, comprising males and females from three
427 developmental stages from each of 10 hemiclonal lines, with three biological
428 replicates per sample type. However, the hemiclone cross produces siblings that
429 do not have the hemiclonal genotype, which are identified as adults with *bw* eye
430 colour among wild-type hemiclonal flies. We therefore carried out additional
431 identification steps for larval and pupal samples, and collected more than twice
432 the number of samples required in order to compensate for non-hemiclonal
433 genotypes. After the initial RNA extraction process (but prior to DNase
434 treatment), a subsample of the extract from larvae/pupae was used in PCR

435 reactions to identify the correct genotypes. Two PCR reactions were carried out:
436 one set of primers was designed for the dominant insertion mutation in the *bw*
437 gene, *bw^D* (F: CTTATCTTTGGAGAGAAGAGA; R: GGATCATCCGTGCATCAAGAC),
438 and the other set for the male fertility factor *kl5* on the Y chromosome (F:
439 GCTGCCGAGCGACAGAAAATAATGACT; R:
440 GTCCCAGTTACGGTTCGGGTTCCATTGT), to make sure the sexes had been
441 identified correctly by phenotype. Primers to a region on chromosome 2R were
442 used as a control reaction (F: AAAAGGTACCCGCAATATAACCCAATAATTT; R:
443 GTCCCAGTTACGGTTCGGGTTCCATTGT). Control and Y chromosome primer
444 sequences were taken from Lott et al. ³⁶.

445
446 After the genotypes had been identified, DNase digestion was carried out on the
447 correct samples, and RNA was suspended in RNase-free water. Library
448 preparation (Illumina TruSeq RiboZero) and Illumina HiSeq sequencing was
449 carried out by AROS Applied Biotechnology (Aarhus, Denmark) according to
450 Illumina v.4 protocols, using 30 sequencing lanes with 6 samples per lane. For
451 each sample, 400ng of total RNA was used and 32-49M reads were achieved per
452 sample. Gene expression data were aligned to the FlyBase version r6.05 of the *D.*
453 *melanogaster* genome ³⁷ and normalised within the R (v3.2.1) BioConductor
454 'QuasR' and 'DESeq' pipeline ³⁸. At this stage, samples were checked for the *bw*
455 gene and the fertility factor *kl5*, and one sample was removed due to having been
456 incorrectly genotyped prior to sequencing (a male pupa had been incorrectly
457 assigned as a female pupa from one of the hemiclinal lines), leaving 179 samples
458 for analysis. The alignment generated read counts for 16727 genes. The dataset
459 was filtered to remove low variance genes, leaving 14008 genes, of which 13501,
460 13602 and 13495 were expressed in larvae, pupae and adults respectively.

461
462 Sex-specific fitness data was recorded as hemiclinal line averages of male and
463 female total reproductive success under competitive conditions, as described in
464 assays in previous studies ^{17,26}. Fitness data was analysed as relative fitness by
465 dividing line-specific fitness means by the maximum average fitness for each sex.
466

467 **Sex-biased and fitness-associated gene expression.** Variation in the read
468 counts for each gene was partitioned in a generalised linear mixed model using
469 the ‘glmer’ function in the R package ‘lme4’³⁹ across sex, line and sex-by-line
470 terms. Initially, a separate model was performed for each gene at each
471 developmental stage. This was due to the difficulties in model convergence
472 trying to run a full model (which would require sex-by-line-by-stage-by-fitness
473 effects) with data from only 10 genetic lines. Models assumed a Poisson
474 distribution and included ID as a random term to account for differences in total
475 number of reads per sample. The significance of variance components was
476 calculated from a $0.5X_0^2+0.5X_1^2$ mixture distribution from a likelihood ratio test
477 comparing the full model with the reduced model (without the component being
478 tested), and *P* values were adjusted according to the false discovery rate (FDR)⁴⁰
479 including the full set of results from all three developmental stages (since most
480 genes were tested at every stage). We then tested if each gene was associated
481 with fitness at each stage, using a GLMM under the same conditions as before but
482 with sex, fitness and fitness*sex. A significant fitness*sex interaction was
483 interpreted as sexually antagonistic (SA) selection, whereas a significant overall
484 fitness term, with a non-significant fitness*sex interaction, was interpreted as
485 sexually concordant (SC) selection.

486
487 **Transcription modules.** We began by calculating the genetic correlation
488 between all pairwise combinations of fitness-related (both SA and SC combined)
489 genes separately in larvae (N=1261), pupae (N=3103) and adults (N=2959). The
490 absolute correlations were used to examine overall gene connectivity (average of
491 all absolute pairwise correlations per gene at each stage), and were then used in
492 a clustering algorithm to identify gene modules²¹. This algorithm works via an
493 optimisation function that finds a pattern of gene modules that maximises
494 correlation within modules while minimising correlation between modules. Each
495 of the identified modules was tested for enrichment with SA genes using
496 hypergeometric tests (*P* values adjusted for FDR). Next, we identified tissue-
497 specific genes for larval and adult tissues using the FlyAtlas *D. melanogaster*
498 microarray database²², and tested modules for enrichment with tissue-specific
499 genes. These tests could only be carried out on modules with >100 genes due to

500 sample size issues. Again, hypergeometric tests were used and P values were
501 FDR-adjusted.

502

503 **Quantitative genetic analyses.** In order to estimate the full univariate between-
504 sex and between-stage genetic variance-covariance matrix for each gene, we
505 used the 'MCMCglmm' package ⁴¹ in R to partition variance in gene expression
506 for the 14008 genes that varied in expression at any stage. The posterior
507 distribution for each model was estimated using weakly informative inverse-
508 Wishart priors for the variance components ($V=\text{diag}(6)/6$, $\nu=6$) and
509 convergence was visually inspected with diagnostic plots. The models included
510 stage and sex as fixed terms, and partitioned genetic variance across a 6x6
511 matrix for each gene, with female- and male-specific larval, pupal and adult
512 genetic variance and all possible covariances. The posterior distribution of these
513 models was used for all univariate quantitative genetic aspects of the analysis.
514 We calculated the intersexual genetic correlation, r_{mf} , from the posterior
515 estimates of variance components ⁴²; and used the absolute sum of the relevant
516 variance components (scaled to the total amount of genetic variation in the full
517 matrix) to compare the amount of between-sex and/or between-stage shared
518 genetic variation across SA and non-SA genes.

519

520 We also examined shared genetic variation between-sex and between-gene for
521 each developmental stage independently. Ideally, this would involve running a
522 multivariate model with expression variance for all genes partitioned between
523 sex and line. However, this would involve estimating a genetic variance-
524 covariance matrix (**G** matrix) with more than 5 million parameters. Instead, we
525 ran 2000 iterations of a multivariate model that randomly sampled 5 genes at a
526 time, and, using the expression of the 5 genes as a multivariate response,
527 partitioned gene expression variance between sex and line. Each model was ran
528 using 'MCMCglmm' ⁴¹ in R, with sex:gene as fixed terms, sex:gene:line as random
529 terms, and sex- and gene-specific residuals. The prior specified a weakly
530 informative inverse-Wishart distribution for the variance components
531 ($V=\text{diag}(10)/10$, $\nu=10$), and model checks were carried out as above. Each
532 iteration of the model generated a posterior distribution of a 10x10 **G** matrix (5

533 genes expressed in 2 sexes). We repeated the analysis for six subsets of genes,
534 which were exclusive within each developmental stage: SA and SC genes in
535 larvae, pupae and adults. Results are summarised from the average **G** matrix
536 calculated from the posterior distribution of all 2000 model iterations.
537 Importantly, this **G** matrix represents the full **G_{mf}** matrix, including the sub-
538 matrix **B**, which summarises the between-sex and between-gene covariance (see
539 Supplementary File 3 for details). We examined genetic variation within **B** using
540 two metrics. First, $\|\mathbf{B}\|$, the matrix norm was calculated to approximate the
541 magnitude of the variation within **B**. The matrix norm was used instead of the
542 matrix trace (that has been used previously to estimate the amount of variance
543 in **G**) since the **B** sub-matrix is composed entirely of covariance estimates and
544 can therefore have negative eigenvalues on the main diagonal. Note, however,
545 that using the sum of the absolute eigenvalues of **B** (i.e. the **B** matrix trace)
546 produces qualitatively identical results. Second, the matrix correlation between
547 the upper and lower halves of **B** was calculated to estimate symmetry of variance
548 within **B**. Finally, we implemented the multivariate breeder's equation:

549 $\Delta\bar{z} = \frac{1}{2}\mathbf{G}\beta$ (Lande 1980). This equation uses the product of the genetic variation

550 in **G** and the vector of linear selection gradients in β to calculate a vector of the
551 predicted response to selection for each trait, $\Delta\bar{z}$. We generated a vector of
552 linear selection gradients that corresponded to each estimate of the **G** matrix in
553 the models described above. For each iteration of the **G** matrix model, selection
554 gradients for the same 5 genes were estimated from a multiple linear regression
555 of the genes against relative fitness for males and females, following Lande ²⁴.
556 This allowed us to calculate sex-specific $\Delta\bar{z}$ based on the sex-specific
557 components of **G** and β . This calculation was carried out using the distribution of
558 2000 estimates, to create a distribution of $\Delta\bar{z}$ estimates, from which confidence
559 intervals could be used to assess significant differences.

560

561 Finally, to include **B** in the calculation of $\Delta\bar{z}$, the multivariate breeder's equation
562 ²⁴ can be expanded as:

563

564
$$\begin{pmatrix} \Delta\bar{z}_{mB} \\ \Delta\bar{z}_{fB} \end{pmatrix} = \frac{1}{2} \begin{pmatrix} \mathbf{G}_m & \mathbf{B} \\ \mathbf{B}^T & \mathbf{G}_f \end{pmatrix} \begin{pmatrix} \beta_m \\ \beta_f \end{pmatrix} \quad (1)$$

565

566 where $\Delta\bar{z}_{mB}$ and $\Delta\bar{z}_{fB}$ represent the predicted response to selection of each sex
567 given both the sex-specific genetic variation in \mathbf{G}_m and \mathbf{G}_f and the shared genetic
568 variation within \mathbf{B} . The predicted divergence between the male and female
569 response to selection was measured as the angle between male and female $\Delta\bar{z}$,
570 with and without \mathbf{B} . The angle was calculated as:

571

572
$$\theta = \cos^{-1} \left(\frac{\Delta\bar{z}_f \cdot \Delta\bar{z}_m}{\|\Delta\bar{z}_f\| \|\Delta\bar{z}_m\|} \right) \quad (2)$$

573 with $\Delta\bar{z}_{mB}$ and $\Delta\bar{z}_{fB}$ substituted for the calculations accounting for shared genetic
574 variation in \mathbf{B} . As before, all calculations used the distribution of 2000 estimates
575 from the original models to enable confidence interval calculation and
576 significance testing.

577

578 **Differential splicing.** We used the cuffdiff function in Cufflinks software ⁴³ to
579 test for evidence of differential splicing between males and females at each stage.
580 This test runs for any gene where alternative isoform expression is found, which
581 in this dataset tested 1636, 2015 and 2822 genes in larvae, pupae and adults
582 respectively. The analysis uses the Jensen-Shannon distribution to test for
583 significant differential splicing between the male and female samples within each
584 developmental stage.

585

586 **Accession codes.** RNA-seq data have been deposited with accession SRP068235

587

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593

594 **AUTHOR CONTRIBUTIONS**

595 F.C.I. and E.H.M. wrote the paper and designed the experiments. T.M.P. and I.F.
596 set up the hemiclone lines and collected fitness data. F.C.I. and C.L.W. collected
597 the samples and performed the molecular work. F.C.I. performed the statistical
598 analyses.

599

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- 709

710 **TABLES AND FIGURES**

711

712 **Table 1.** Overall numbers of genes that were (A) significantly sex-biased, (B) had
 713 significant between-line (genetic) and sex*line (sex-specific genetic) variation,
 714 and (C) significantly associated with sexually antagonistic (fitness*sex) or
 715 sexually concordant (fitness with no fitness*sex interaction) fitness. All results
 716 are taken from the linear models described in the text. In total, 13501, 13602
 717 and 13495 genes were tested for larvae, pupae and adults respectively. The
 718 percentages of these totals are shown italicised in brackets.

	Larvae	Pupae	Adults
(A) Sex-biased	11262 (83.4)	10705 (78.7)	12133 (89.9)
Sex-biased (fold change > 2)	4535 (33.6)	2613 (19.2)	8252 (61.2)
Female-biased	1200 (8.9)	3620 (26.6)	5696 (42.2)
Female-biased (fold change > 2)	131 (1.0)	324 (2.4)	3525 (26.1)
Male-biased	10062 (74.5)	7085 (52.1)	6437 (47.7)
Male-biased (fold change > 2)	4404 (32.6)	2289 (16.8)	4727 (35.1)
(B) Genetic variation	2870 (21.3)	4706 (34.6)	4912 (36.4)
Sex-specific genetic variation	192 (1.4)	3110 (22.3)	2043 (15.1)
(C) Sexually antagonistic	841 (6.2)	610 (4.5)	1300 (9.6)
Sexually concordant	420 (3.1)	2493 (18.3)	1659 (12.3)

719

720 **Figure 1.** Venn diagrams of the overlap between (A) sexually antagonistic and
 721 (B) sexually concordant candidate genes called in larvae (L), pupae (P) and
 722 adults (A). In total, 2571 genes were SA in at least one stage (18.4% of the 14008
 723 genes tested), and 4148 genes were SC in at least one stage (29.6%).

724

725 **Figure 2.** Connectivity of all fitness-related genes for each stage, as the average
 726 absolute genetic correlation of all pairwise gene combinations within each stage.
 727 White boxes represent the overall connectivity for all fitness-related genes in
 728 larvae (L), pupae (P) and adults (A). Smaller boxes represent connectivity of
 729 sexually antagonistic (dark grey) and sexually concordant (pale grey) genes
 730 separately. Numbers of genes included in each box are shown. Notches in

731 boxplots represent 95% CI approximations, as $\pm 1.58 \cdot \text{IQR} / \sqrt{N}$. Asterisks indicate
732 significance of the difference between SA/SC genes at each stage.

733

734 **Figure 3.** Modules of correlated gene expression for all fitness-related genes in
735 (A) larvae (N=1261); (B) pupae (N=3103); and (C) adults (N=2959). Colours
736 within modules represent genetic correlations between all pairwise
737 combinations of genes; colours between modules represent the average genetic
738 correlation between modules. Outlined modules are those that tested significant
739 for enrichment with sexually antagonistic genes. Numbered modules are those
740 with >100 genes that were tested for enrichment with tissue-specific genes.

741

742 **Figure 4.** Histograms of the between-sex and between-stage genetic variance-
743 covariance matrix components for individual genes, partitioned between
744 variance components as shown in the inset matrix diagrams. (A) Sum of the sex-
745 specific, stage-specific genetic variance. (B) Sum of the absolute values of the
746 between-sex genetic covariance within each stage. (C) Sum of the absolute values
747 of the between-stage genetic covariance within both sexes. (D) Sum of the
748 absolute values of the between-sex, between-stage genetic covariance. Only
749 genes with significant genetic variance for at least one developmental stage are
750 included (N=8761). Genes that are sexually antagonistic in at least one stage
751 (N=2056) are shown in dark grey and genes that are not SA at any stage are
752 shown in pale grey (N=6705). Notches in boxplots represent $\pm 1.58 \cdot \text{IQR} / \sqrt{N}$.
753 Asterisks indicate significance of the difference between SA/non-SA genes at
754 each stage.

755

756 **Figure 5.** Intersexual genetic correlation (r_{mf}) for sexually antagonistic (dark
757 grey) and non-sexually antagonistic (pale grey) genes in larvae (L), pupae (P)
758 and adults (A). Only genes with significant genetic variation at each stage are
759 shown, and the numbers of genes included in each box are shown. Notches in
760 boxplots represent $\pm 1.58 \cdot \text{IQR} / \sqrt{N}$. Asterisks indicate significance of the
761 difference between SA/non-SA genes at each stage.

762

763 **Figure 6.** Genetic variation in the average **B** matrix (between-sex and between-
764 gene) for SC and SA genes in larvae (L), pupae (P) and adults (A). (A) The **B**
765 matrix norm, $||\mathbf{B}||$, as a measure of the overall magnitude of genetic variation in
766 **B** for SC (pale grey) and SA (dark grey) genes at each stage. Boxes represent the
767 estimates from all 2000 iterations of the model, notches in boxplots represent
768 $\pm 1.58 \cdot \text{IQR} / \sqrt{N}$, and asterisks indicate significant difference between SA/SC
769 genes at each stage. (B) The symmetry of the **B** matrix, as the matrix correlation
770 between the two halves of **B**, for SC (pale grey) and SA (dark grey) genes at each
771 stage. Boxes represent the estimates from all 2000 iterations of the model,
772 notches in boxplots represent $\pm 1.58 \cdot \text{IQR} / \sqrt{N}$, and asterisks indicate significance
773 of the difference between SA/SC genes at each stage. (C-E) The angle between
774 the male and female predicted response to selection without the **B** matrix (solid
775 arrows) and adjusted for the inclusion of the **B** matrix (dashed arrow) for SC
776 (pale grey) and SA (dark grey) genes in larvae (C), pupae (D) and adults (E).
777

778 **Figure 7.** Evidence for differential splicing between males and females in (A)
779 larvae (1636 genes tested); (B) pupae (2015 genes); and (C) adults (2822 genes),
780 as histograms of the square root of the Jensen-Shannon distance. Sexually
781 antagonistic genes are shown in dark grey; non-sexually antagonistic genes in
782 pale grey. The numbers of sexually antagonistic genes tested was generally low
783 (151, 63 and 306 genes respectively for larvae, pupae and adults). Notches in
784 boxplots represent $\pm 1.58 \cdot \text{IQR} / \sqrt{N}$. Asterisks indicate significance of the
785 difference between SA/non-SA genes at each stage. (D) The square root of the
786 Jensen-Shannon distance plotted against the intersexual genetic correlation (r_{mf})
787 for larvae (green points), pupae (red) and adults (blue).
788

789 **SUPPLEMENTARY MATERIAL**

790

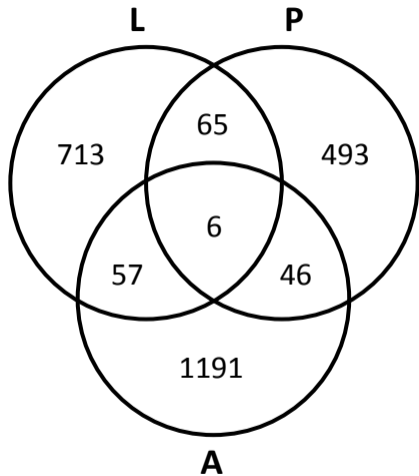
791 **Supplementary File 1.** List of Entrez Gene IDs tested at each developmental
792 stage, annotated with results of significance tests for fitness association, sex-
793 biased expression and genetic variation.

794

795 **Supplementary File 2.** Results from analysis of gene clusters. Gene modules and
796 connectivity of genes within modules are given for each fitness-associated (SA or
797 SC) gene at each developmental stage (identified by Entrez Gene IDs). Results of
798 hypergeometric tests (adjusted for $FDR < 0.05$) for cluster enrichment with (A) SA
799 genes and (B) tissue-specific genes are also provided.

800

801 **Supplementary File 3.** Summary of the structure of the full \mathbf{G} matrix, \mathbf{G}_{mf} , and
802 how it was used in the multivariate analyses.

A.**B.**