Article

Dynamics of sex-biased gene expression during development in the stick insect *Timema californicum*

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Abstract

Sexually dimorphic phenotypes are thought to arise primarily from sex-biased gene expression during development. Major changes in developmental strategies, such as the shift from hemimetabolous to holometabolous development, are therefore expected to have profound consequences for the dynamics of sex-biased gene expression. However, no studies have previously examined sex-biased gene expression during development in hemimetabolous insects, precluding comparisons between developmental strategies. Here we characterized sex-biased gene expression at three developmental stages in a hemimetabolous stick insect (*Timema californicum*): hatchlings, juveniles, and adults. As expected, the proportion of sex-biased genes gradually increased during development, mirroring the gradual increase of phenotypic sexual dimorphism. Sex-biased genes identified at early developmental stages were generally consistently male- or female- biased at later stages, suggesting their importance in sexual differentiation. Additionally, we compared the dynamics of sex-biased gene expression during development in *T. californicum* to those of the holometabolous fly *Drosophila melanogaster* by reanalyzing publicly available RNA-seq data from third instar larval, pupal and adult stages. In *D. melanogaster,* 84% of genes were sex-biased at the adult stage (compared to only 20% in *T. californicum*), and sex-biased gene expression increased abruptly at the adult stage when morphological sexual dimorphism is manifested. Our findings are consistent with the prediction that the dynamics of sex-biased gene expression during development differ extensively between holometabolous and hemimetabolous insect species.

Introduction

Males and females often have divergent evolutionary interests, resulting in sex-specific selection pressures and ultimately the evolution of sexually dimorphic phenotypes (Lande 1980; Khila *et al.* 2012). Studies investigating how sexually dimorphic phenotypes are generated have shown the importance of differential gene expression between the sexes, suggesting that sex-specific selection is the major driving force behind the evolution of sex-biased gene expression (Mank 2017). The relationship between sexual dimorphism and sex-biased gene expression has been largely studied at the adult stage when sexual dimorphism is completely manifested and reproductive interests between males and females are most different (Mank 2017). However, sex-biased gene expression has also been found at early developmental stages of many species, well before any phenotypic sexual dimorphism becomes apparent (Lowe *et al.* 2015; Paris *et al.* 2015). This suggests that expression patterns in early developmental stages are also under sex-specific selection pressures (Mank *et al.* 2010; Hale *et al.* 2011; Zhao *et al.* 2011; Perry *et al.* 2014; Ingleby *et al.* 2015).

Because we lack detailed studies of sex-biased gene expression during development, we do not know how consistent or dynamic the expression of the sex-biased genes is, nor how these dynamics relate to changes in phenotypic sexual dimorphism. For example, although we expect to see an overall increase in sex-biased gene expression during development (Ingleby *et al.* 2015; Mank 2017), it is not clear whether this results from the gradual increase of sex-bias of a set of genes or if sex-biased genes at early stages are largely different to those at later stages (Mank 2017). Furthermore, sex-biased gene expression can be considered to reflect a broad measure of sexual dimorphism, including physiological and behavioral traits, and may

67 therefore be more representative than dimorphism quantified using external
68 morphology. However, whether the extent of sexual dimorphism and of sex-biased
69 gene expression are generally correlated remains poorly known.

70 In insects there are two major developmental strategies, and developmental
71 patterns of sex-biased gene expression could differ between them. Holometabolous
72 insects have morphologically and ecologically distinct larval, pupal, and adult stages,
73 and phenotypic sexual dimorphism is commonly prominent only at the adult stage.
74 Contrastingly, hemimetabolous insects go through gradual morphological changes
75 and sexual differentiation, and the nymphal stages morphologically resemble adults
76 (Chen *et al.* 2010). These distinct dynamics for sexual morphological differentiation
77 are expected to be mirrored by sex-biased gene expression, with abrupt versus
78 gradual increases in the proportion of sex-biased genes during development.
79 However, studies of sex-biased gene expression during development have thus far
80 only been conducted in holometabolous insects (Magnusson *et al.* 2011; Rago *et al.*
81 2020), with the main focus on *Drosophila* (Perry *et al.* 2014; Ingleby *et al.* 2015),
82 precluding comparisons between species with different developmental strategies. For
83 example, more than 50% of the transcriptome is sex-biased in pre-gonad tissue in
84 juvenile stages of *Drosophila melanogaster* (Perry *et al.* 2014) but similar data are not
85 available for hemimetabolous species. Furthermore, sex-biased genes in reproductive
86 tissues provide only partial insight into total sexual dimorphism, since it excludes
87 differences beyond sexual organs, such as secondary sexual traits.

88 Here, we studied the dynamics of sex-biased gene expression across
89 development in a hemimetabolous insect, *Timema californicum. Timema* are sexually
90 dimorphic walking-stick insects, found in California (Vickery and Sandoval 2001).
91 Males are smaller than females, with different coloration, and have conspicuous cerci

92 used for holding on to the female during copulation. We selected three developmental
93 stages (Fig. 1a): the hatchling stage where sexes are phenotypically identical, the third
94 juvenile stage with minor phenotypic differences, and the adult stage, with pronounced
95 sexual dimorphism. We investigated whether sex-biased genes are recruited in a
96 stage-specific manner, and if the magnitude of sex-bias increases with development.
97 We further explored sequence evolution rates of sex-biased genes in pre-adult stages,
98 to determine if they show elevated rates as commonly observed for sex-biased genes
99 at adult stages in many species (Ellegren and Parsch 2007; Grath and Parsch 2012;
100 Perry *et al.* 2014).

101 We then compared the dynamics of sex-biased gene expression during
102 development in the hemimetabolous stick insect to those of the holometabolous fly
103 *Drosophila melanogaster* by reanalyzing publicly available RNA-seq data from 3rd.
104 instar larvae, pupal and adult stages, using the same pipeline as for *T. californicum.*
105 In *D. melanogaster*, both larval and pupal stages have little morphological sexual
106 dimorphism, while the adult stage has extensive dimorphism (Fig. 1b). We
107 hypothesized that sex biased gene expression follows the establishment of
108 morphological sexual dimorphism, with a gradual increase in sex-biased gene
109 expression during development in *T. californicum*, and an abrupt increase at the adult
110 stage in *D. melanogaster*.

111 We showed that the proportion of sex-biased genes gradually increases during
112 development in *T. californicum*, mirroring the gradual differentiation of phenotypic
113 sexual dimorphism in hemimetabolous insects. Sex-biased genes from early
114 developmental stages largely remained sex-biased at later stages. Finally, our findings
115 are consistent with the prediction that the dynamics of sex-biased gene expression

116 during development differ extensively between holometabolous and hemimetabolous
117 insect species.
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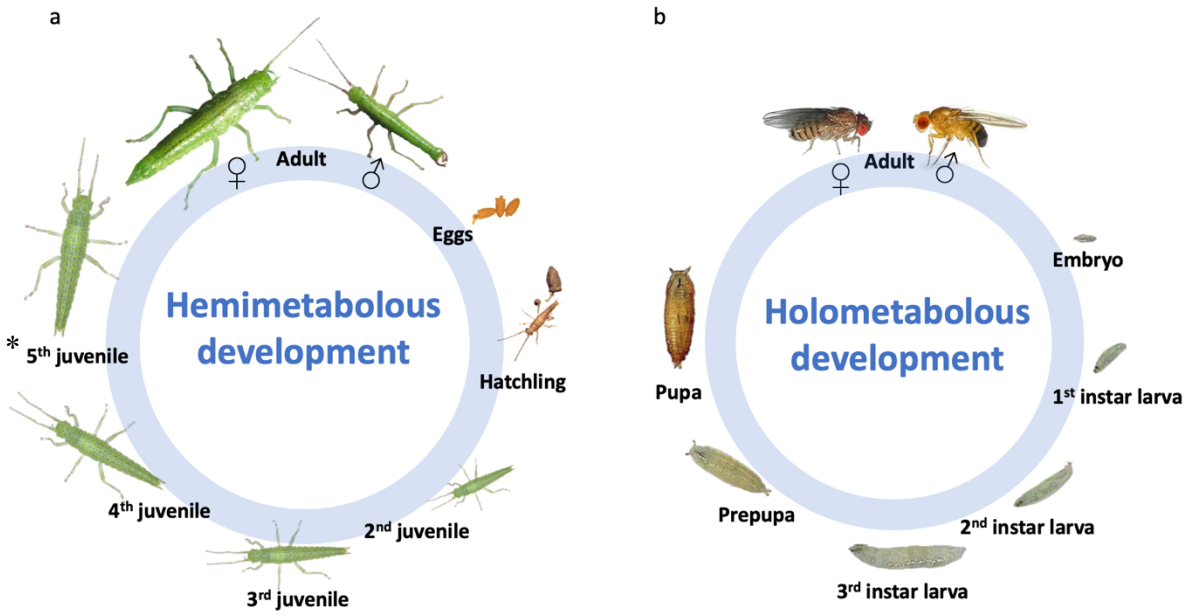

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120

121 **Fig. 1 |** Life cycles of (a) the stick insect *T. californicum* and (b) the fly *D. melanogaster.*
122 *represents a stage present only in females, as *Timema* males have one moult less
123 than females. Hatchling and egg photographs of *Timema* were kindly provided by Bart
124 Zijlstra (http://www.bartzijlstra.com), juvenile and adult stages by Jelisaveta Djordjevic.
125 Life cycle of *D. melanogaster* modified from Weigmann *et al.* (2003).
126

127 ## Results

128 ### Sex-biased gene expression increases during development in *T. californicum*

129 Sex-biased gene expression gradually increased during the three developmental
130 stages, with 0.2% (26) of the expressed genes sex-biased at the hatchling stage, 4.7%

131 (568) at the juvenile, and 20.3% (2485) at the adult stage (Fig. 2). There are
132 significantly fewer sex-biased genes both at the hatchling stage compared to the
133 juvenile stage ($\chi^2_{(1)}$ = 498.11, $p_{adj.}$= 2.2x 10^{-16}) and at the juvenile compared to the
134 adult stage ($\chi^2_{(1)}$ = 1347, $p_{adj.}$= 2.2x 10^{-16}). In addition, the two effect sizes are similar;
135 $h_{(hatchling\text{-}juvenile)}$ = 0.34, $h_{(juvenile\text{-}adult)}$ = 0.49 (Cohen 1988), supporting a gradual rather
136 than abrupt increase. The hatchling stage had more female than male biased genes
137 ($\chi^2_{(1)}$ =11.13, p= 0.0004), while the juvenile and adult stages had more male biased
138 genes ($\chi^2_{juvenile\ (1)}$ =467.1, $\chi^2_{adult\ (1)}$ =85.915, p< 2.2x 10^{-16}) (Supplemental Table 4). All
139 except one of the sex-biased genes at the two preadult stages had strong sex-bias
140 (>2 FC), while at the adult stage 77% had strong sex-bias (>2 FC) (Fig. 2, see also
141 Supplemental Table 4 and Fig 8.). The intensity of sex-bias varied greatly during
142 development, with 1137 genes showing a significant sex by development interaction.

143 To characterize the dynamics of sex-biased expression during development, we then
144 classified the 2671 genes which are sex-biased at one or more developmental stages
145 into 13 categories, dependent on their expression patterns during development
146 (Supplemental Fig. 1). This classification showed that sex-biased genes are added
147 gradually during development, with genes sex-biased at earlier stages generally
148 remaining sex-biased in the same direction at later stages (Supplemental Fig. 1 and
149 2). Only a single gene shifted from male-biased to female-biased expression
150 (Supplemental Fig. 1). Out of 26 sex-biased genes at the hatchling stage, 18 (69%)
151 were also significantly sex-biased in at least one of the later stages, with 9 (35%)
152 remaining sex-biased throughout development (Fig. 3), while out of 568 sex-biased
153 genes at the juvenile stage, 390 (69%) stayed sex-biased at the adult stage (Fig. 3).
154 This classification is conservative, as it depends on the sex-biased gene expression
155 detection threshold, meaning sex-biased gene expression may be missed in some

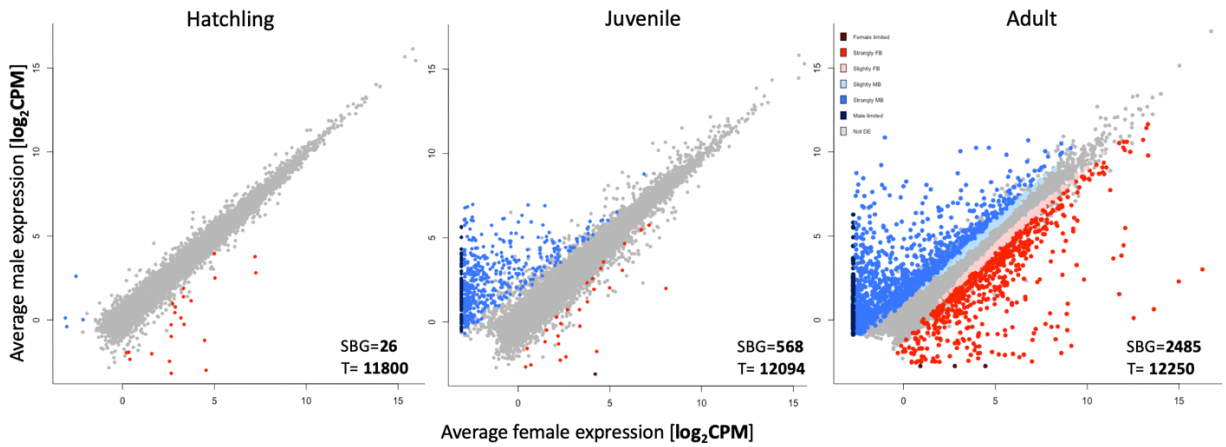
156 stages inflating the number of differences we see. This is supported by the fact that
157 only around 65% of genes that are sex-biased in two stages show a significant sex by
158 development stage interaction (Supplemental table 8, also see Supplemental Fig. 1).
159 Furthermore, the fold changes of sex-biased genes were strongly correlated between
160 all developmental stages (ρ = 0.75 - 0.9 Supplemental fig 3).

161 Sex-biased genes at the hatchling stage are particularly interesting because they
162 reveal sexual differentiation prior to visible morphological differences. We therefore
163 looked for functional annotations of the 26 genes sex-biased at the hatchling stage in
164 the *T. californicum* reference genome (Jaron *et al.* 2020). However, only 16 out of the
165 26 sex-biased genes had functional annotations and only one (*vitellogenin receptor*)
166 had a clear link to sexual differentiation (Fig. 3b). While key genes known to play a
167 role in insect sex-determination and differentiation pathways (*doublesex*, *transformer-*
168 *2* and *sex-lethal*) were expressed at the hatchling stage, *doublesex* had very low
169 expression, preventing analysis of sex-bias, and *transformer-2* and *sex-lethal* did not
170 feature sex-biased expression.

171 **Sex-biased genes are enriched for development-related processes**

172 Genes sex-biased at the hatchling stage were enriched for GO-terms related to
173 developmental processes (e.g., “regulation of cell development”, “regulation of cell
174 proliferation”, “cuticle development”), and GO-terms related to female specific
175 functions such as oogenesis (“oocyte construction”, “oocyte development”)
176 (Supplemental Fig. 5 a, and Supplemental Table 10). At the juvenile stage, sex-biased
177 genes were enriched for GO-terms related to metabolic processes, in particular to
178 catabolism (e.g., “lipid catabolic processes”, “cellular catabolic processes”, “regulation

179 of catabolic processes") (Supplemental Fig. 5 b, and Supplemental table 11). At the
180 adult stage, sex-biased genes were enriched for GO-terms related to diverse
181 metabolic and physiological processes with no clear association to sexual
182 differentiation (Supplemental Fig. 5 c, and Supplemental table 12); however, several
183 terms were related to chemosensory and olfactory behavior, which may play a role in
184 mate detection. Furthermore, sex-biased genes at the adult stage were enriched for
185 pigmentation (e.g., "developmental pigmentation", "eye pigmentation"), which is a
186 sexually dimorphic trait in *T. californicum*.



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188 **Fig. 2 |** Gene expression (log_2CPM) in *T. californicum* males and females at the
189 hatchling (left), juvenile (middle) and adult stage (right). The number of differentially
190 expressed genes (SBG) is shown at the bottom right corner of each plot, as well as
191 the total number of expressed genes at each stage (T). Genes are classified based
192 on their sex-bias into seven categories: "slight FB"- female bias (<2 FC), "strong FB"-
193 female bias (>2 FC), "female limited"- with no expression in males, "slight MB"- male
194 bias (<2 FC), "strong MB"- male bias (>2 FC), "male limited"- no expression in females,
195 "Not DE"- not differentially expressed genes.

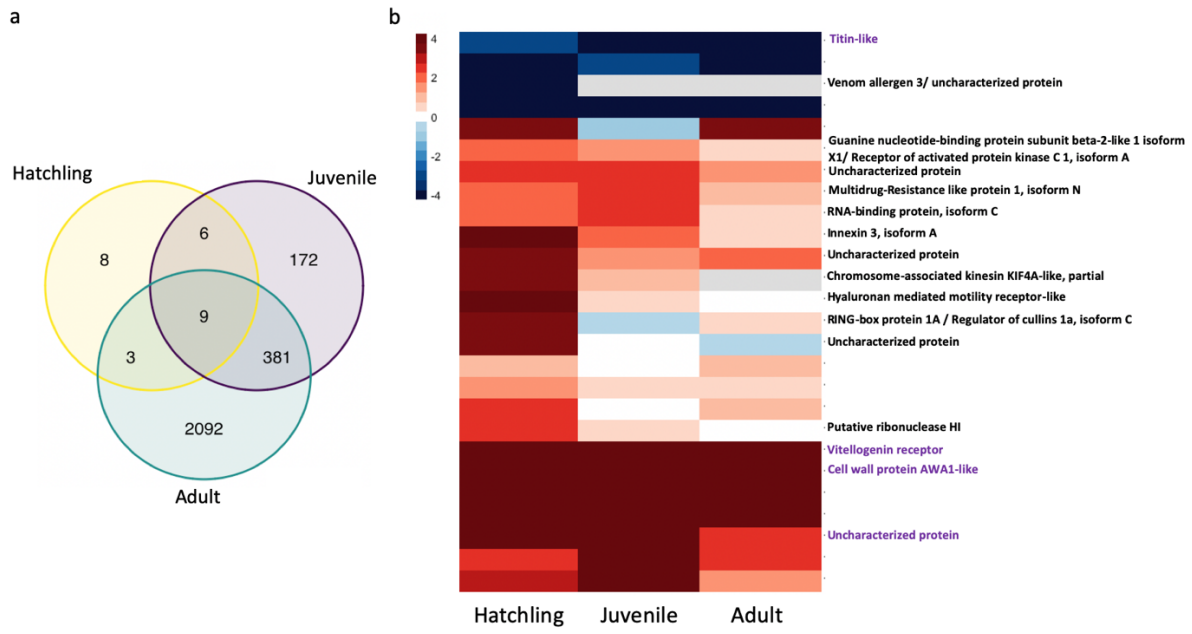
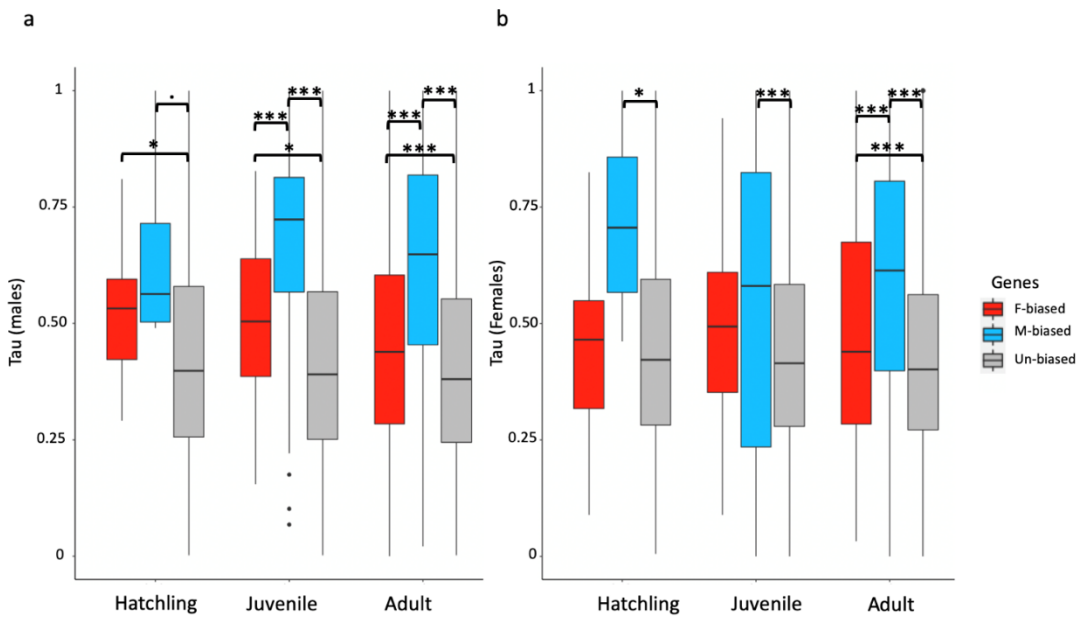


Fig. 3 | Sex-biased genes shared across development. (**a**) Venn-diagram of sex-biased genes in three developmental stages: hatchling (yellow), juvenile (purple), and adult (green). The number of genes shared between all three stages was greater than expected by chance (observed overlap= 9, expected overlap= 0.21, Exact test of multi set interactions: p_{adj} = 5x10^{-13}). Note that all pairwise overlaps also contained more genes than expected by chance (detailed results in Supplemental Table 7). (**b**) A heatmap showing the 26 sex-biased genes at the hatchling stage with their annotations and their expression in three developmental stages. Genes in red are female-biased, blue are male-biased. Saturation of the colors increases with the $\log_2$FC. Genes without a label have no annotation, annotation in purple is for genes that were sex-biased in all three stages

Sex-biased genes have more stage-specific expression

Expression levels of sex-biased genes tend to be specific to only one developmental stage while unbiased genes have a more constant expression level across development (Fig. 4, Supplemental Fig. 4). Male-biased genes have the most stage

212 specific expression in both males and females. Female-biased genes are only slightly
213 more stage specific than un-biased genes, but the trend is consistent and the
214 difference is significant notably in both male and female adults.



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216 **Fig. 4 |** Tau index of gene expression at three developmental stages in males (a) and
217 females (b). Tau range from zero (similarly expressed during development) to one
218 (gene expressed in only one stage). Three gene categories are depicted with different
219 colors; female-biased in red, male-biased in blue, and un-biased in grey. Boxplots
220 represent the median, lower and upper quartiles, and whiskers the minimum and
221 maximum values (in the limit of 1.5x interquartile range). Adjusted p values of Wilcoxon
222 rank sum tests are summarized above the box plots (*** = $p < 0.001$, * = $p < 0.05$, ·
223 $p=0.06$).

224 **Sex-biased genes have faster sequence evolution rates**

225

226 Male-biased genes have faster rates of sequence evolution (dN/dS) compared to both
227 un-biased and female-biased genes, at juvenile and adult stages (Fig. 5). Female-

228 biased genes do not evolve significantly faster than un-biased genes, at any of the
229 three developmental stages.

230 We performed partial correlation analyses to test the effect of sex bias strength
231 on sequence evolutionary rate, controlling for the average expression level of the
232 gene, and GC-content (Supplemental Table 6). For both male-biased and female-
233 biased genes, stronger sex-bias is associated with faster sequence evolution at the
234 adult stage (Fig. 6). Partial correlations are not significant at juvenile or hatchling
235 stages.

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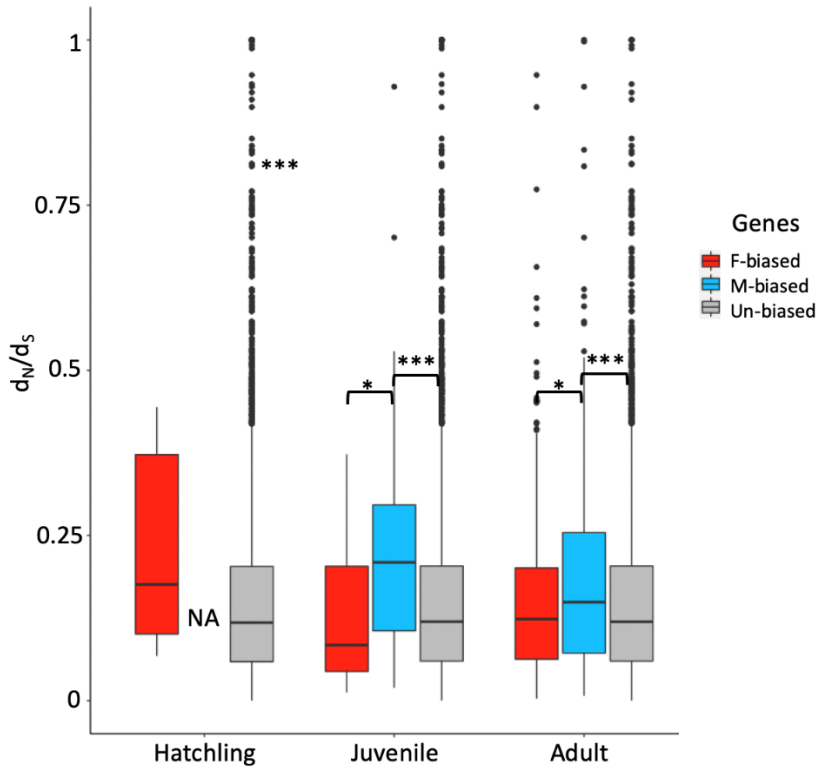

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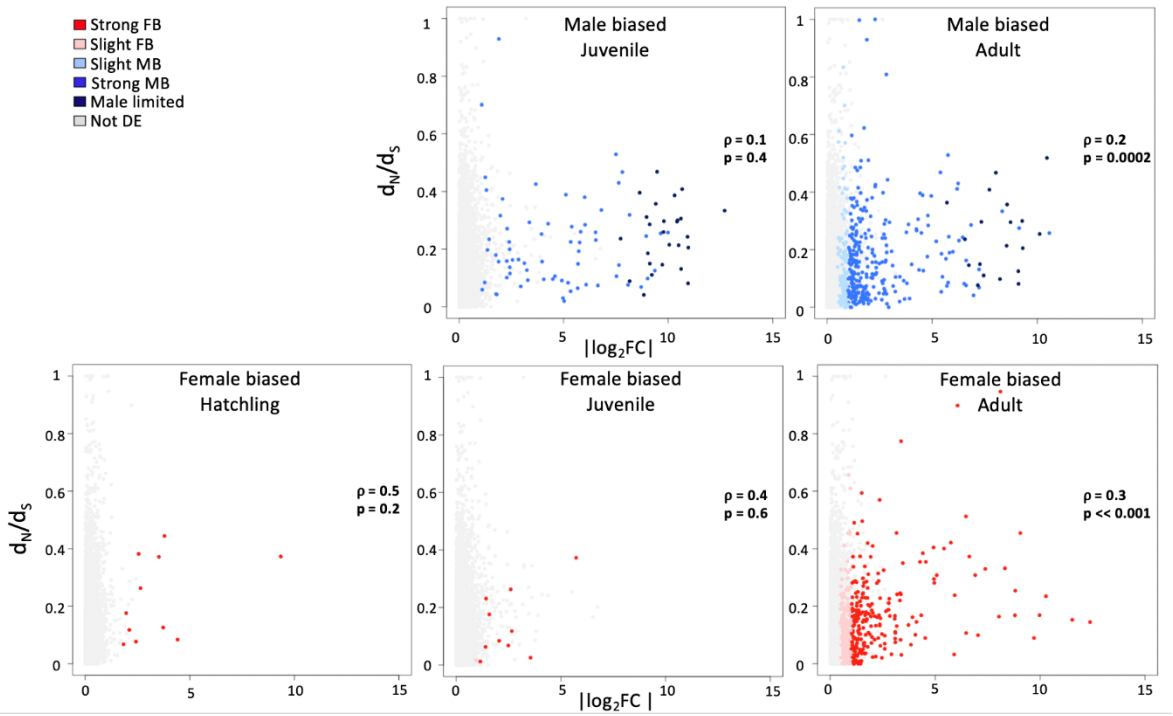
238 **Fig. 5 |** Divergence rates (the ratio of nonsynonymous to synonymous substitutions,
239 d_N/d_S), at three developmental stages; hatchling, juvenile and adult stage. Boxplots
240 represent the median, lower and upper quartiles, and whiskers the minimum and
241 maximum values (in the limit of 1.5x interquartile range). Adjusted p values of Wilcoxon

242 rank sum tests are summarized above the box plots (*** = p < 0.001, * = p < 0.05).
243 Note that the four male-biased genes at the hatchling stage had no identified ortholog
244 in other *Timema* species and therefore no associated d_N/d_S value.



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246 **Fig. 6 |** Partial Spearman's rank correlations between the divergence rate of genes,
247 d_N/d_S, and their strength of the sex bias $|\log_2 FC|$ at three developmental stages. Note
248 that all genes are displayed on each plot but only male- respectively female-biased
249 ones are used for correlation tests. Adjusted p values and partial correlation
250 coefficients are shown in each plot.

251 **Sex-biased gene expression increases abruptly in adult *D. melanogaster***

252 In order to compare the dynamics of sex-biased gene expression during development
253 between hemimetabolous and holometabolous insects, we reanalyzed publicly
254 available data from *D. melanogaster* (Ingleby *et al.* 2016), using the same pipeline as
255 for *T. californicum.* This experiment was chosen for comparison as it has a similar

256 design, i.e. whole-body RNA-seq data from several developmental stages. In addition,
257 although *D.* melanogaster from this experiment were lab-reared, they had a similar
258 between sample variance as *T. californicum* (biological coefficient of variation at the
259 adult stage: *D. melanogaster* = 0.274, *T. californicum* = 0.348).

260 In contrast to the gradual increase in the amount of sex-biased expression during
261 development observed in *T. californicum,* sex-biased gene expression increased
262 abruptly at the adult stage in *D. melanogaster* (Fig. 7 and Fig. 8.), from 22% to 84% of
263 sex-biased genes (20% to 69% restricting to > 2 FC) (Fig. 8). There were significantly
264 fewer sex-biased genes both at the larval compared to the pupal stage ($\chi^2_{(1)}$ = 19.445,
265 $p_{adj.}$ = 5.18 x 10^{-6}) and at the pupal compared to the adult stage ($\chi^2_{(1)}$ = 9607.2, $p_{adj.}$=
266 4.40 x 10^{-16}). The two effect sizes were very different, with a more extensive shift from
267 pupa to adult ($h_{(larva\text{-}pupa)}$ = 0.06, $h_{(pupa\text{-}adult)}$ = 1.35) supporting the abrupt increase.
268 Moreover, adult *D. melanogaster* had a significantly larger proportion of sex-biased
269 genes than *T. californicum* ($\chi^2_{(1)}$ =10061, p< 2.2 x 10^{-16}). Overall, at each
270 developmental stage, there were more male than female-biased genes (Supplemental
271 table 5). The majority of sex-biased genes had a strong sex bias at every
272 developmental stage (> 2 FC: 83%, 94% and 82%, in larval, pupal, and adult stage,
273 respectively) (Supplemental table 5). Out of 2113 sex-biased genes at the larval stage,
274 1262 (60%) were significantly sex-biased in at least one of the later stages, with 1226
275 (58%) remaining sex-biased throughout development (Fig. 7b), while out of 2643 sex-
276 biased genes at the pupal stage, 2423 (92%) stayed sex-biased at the adult stage
277 (Fig. 7b). The fold changes of sex-biased genes were also low to moderately
278 correlated between larvae and pupae or adults (ρ = 0.30 and 0.69; Supplemental Fig.
279 3) but strongly correlated between pupae and adults (ρ = 0.85; Supplemental Fig. 3).

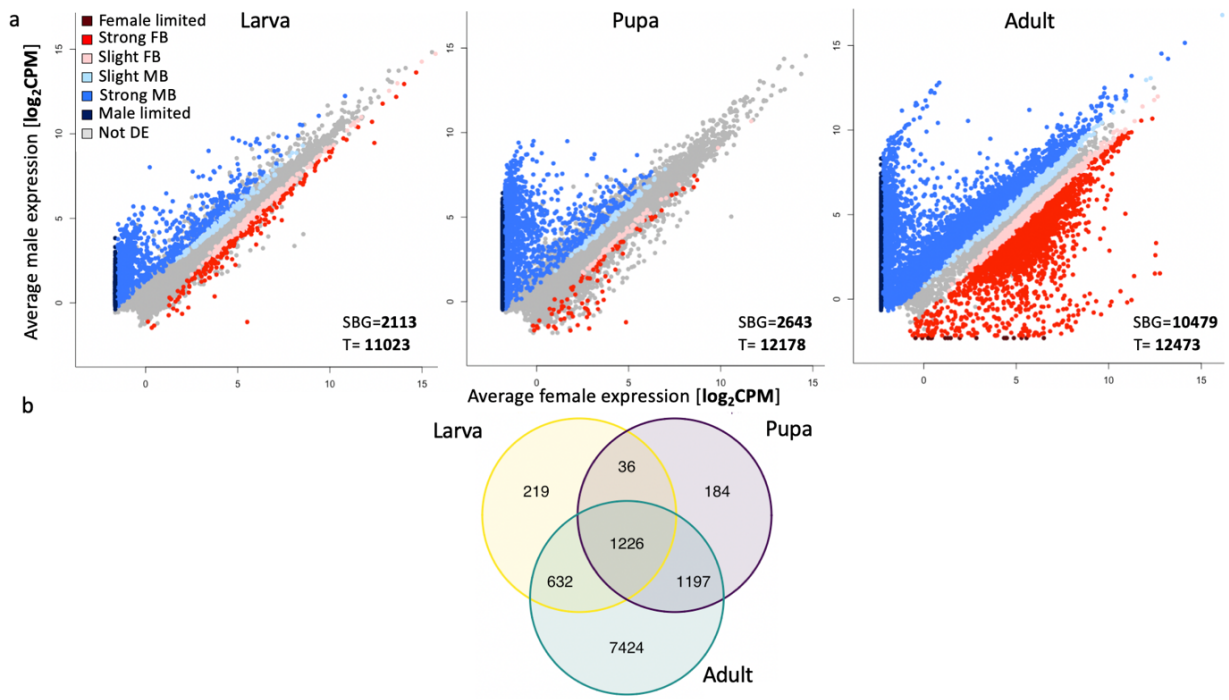

Fig. 7 | (a) Gene expression (log_2CPM) in males and females at the larval (left), pupal (middle) and adult stage (right). The number of differentially expressed genes (SBG) is shown at bottom right corner of each plot, as well as the total number of expressed genes at each stage (T). Genes are classified based on their sex-bias into seven categories: “slight FB”- female bias (<2 FC), “strong FB”- female bias (>2 FC), “female limited”- with no expression in males, “slight MB”- male bias (<2 FC), “strong MB”- male bias (>2 FC), “male limited”- no expression in females, “Not DE”- not differentially expressed genes. **(b)** Venn-diagram of sex-biased genes in three developmental stages: larval (yellow), pupal (purple), and adult (green). The number of genes shared between all three stages was greater than expected by chance (observed overlap= 1226, expected overlap= 334.7), Exact test of multi set intersections: p-value≈ 0).

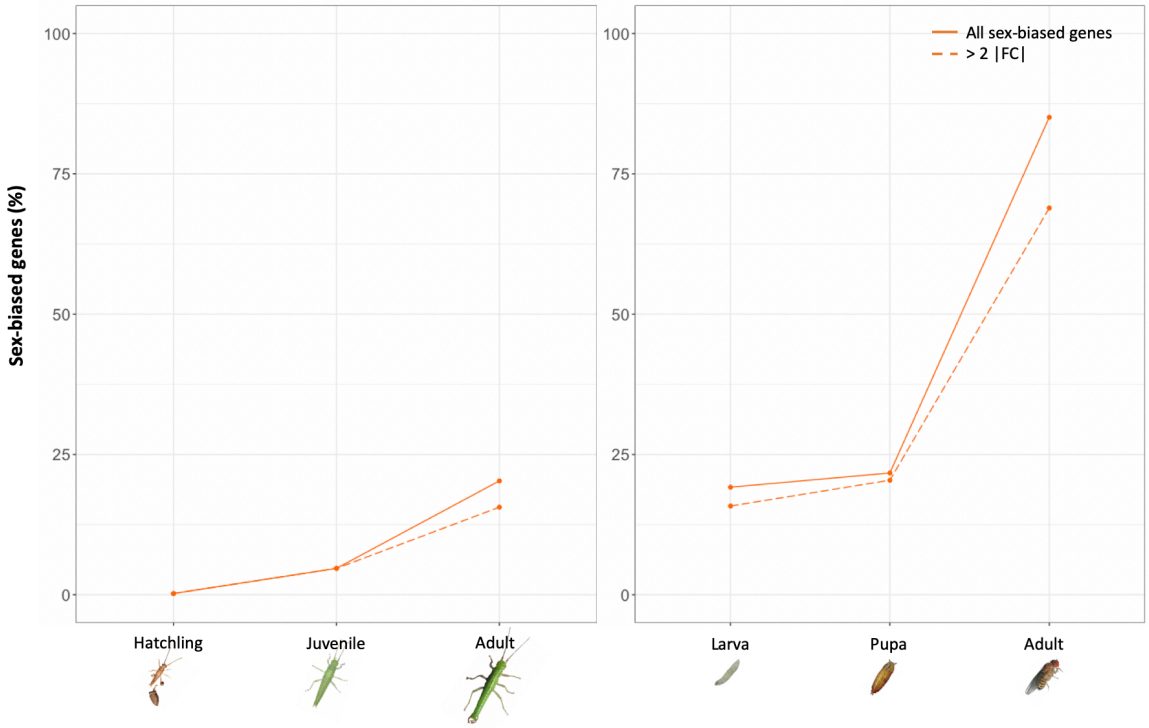

Fig. 8 | Percentage of sex-biased genes (SBG) in *T. californicum* (left panel) and *D. melanogaster* (right panel) at three developmental stages. Note that the immature developmental stages in the two species are not homologous but serve to illustrate the gradual *versus* abrupt increase in sex-biased gene expression in adults.

Discussion

In the stick insect *T. californicum,* gene expression differs between males and females before morphological sexual dimorphism. During development, most of the sex-biased genes are added gradually and cumulatively, meaning that once a gene gains sex-biased expression in *T. californicum,* it generally remains sex-biased at later developmental stages. In addition, genes were consistent in their sex-bias direction; only a single gene shifted from male- to female-biased expression. Surprisingly, such a gradual and consistent addition of sex-bias does not appear to be a general pattern

306 over animal development. For example, in humans and mosquitos, some stages have
307 bursts of sex-biased gene expression, suggesting that specific developmental stages
308 contribute discretely to sexual differentiation, and that sexual phenotypes are not
309 gradually established (Magnusson *et al.* 2011; Shi *et al.* 2016).

310 Although only 26 genes were sex-biased in *T. californicum* at the hatchling
311 stage, they all had a strong sex-bias and nine of them stayed significantly sex-biased
312 at both the juvenile and adult stages. As such these 26 genes likely represent key
313 genes involved in the sexual differentiation of *Timema*. Most of the sex-biased genes
314 at the hatchling stage were female-biased. This might suggest that the process of
315 building a female phenotype begins earlier in development than the one of building a
316 male phenotype. Later developmental stages had more male-biased than female-
317 biased genes. A similar shift from female- to male-bias in gene expression during
318 development was found in *Nasonia* jewel wasps, where little male-biased expression
319 was detected until the pupal stage, during the activation of spermatogenesis. Similar
320 to *Timema*, the shift was interpreted as a different developmental timing of the two
321 sexes (Rago *et al.* 2020). In *Timema*, male-biased genes were also expressed in a
322 relatively stage-specific manner, in contrast to a more constant expression of female-
323 biased genes. This further supports the idea that *Timema* individuals first develop
324 along the female trajectory, i.e. that the female phenotype is the "default" in *Timema*,
325 and may indicate greater pleiotropy of female-biased genes. Additional support for this
326 interpretation comes from the lower sequence divergence rates of female-biased than
327 male-biased genes, implicating stronger selective constraints on female- than male-
328 biased genes. In addition, we observed that genes with greater sex-bias have faster
329 sequence divergence rates. This is largely due to relaxed purifying selection, with
330 positive selection contributing little to the accelerated evolutionary rate of sex-biased

331 genes. These findings are consistent with the idea that sex-biased genes evolve from
332 genes with few evolutionary constraints and that are relatively “dispensable” (Mank
333 and Ellegren 2009; Catalan *et al.* 2018).

334 Sex-biased genes in *Timema* hatchlings were enriched for processes related
335 to oogenesis. Moreover, one of the most consistently and strongly female-biased
336 genes across development was annotated as *vitellogenin receptor*. In insects,
337 *vitellogenin receptor* transfers yolk protein precursors into the oocytes and is
338 necessary for oocyte and early embryo development (Raikhel and Dhadialla 1992;
339 Cho and Raikhel 2001). The role of these processes in *Timema* hatchlings remain to
340 be investigated but *vitellogenin receptor* expression may indicate a very early onset of
341 oocyte development in *Timema*, consistent with studies in other stick insects (Taddei
342 *et al.* 1992). Sex-biased genes in juvenile and adult stages were not enriched for
343 processes obviously related to sexual traits. However, some processes such as
344 pigmentation and chemosensory/olfactory behavior could have a sex-related role.
345 Pigmentation is a sexually dimorphic trait in *Timema* (Sandoval 2008), while
346 chemosensory and olfactory behaviors are important for mate recognition and mating
347 (Nosil *et al.* 2007; Schwander *et al.* 2013). We also checked the expression of two key
348 genes involved in insect sex-determination pathways: *transformer* and *doublesex*. In
349 holometabolous insects, *transformer* acts as a splicing regulator of *doublesex*, a sex
350 differentiation master switch gene (Geuverink and Beukeboom 2014). The *doublesex-*
351 *transformer* role in sex-differentiation is conserved among Diptera, Coleoptera and
352 Hymenoptera (Verhulst *et al.* 2010; Wexler *et al.* 2019), and likely play the same role
353 in sex differentiation in hemimetabolous insects (Zhuo *et al.* 2018; Wexler *et al.* 2019).
354 In *T. californicum,* we found that *doublesex* was expressed in all three developmental
355 stages, but its expression was too low to examine sex-bias. *Transformer* is not

356 annotated in the available *T. californicum* genome; a *transformer-2 homolog* is
357 annotated but did not feature sex-bias at any of the three developmental stages. As
358 such, whether or not these classic sex-determining genes influence sexual
359 differentiation in *Timema* is unclear, and requires future studies.

360 We hypothesized that the dynamics of sex biased gene expression would be
361 strongly affected by hemimetabolous *vs* holometabolous development in insects, with
362 a gradual increase of sex-biased gene expression during development in the former
363 and an abrupt increase at the adult stage in the latter. Hemimetabolous insects such
364 as *T. californicum* have multiple nymphal stages that progressively resemble the adult
365 stage, together with a gradual increase in sexual dimorphism. On the other hand, a
366 holometabolous insect like *D. melanogaster* has more monomorphic larval stages and
367 pupae, with no resemblance to the sexually dimorphic adult stage. The change in
368 sexually dimorphic gene expression across development indeed mirrored these
369 different developmental patterns, with the proportion of sex-biased genes increasing
370 gradually in *T. californicum* as sexual dimorphism became more pronounced, whereas
371 sexually dimorphic gene expression increased abruptly at the adult stage in *D.*
372 *melanogaster*.

373 Although the change in sexually dimorphic gene expression across
374 development fits with our expectations, we were surprised to find a much higher
375 proportion of sex-biased genes in *D. melanogaster* than in *T. californicum* overall. This
376 difference is clearest at the adult stage when most of the expressed genes in *D.*
377 *melanogaster* were sex-biased (84%), compared to only 20% in *T. californicum*.
378 Similar to *Drosophila*, other holometabolous insects also featured sex-bias for more
379 than half of the genes expressed at the adult stage (Baker *et al.* 2011; Rago *et al.*

380 2020), while all studied hemimetabolous insects had a much smaller proportion of sex-
381 biased genes (<10%) (Pal and Vicoso 2015). Why there is such a difference in the
382 prevalence of sex-biased genes between species with different developmental
383 strategies is still an open question. One potential explanation is that the proportion of
384 sex-biased genes at the adult stage may be less constrained under holo- than
385 hemimetabolous development. For example, the complete reorganization of cells and
386 tissues during metamorphosis in holometabolous insects may allow for more drastic
387 phenotypic changes between the sexes, while incomplete metamorphosis constrains
388 the evolution of distinct sexual phenotypes. Data on additional species and specific
389 tissues rather than whole bodies are required to confirm that this difference is a
390 general phenomenon and not simply due idiosyncrasies of the few species yet
391 investigated. Holometabolous insects studied are often established lab-models, with
392 high fecundity, and may thus be characterized by comparatively large gonads, which
393 would result in large proportions of sex-biased genes in adults. By contrast, all
394 hemimetabolous insects studied for sex-biased expression are non-model species.
395 However, the choice of model species is unlikely to be the sole explanation for the
396 different amount of sex bias in holo- and hemimetabolous species. Indeed, in the
397 cricket *Gryllus bimaculatus* and in *Timema* stick insects, even the most sexually
398 differentiated tissue (gonads) feature sex-bias of fewer than 30% of genes (Parker *et*
399 *al.* 2019; Whittle *et al.* 2020), suggesting that extensive differences are not completely
400 driven by large gonads. Finally, while whole body analysis such as we have performed
401 here provides a window into ontogeny, it will necessarily miss tissue specific regulation
402 of sex-biased gene expression (Montgomery and Mank 2016). Future studies should
403 therefore study sex-biased gene expression during development by examining a panel
404 of different tissues or cell types.

405 Overall, our results describe the dynamics of sex-biased gene expression
406 during development in a hemimetabolous insect. Generating sexually dimorphic
407 phenotypes is a developmental process, and we show that dynamics of sex-biased
408 gene expression mirror this development with sex-biased genes being added
409 gradually during development, and with the majority of genes sex-biased at early
410 stages remaining sex-biased in later stages.

411 Material and methods

412 Sample collection and preservation

413 *T. californicum* eggs hatch in early winter. Upon hatching, insects moult several times
414 before reaching maturity in late spring (Sandoval 1994). Developmental stages
415 (especially adults and hatchlings) do not co-occur temporally, hence samples from
416 different stages cannot be collected simultaneously. Adults (4 males, 4 females) and
417 juveniles (4 males, 3 females) were collected in California (Saratoga County) in 2015
418 and 2016, respectively. They were fed with artificial medium for two days to prevent
419 contamination with plant cells from the gut, and subsequently frozen at -80°C.
420 Hatchlings were obtained from the eggs laid by field-collected adults in 2015.
421 Hatchlings were flash frozen and stored at -80 °C. Sexes cannot be distinguished
422 morphologically at the hatchling stage, thus single individuals were extracted to allow
423 for later identification of sex via genotyping (see below) and 5 male and 4 female
424 hatchlings were used.

425 RNA extraction and sequencing

426 Individuals (whole-bodies) from all developmental stages were mechanically
427 homogenized with beads (Sigmund Linder) in liquid nitrogen. We then added 900 ul

428 Trizol (Life Technologies), followed by 180 ul chloroform and 350 ul ethanol. The
429 aqueous layer was then transferred to RNeasy MinElute Columns (Qiagen). Upon
430 RNA extraction, samples were treated with DNase Turbo Kit (Life Tech) following the
431 manufacturer's protocol. Total RNA from the hatchlings was extracted with
432 MagMaxTM Express Robot (AB Applied Biosystems) using a MagMAXTM-96 Total
433 RNA Isolation Kit from Ambion (Life Technologies) following the manufacturer's
434 protocol, but without DNAse at this step to preserve DNA for the sex determination of
435 hatchlings via genotyping (see below). Following RNA extraction, hatchling samples
436 were treated with DNase Turbo Kit (Life Tech) following the manufacturer's protocol.
437 The quality and quantity of the extracted RNA was then measured using a NanoDrop
438 and Bioanalyzer. Library preparations (one for each individual) were done using the
439 Illumina TruSeq Stranded Total RNA kit, upon which samples were sequenced
440 together in six lanes. Paired-end sequencing with a read length of 100 bp was done
441 on a HiSeq2000 platform at the GTF (Genomic Technologies Facility, Centre of
442 Integrative Genomics, Lausanne, Switzerland).

443 **Hatchling sex identification**

444 To identify the sex of *T. californicum* hatchlings, we developed microsatellite markers
445 on the *T. californicum* X chromosome scaffolds reported by Parker et al. (in prep).
446 *Timema* have an XX/X0 sex determination system (Schwander and Crespi 2009)
447 meaning X-linked regions will be present in two copies in females and one copy in
448 males. Consequently, females can feature heterozygous genotypes for polymorphic
449 X-linked markers, while males are invariably hemizygous. Candidate microsatellite
450 markers were designed with msatcommander (v. 1.08, default options) (Faircloth
451 2008). A selection of four candidate markers were tested for polymorphism and sex-
452 linkage using 15 adult males and 15 adult females in two Multiplex PCR Kit reactions

453 (Qiagen; see Supplemental Table 1 for primer sequences and Supplemental Table 2
454 for PCR conditions). Microsatellite alleles were then determined with an ABI3100
455 machine (Applied Biosystem) and Genemapper v.4.1 (Applied Biosystems 2009).
456 Across microsatellite markers, all 15 tested females were heterozygous at minimum
457 two markers, whereas males were invariably hemizygous at all markers, confirming
458 the predictions for *Timema* X-linked markers (Supplemental table 2). We thus
459 genotyped the nine hatchlings at the four microsatellite markers to identify their sex,
460 and were able to identify four females and five males that were used for the
461 transcriptome study (Supplemental table 2).

462 **Raw data quality control, mapping and read counting**

463 The quality of the reads was checked using FastQC v.0.11.2 (Andrews 2014). We
464 used Cutadapt v. 2.3 with Python v. 3.5.2 (Martin 2011) to remove adapter sequences.
465 Low quality bases at both ends of the reads were trimmed when below a Phred score
466 of 10. Bases with an average Phred score below 20 in a 4bp sliding window were
467 trimmed. Finally, reads with a length below 80 bp were removed Trimmomatic v. 0.36
468 (Bolger *et al.* 2014). Trimmed reads were then mapped to the reference *T. californicum*
469 genome from Jaron *et al.* (2020) using STAR v. 2.6.0c (Dobin *et al.* 2013). HTSeq
470 v.0.9.1 (Anders *et al.* 2014) was used to count the number of reads uniquely mapped
471 to each gene, with the following options (htseq-count -f bam -r name -s reverse -t gene
472 -i ID -m union --nonunique none).

473 ***Drosophila melanogaster* data**

474

475 *D. melanogaster* RNA-seq whole body data from Ingleby *et al.* (2016) was downloaded
476 from SRA (accession number SRP068235). To mirror the data available for *Timema*

477 and to facilitate comparisons, four replicates per sex and three developmental stages
478 from different hemiclonal lines were used. A complete list of samples is provided in
479 the supplementary materials (Supplemental Table 3). Although reads were deposited
480 as paired-end, we found that most of them were unpaired, thus we used only the
481 forward reads for analysis. Reads were trimmed, mapped to the reference genome
482 (FlyBase, r6.23) (Gramates *et al.* 2017) and counted using the methods described
483 above.

484 **Differential gene expression analysis**

485 Differential expression between the sexes was performed using edgeR v.3.16.5
486 (Robinson *et al.* 2009; McCarthy *et al.* 2012) in RStudio v.1.2.5019 (Team 2019). This
487 analysis fits a generalized linear model following a negative binomial distribution to the
488 expression data and calculates a p-value associated with the hypothesis that gene
489 counts differ between experimental groups. EdgeR implements a normalization
490 method (trimmed mean of M values, TMM) to account for composition bias. We
491 analyzed data separately for each developmental stage. We filtered out genes with
492 low counts; we required a gene to be expressed in a majority of male or female libraries
493 dependent on the number of replicates per sex (i.e. a minimum three libraries when
494 replicate number is >= 4, two libraries when replicate number is = 3), with expression
495 level > 0.5 CPM (counts per million). To test for differential gene expression between
496 the sexes, we used a generalized linear model with quasi-likelihood F- test, with
497 contrasts for each stage between females and males (Chen *et al.* 2016). To correct
498 for multiple testing, we applied the Benjamini-Hochberg method (Benjamini and
499 Hochberg 1995) with an alpha of 5%. Genes with significantly higher expression in
500 males were considered as male-biased, genes with significantly higher expression in

501 females as female-biased, and the genes without significant differential expression
502 between sexes as un-biased. To identify genes showing a significant sex by
503 developmental stage interaction we used a similar GLM modelling approach but with
504 all stages included. We used SuperExactTest v.0.99.4 (Wang *et al.* 2015) to test if the
505 overlap of sex-biased genes between the three developmental stages was greater
506 than what is expected by chance. To visualize the overlap of sex-biased genes
507 between developmental stages we used VennDiagram v.1.6.20 (Chen and Boutros
508 2011). We visualized the $\log_2$FC sex-biased gene expression across developmental
509 stages, using pheatmap v.1.0.12 (Kolde 2018). We tested the Spearman's correlation
510 of sex-bias ($\log_2$FC) between the developmental stages, using cor.test from the R
511 package stats v 4.1.1. To describe the increases in the number of sex-biased genes
512 during development, we calculated the effect sizes for each of the two proportions of
513 neighboring developmental stages, using the pwr v. 1.3-0 (Cohen 1988).

514 **Stage specific gene expression**

515 Median gene expression (CPM) values were calculated for each developmental stage
516 and sex. These values were used to calculate Tau, an index of specificity of gene
517 expression during development (Yanai *et al.* 2004; Liu and Robinson-Rechavi 2018)
518 Values of Tau range from zero (broadly expressed during development) to one (gene
519 expressed in only one stage). Tau is generally used to quantify the tissue-specificity
520 of gene expression (Yanai *et al.* 2004). Here we applied the same principle and
521 formula to calculate stage specificity. To visualize the results we made boxplots using
522 ggplot2 v. 3.3.2 (Wickham 2016).
523

524 **Divergence rates**

525 We calculated values of sequence divergence rates (d_N/d_S) along the branch leading
526 to *T. californicum* after the split with *T. poppensis* as described in (Jaron *et al.* 2020).
527 Briefly, branch-site models with rate variation at the DNA level (Davydov *et al.* 2019)
528 were run using the Godon software (https://bitbucket.org/Davydov/godon/, version
529 2020-02-17, option BSG --ncat 4) for each gene with an ortholog found in at least 6
530 species of *Timema* (including *T. poppensis*). Godon estimates the proportion of sites
531 evolving under purifying selection (p0), neutrality (p1), and positive selection. We used
532 only sites evolving under purifying selection or neutrality to calculate dN/dS. To test
533 for differences in sequence divergence rates (d_N/d_S) between different gene
534 categories (female-biased, male-biased and un-biased), we used Wilcoxon tests
535 (ggpubr v.0.2.5, in R) with p-values adjusted for multiple comparisons using the
536 Benjamini-Hochberg method (Haynes 2013). Note that some of the *T. californicum*
537 sex-biased genes had no identified ortholog in other *Timema* species, thus we do not
538 have sequence evolution rates for all sex-biased genes.

539

540 Furthermore, we used a partial correlation between the strength of the sex bias
541 ($\log_2$FC) and the sequence divergence rate (d_N/d_S) on sex-biased genes only, while
542 controlling for the average expression level (CPM) of the gene, and GC- content (ppcor
543 v.01 (Kim 2015), in R) in order to determine if genes with higher $\log_2$FC values have
544 faster sequence divergence rates. Per gene GC content was calculated for the coding
545 sequences from the *T. californicum* genome.

546

547 **Functional analysis of sex-biased genes**

548 We performed Gene Ontology (GO) enrichment analysis using TopGO v.2.26.0 on
549 sex-biased genes obtained with EdgeR (see above) at each developmental stage. We
550 used a functional annotation derived from blasting sequences to *Drosophila*
551 *melanogaster* database. Only GO terms with minimum ten annotated genes were
552 used. Enrichment of terms was determined using a weighted Kolmogorov-Smirnov-
553 like statistical test, equivalent to the gene set enrichment analysis (GSEA method).
554 We applied the "elim" algorithm, which considers the Gene Ontology hierarchy, i.e. it
555 first assesses the most specific GO terms, and then more general ones (Alexa *et al.*
556 2006). Our analysis focused on gene sets in the Biological Processes (BP), GO
557 category. We considered terms as significant when $p < 0.05$. Enriched GO terms were
558 then semantically clustered using ReviGO (Supek *et al.* 2011) to aid interpretation.

559

560 ## Data availability

561 *Timema californicum* raw reads have been deposited in SRA under PRJNA678950
562 bioproject, with accession codes SRR13084978-SRR13085001 (Supplemental table
563 9). *Timema californicum* mapped read counts are provided in Supplemental table 13
564 and fold changes in Supplemental table 14. *Drosophila melanogaster* mapped read
565 counts are provided in Supplemental table 15.

566

567 ## Code availability

568 Scripts with code for the analysis in this study are available at
569 https://github.com/JelisavetaDjordjevic/Sex_biased_gene_expression

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