Article

Dynamics of sex-biased gene expression over 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 over 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 over 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 over 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 support the prediction that the dynamics of sex-biased gene expression over 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 (Chauhan *et al.* 2016; 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 (Pointer *et al.* 2013; Chauhan *et al.* 2016). 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 (Ingleby *et al.* 2015).

Because we lack detailed studies of sex-biased gene expression over 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 over 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? Furthermore, sex-biased gene expression can be considered to reflect a broad measure of sexual dimorphism, including physiological and behavioral traits, and may therefore be more representative than dimorphism quantified using external

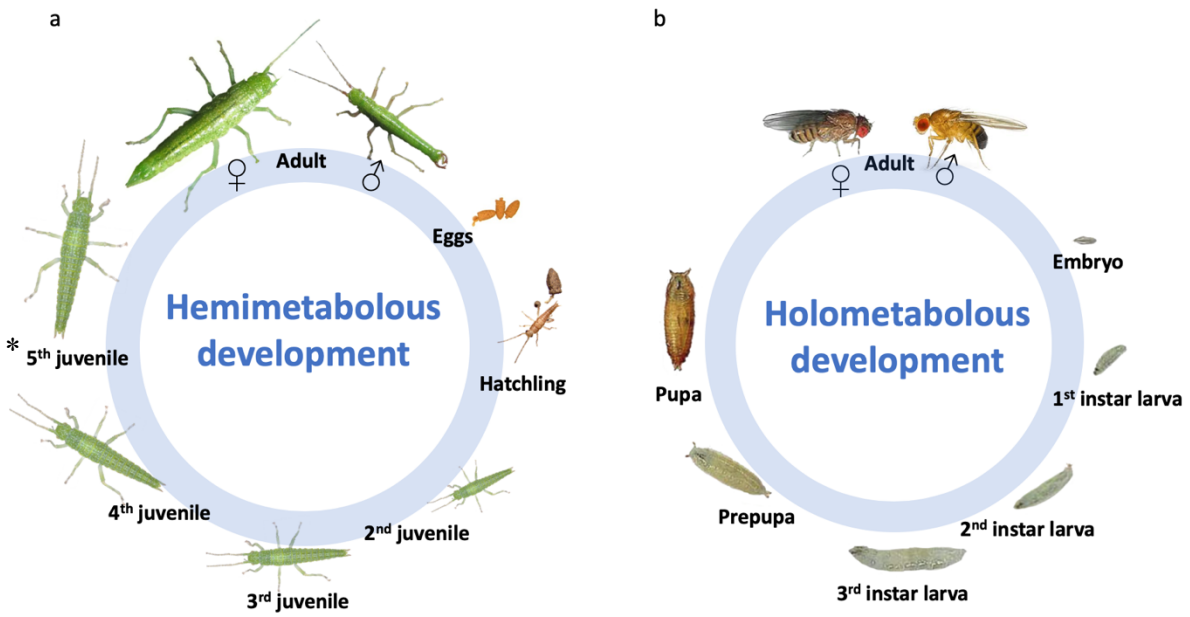
64 morphology. However, whether the extent of sexual dimorphism and of sex-biased
65 gene expression are generally correlated remains poorly known.

66 In insects there are two major developmental strategies, and developmental
67 patterns of sex-biased gene expression could differ between them. Holometabolous
68 insects have morphologically and ecologically distinct larval, pupal, and adult stages,
69 and phenotypic sexual dimorphism is commonly prominent only at the adult stage.
70 Contrastingly, hemimetabolous insects go through gradual morphological changes
71 and sexual differentiation, and the nymphal stages morphologically resemble adults
72 (Chen *et al.* 2010). These distinct dynamics for sexual morphological differentiation
73 are expected to be mirrored by sex-biased gene expression, with abrupt versus
74 gradual increases in the proportion of sex-biased genes over development. However,
75 studies of sex-biased gene expression during development have thus far only been
76 conducted in holometabolous insects (Magnusson *et al.* 2011; Rago *et al.* 2020), with
77 the main focus on *Drosophila* (Perry *et al.* 2014; Ingleby *et al.* 2015), precluding
78 comparisons between species with different developmental strategies.

79 Here, we studied the dynamics of sex-biased gene expression across
80 development in hemimetabolous insects, *Timema californicum.* These are sexually
81 dimorphic walking-stick insects, found in California (Vickery and Sandoval 2001).
82 Males are smaller than females, with different coloration, and have conspicuous cerci
83 used for holding on to the female during copulation. We selected three developmental
84 stages (Fig. 1a): the hatchling stage where sexes are phenotypically identical, the third
85 juvenile stage with minor phenotypic differences, and the adult stage, with pronounced
86 sexual dimorphism. We investigated whether sex-biased genes are recruited in a
87 stage-specific manner, and if the magnitude of sex-bias increases with development.
88 We further explored sequence evolution rates of sex-biased genes in pre-adult stages,

to determine if they show elevated rates as commonly observed for sex-biased genes at adult stages in many species (Ellegren and Parsch 2007; Grath and Parsch 2012; Perry *et al.* 2014).

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



105 **Fig. 1.** Life cycles of (a) the stick insect *T. californicum* and (b) the fly *D. melanogaster.*
106 *represents a stage present only in females, as *Timema* males have one moult less
107 than females. Hatchling and egg photographs of *Timema* were kindly provided by Bart
108 Zijlstra (http://www.bartzijlstra.com), juvenile and adult stages by Jelisaveta Djordjevic.
109 Life cycle of *D. melanogaster* modified from Weigmann *et al.* (2003).
110

111 ## Results

112 ### Sex-biased gene expression increases over development in *T. californicum*

113 Sex-biased gene expression gradually increased over the three developmental
114 stages, with 0.2% (26) of the expressed genes sex-biased at the hatchling stage, 4.7%
115 (568) at the juvenile, and 20.3% (2485) at the adult stage (Fig. 2). The hatchling stage
116 had more female than male biased genes ($x^2_{(1)}$=12.46, p<0.05), while the juvenile and
117 adult stages had more male biased genes ($x^2_{\text{juvenile (1)}}$ =457.92, $x^2_{\text{adult (1)}}$ = 77.554,
118 p<0.05) (Supplemental Table 4). The majority (85% and 91%) of sex biased genes at
119 the two preadult stages had strong sex bias (≥2 $\log_2$FC, P_{adj} <0.05), in contrast to the
120 adult stage where sex bias was slight (57% of the sex-biased genes had <2 $\log_2$FC,
121 P_{adj} <0.05) (Fig. 2). The intensity of sex-bias varied greatly over development with 1137
122 genes showing a significant sex by development interaction (P_{adj}<0.05).

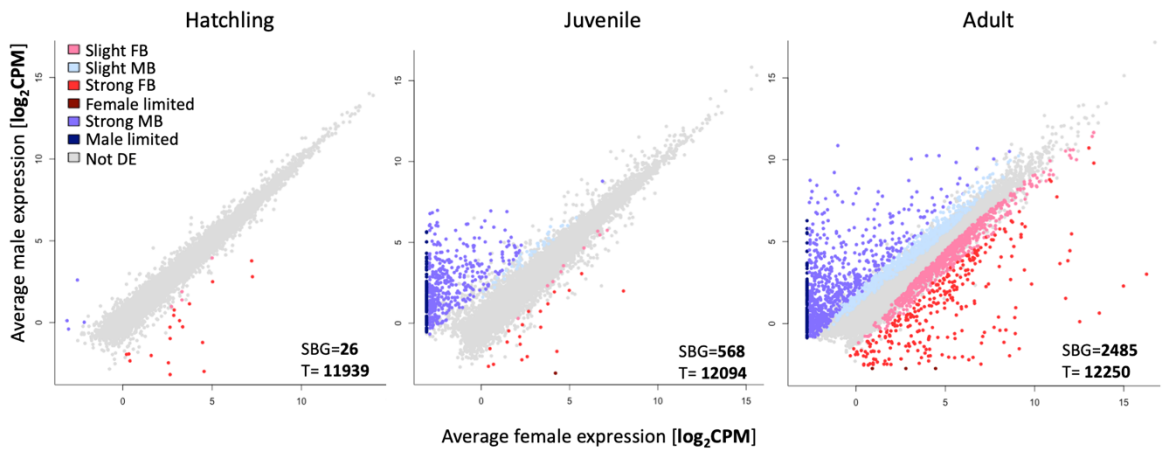
123 To characterize the dynamics of sex-biased expression over development, we then
124 classified the 2671 genes which are sex-biased at one or more developmental stages
125 into 13 categories, dependent on their expression patterns over development
126 (Supplemental Fig. 1). This classification showed that sex-biased genes are added
127 gradually over development, with genes sex-biased at earlier stages generally

128 remaining sex-biased in the same direction at later stages (Supplemental Fig. 2). Only
129 a single gene shifted from male-biased to female-biased expression (Supplemental
130 Fig. 1). Out of 26 sex-biased genes at the hatchling stage, 18 (69%) were also
131 significantly sex-biased in at least one of the later stages, with 9 (35%) remaining sex-
132 biased throughout development (Fig. 3), while out of 568 sex-biased genes at the
133 juvenile stage, 390 (69%) stayed sex-biased at the adult stage (Fig. 3). This
134 classification is conservative, as it depends on the sex-biased gene expression
135 detection threshold, meaning sex-biased gene expression may be missed in some
136 stages inflating the number of differences we see. This is supported by the fact that
137 only around 65% of genes that are sex-biased in two stages show a significant sex by
138 development stage interaction (Supplemental table 8, also see Supplemental Fig. 1).

139 Sex-biased genes at the hatchling stage are particularly interesting because they
140 reveal sexual differentiation prior to visible morphological differences. We therefore
141 looked for functional annotations of the 26 genes sex-biased at the hatchling stage in
142 the *T. californicum* reference genome (Jaron *et al.* 2020). However, only 16 out of the
143 26 sex-biased genes had functional annotations and only one (*vitellogenin receptor*)
144 had a clear link to sexual differentiation (Fig. 3b). While key genes known to play a
145 role in insect sex-determination and differentiation pathways (*doublesex*, *transformer-*
146 *2* and *sex-lethal*) were expressed at the hatchling stage, *doublesex* had very low
147 expression, preventing analysis of sex-bias, and *transformer-2* and *sex-lethal* did not
148 feature sex-biased expression.

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

150 Genes sex-biased at the hatchling stage were enriched for GO-terms related to
151 developmental processes (e.g., “regulation of cell development”, “regulation of cell
152 proliferation”, “cuticle development”), and GO-terms related to female specific
153 functions such as oogenesis (“oocyte construction”, “oocyte development”)
154 (Supplemental Fig. 3a, and Supplemental Table 10). At the juvenile stage, sex-biased
155 genes were enriched for GO-terms related to metabolic processes, in particular to
156 catabolism (e.g., “lipid catabolic processes”, “cellular catabolic processes”, “regulation
157 of catabolic processes”) (Supplemental Fig. 3b, and Supplemental table 11). At the
158 adult stage, sex-biased genes were enriched for GO-terms related to diverse
159 metabolic and physiological processes with no clear association to sexual
160 differentiation (Supplemental Fig. 3c, and Supplemental table 12); however, several
161 terms were related to chemosensory and olfactory behavior, which may play a role in
162 mate detection. Furthermore, sex-biased genes at the adult stage were enriched for
163 pigmentation (e.g., “developmental pigmentation”, “eye pigmentation”), which is a
164 sexually dimorphic trait in *Timema californicum*.



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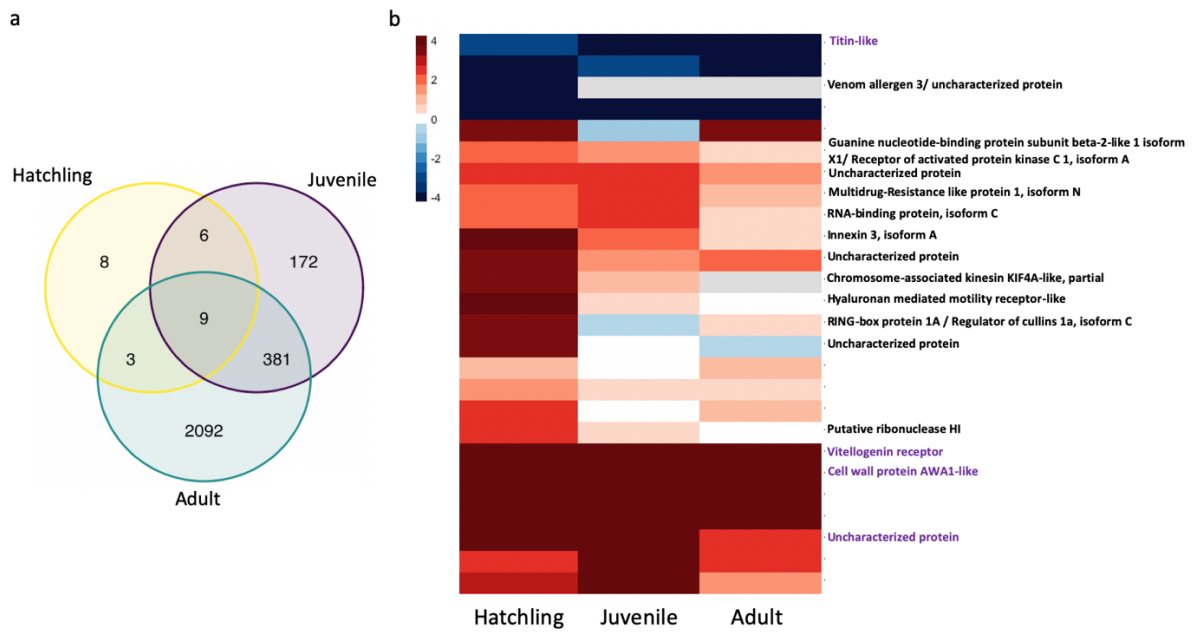
166 **Fig. 2 |** Gene expression (log_2CPM) in *T. californicum* males and females at the
167 hatchling (left), juvenile (middle) and adult stage (right). The number of differentially

168 expressed genes (SBG) is shown at the bottom right corner of each plot, as well as
169 the total number of expressed genes at each stage (T). Genes are classified based
170 on their sex-bias into seven categories: “slight FB”- female bias (<2 log_2FC), “strong
171 FB”- female bias (≥2 log_2FC), “female limited”- with no expression in males, “slight
172 MB”- male bias (<2 log_2FC), “strong MB”- male bias (≥2 log_2FC), “male limited”- no
173 expression in females, “Not DE”- not differentially expressed genes.



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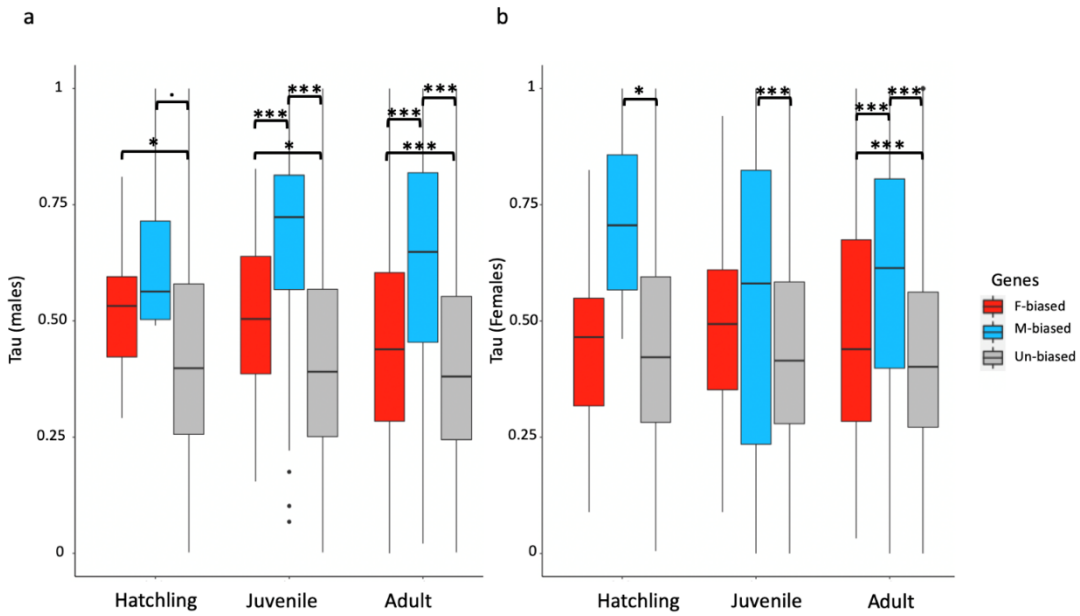
175 **Fig. 3 | Sex-biased genes shared across development**. (**a**) Venn-diagram of sex-
176 biased genes in three developmental stages: hatchling (yellow), juvenile (purple), and
177 adult (green). The number of genes shared between all three stages was greater than
178 expected by chance (observed overlap= 9, expected overlap= 0.21, Exact test of multi
179 set interactions: $p= 4.66x10^{-13}$). (**b**) A heatmap showing the 26 sex-biased genes at
180 the hatchling stage with their annotations and their expression in three developmental
181 stages. Genes in red are female-biased, blue are male-biased. Saturation of the colors
182 increases with the log_2FC. Genes without a label have no annotation, annotation in
183 purple is for genes that were sex-biased in all three stages

184 **Sex-biased genes have more stage-specific expression**

185 Expression levels of sex-biased genes tend to be specific to only one developmental
186 stage while unbiased genes have a more constant expression level across
187 development (Fig. 4). Male-biased genes have the most stage specific expression in
188 both males and females. Female-biased genes are only slightly more stage specific
189 than un-biased genes, but the trend is consistent and the difference is significant
190 notably in both male and female adults.



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

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

200

201 Male-biased genes have faster rates of sequence evolution (dN/dS) compared to both
202 un-biased and female-biased genes, at juvenile and adult stages (Fig. 5). At the
203 hatchling stage, female-biased genes evolve significantly faster than un-biased genes,
204 but not at the other two developmental stages. Elevated rates of sequence evolution
205 are likely the result of reduced purifying selection, but stronger positive selection may
206 also contribute to fast sequence evolution in some genes. To test this possibility, we
207 repeated our analysis after excluding 19 (11 F-biased and 8 M-biased) genes for which
208 a previous study (Jaron *et al.* 2020) detected signatures of positive selection.
209 Excluding these 19 genes did not change our results (Supplemental Table 7a). More
210 generally, we did not find an enrichment of positively selected genes among those with
211 sex-biased expression (Fisher's exact test; p-value=0.39, odds ratio: 1.1)
212 (Supplemental Table 7b).

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

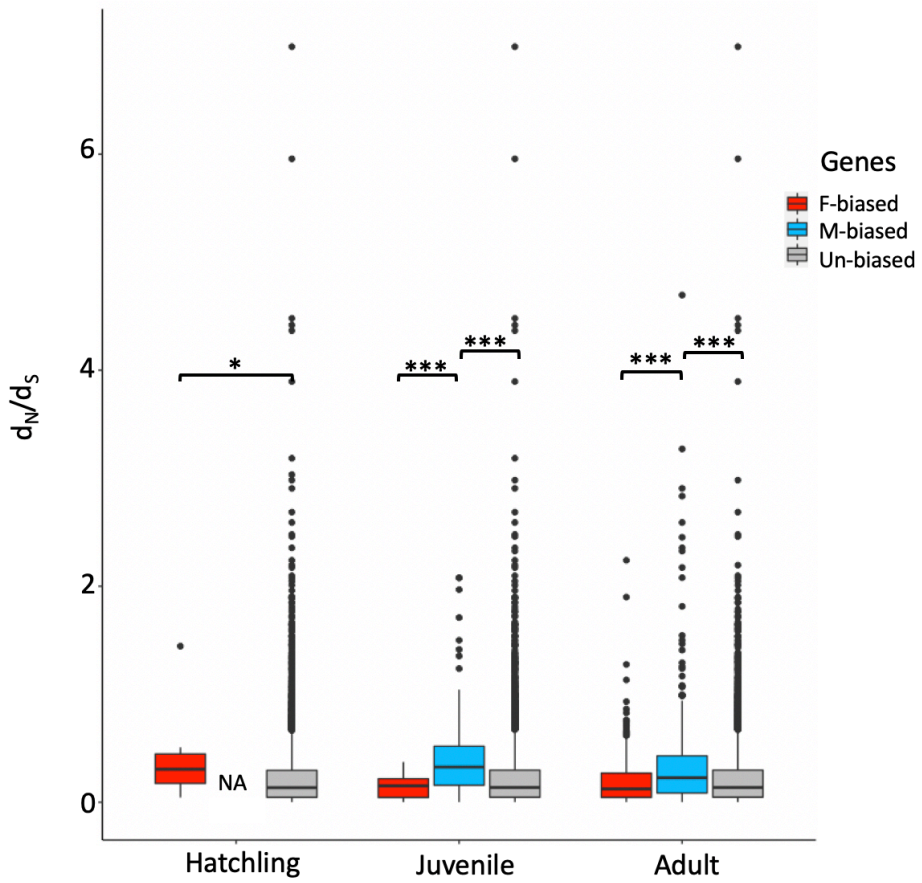
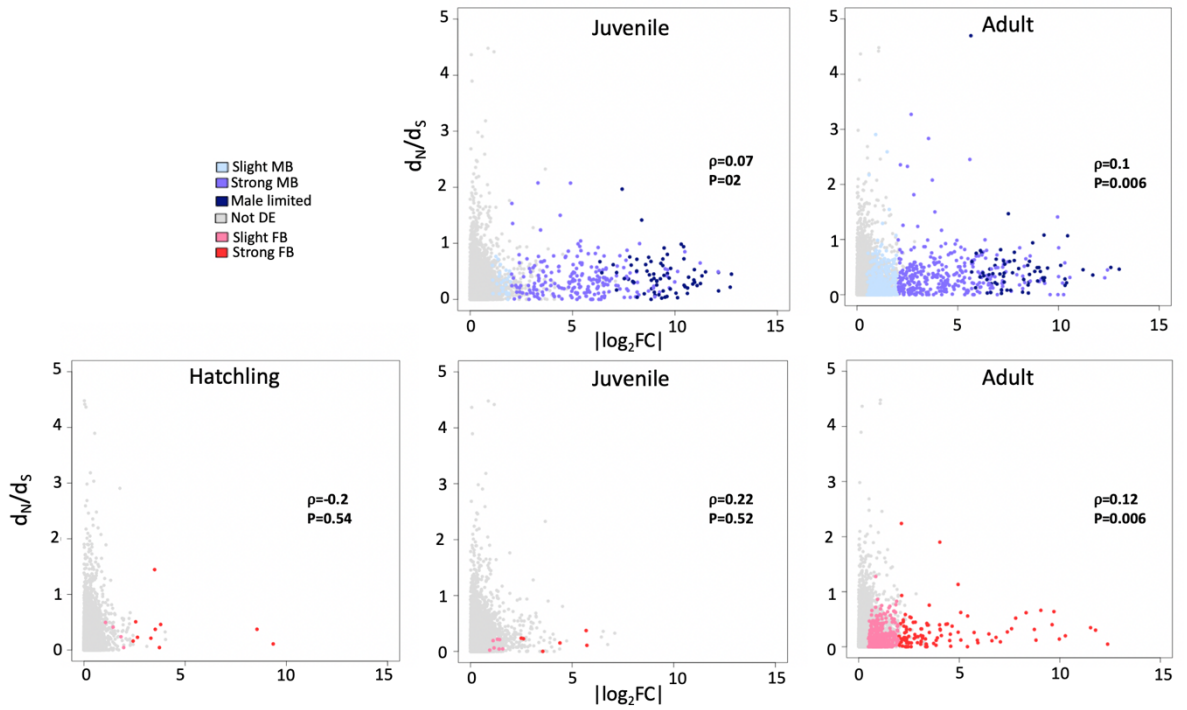


Fig. 5 | Divergence rates (the ratio of nonsynonymous to synonymous substitutions, d_N/d_S), at three developmental stages; hatchling, juvenile and adult stage. Boxplots represent the median, lower and upper quartiles, and whiskers the minimum and maximum values (in the limit of 1.5x interquartile range). Adjusted p values of Wilcoxon rank sum tests are summarized above the box plots (*** = $p < 0.001$, * = $p < 0.05$).



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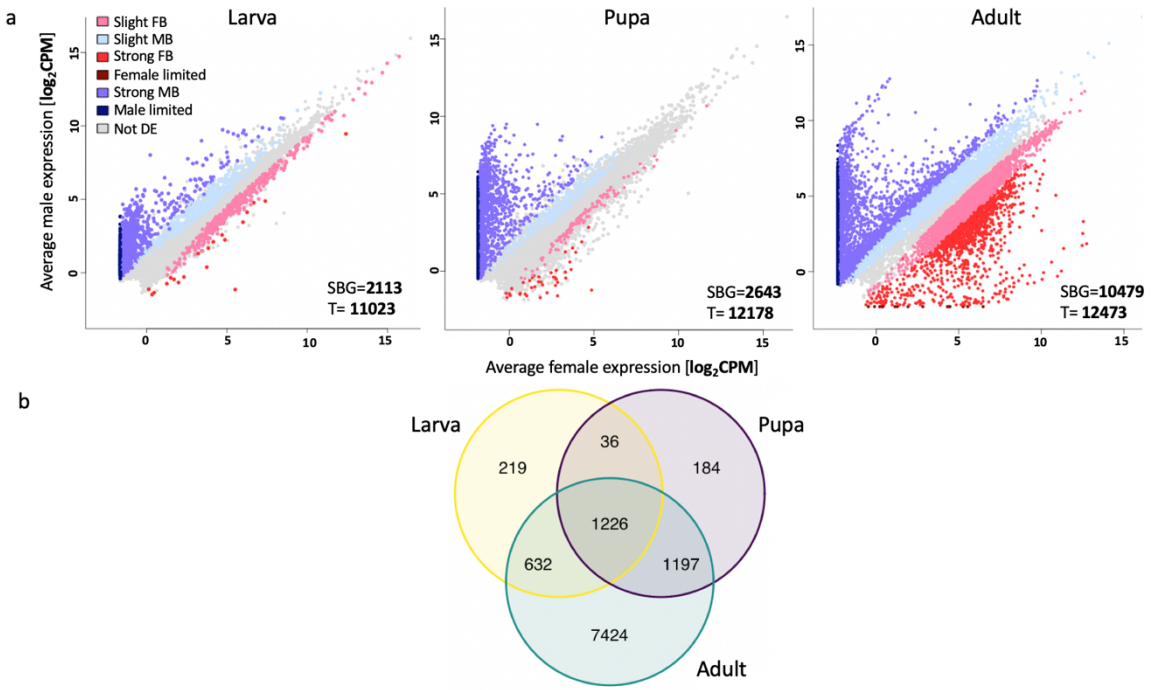
225 **Fig. 6 |** Spearman's rank correlations between the divergence rate of genes, d_N/d_S,
226 and their strength of the sex bias $|\log_2 FC|$ at three developmental stages. Note that all
227 genes are displayed on each plot but only male- respectively female-biased ones are
228 used for correlation tests.

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

230 In order to compare the dynamics of sex-biased gene expression over development
231 between hemimetabolous and holometabolous insects, we reanalyzed publicly
232 available data from *D. melanogaster* (Ingleby *et al.* 2016), using the same pipeline as
233 for *T. californicum.* This experiment was chosen for comparison as it has a similar
234 design, i.e. whole-body RNA-seq data from several developmental stages. In addition,
235 although *D.* melanogaster from this experiment were lab-reared, they had a similar
236 between sample variance as *T. californicum* (biological coefficient of variation at the
237 adult stage: *D. melanogaster* = 0.274, *T. californicum* = 0.348).

238 In contrast to the gradual increase in the amount of sex-biased expression over
239 development observed in *T. californicum,* sex-biased gene expression increased
240 abruptly at the adult stage in *D. melanogaster* (Fig. 8), from 22% to 84% of sex-biased
241 genes (Fig. 7). Overall, at each developmental stage, there were more male than
242 female-biased genes (Supplemental table 5). The majority (85% and 63%) of sex-
243 biased genes at the two preadult stages had strong sex bias (≥2 $\log_2$FC, P_{adj} <0.05) in
244 contrast to the adult stage where weaker sex-bias was slightly more common (50.5%
245 of the sex-biased genes had <2 $\log_2$FC, P_{adj} <0.05) (Supplemental table 5). Out of
246 2113 sex-biased genes at the larval stage, 1262 (60%) were significantly sex-biased
247 in at least one of the later stages, with 1226 (58%) remaining sex-biased throughout
248 development (Fig. 7b), while out of 2643 sex-biased genes at the pupal stage, 2423
249 (92%) stayed sex-biased at the adult stage (Fig. 7b).



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251 **Fig. 7 | (a)** Gene expression ($\log_2$CPM) in males and females at the larval (left), pupal
252 (middle) and adult stage (right). The number of differentially expressed genes (SBG)
253 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 $\log_2$FC), “strong FB”- female bias (≥2 $\log_2$FC), “female limited”- with no expression in males, “slight MB”- male bias (<2 $\log_2$FC), “strong MB”- male bias (≥2 $\log_2$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 interactions: p-value≈ 0).

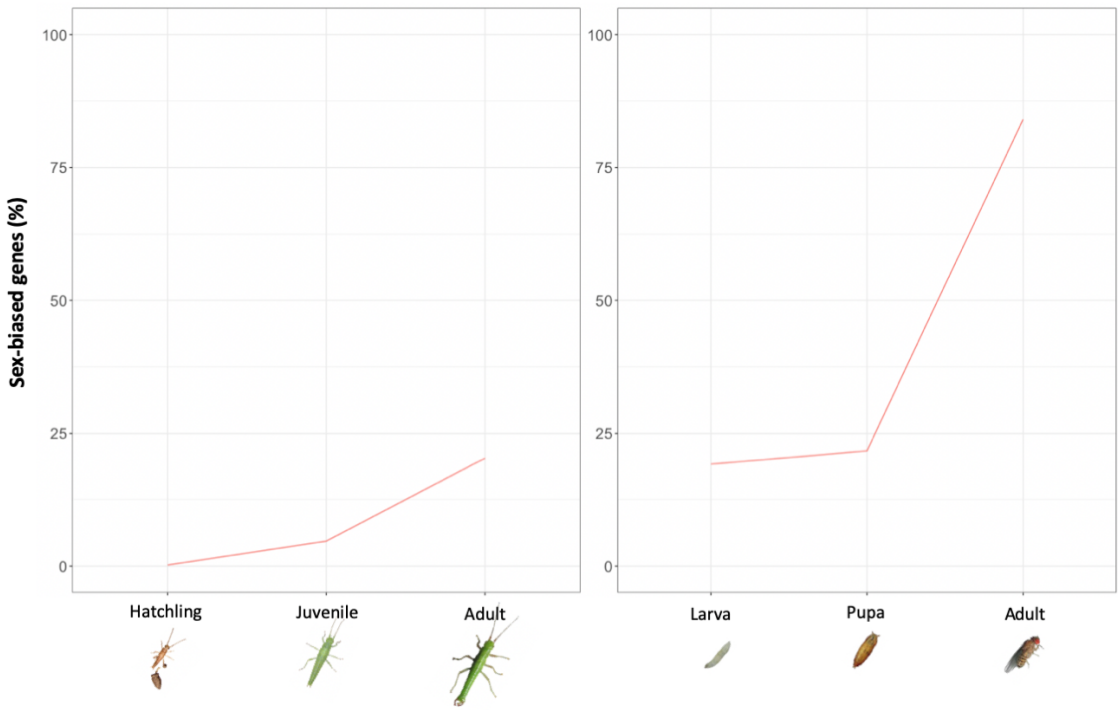

Fig. 8 | Percentage of sex-biased genes (SBG) in *T. californicum* (left panel) and *D. melanogaster* (right panel) at three developmental stages. Note that the immature developmental stages in the two species are not homologous but serve to illustrate the gradual *versus* abrupt increase in sex-biased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

268 ## Discussion

269 In the stick insect *T. californicum,* gene expression differs between males and
270 females before morphological sexual dimorphism. Over development, most of the sex-
271 biased genes are added gradually and cumulatively, meaning that once a gene gains
272 sex-biased expression in *T. californicum* it generally remains sex-biased at later
273 developmental stages. In addition, genes were consistent in their sex-bias direction;
274 only a single gene shifted from male- to female-biased expression. Surprisingly, such
275 a gradual and consistent addition of sex-bias does not appear to be a general pattern
276 over animal development. For example, in humans and mosquitos, some stages have
277 bursts of sex-biased gene expression, suggesting that specific developmental stages
278 contribute discretely to sexual differentiation, and that sexual phenotypes are not
279 gradually established (Magnusson *et al.* 2011; Shi *et al.* 2016).

280 Although only 26 genes were sex-biased in *T. californicum* at the hatchling
281 stage, they all had a strong sex-bias and nine of them stayed significantly sex-biased
282 at both the juvenile and adult stages. As such these 26 genes likely represent key
283 genes involved in the sexual differentiation of *Timema*. Most of the sex-biased genes
284 at the hatchling stage were female-biased. This might suggest that the process of
285 building a female phenotype begins earlier in development. Later developmental
286 stages had more male-biased than female- biased genes. A similar shift from female-
287 to male-bias in gene expression during development was found in *Nasonia* jewel
288 wasps, where little male-biased expression was detected until the pupal stage, during
289 the activation of spermatogenesis. Similar to *Timema*, the shift was interpreted as a
290 different developmental timing of the two sexes (Rago *et al.* 2020). In *Timema*, male-
291 biased genes were also expressed in a relatively stage-specific manner, in contrast to

292 a more constant expression of female-biased genes. This further supports the idea
293 that *Timema* individuals first develop along the female trajectory, i.e. that the female
294 phenotype is the "default" in *Timema*, and may indicate greater pleiotropy of female-
295 biased genes. Additional support for this interpretation comes from the lower
296 sequence divergence rates of female-biased genes than male-biased genes,
297 implicating stronger selective constraints on female- than male- biased genes. In
298 addition, we observed that genes with greater sex-bias have faster sequence
299 divergence rates. This is largely due to relaxed purifying selection, with positive
300 selection contributing little to the accelerated evolutionary rate of sex-biased genes.
301 These findings are consistent with the idea that sex-biased genes evolve from genes
302 with few evolutionary constraints and that are relatively "dispensable" (Mank and
303 Ellegren 2009; Catalan *et al.* 2018).

304 Sex-biased genes in *Timema* hatchlings were enriched for processes related
305 to oogenesis. Moreover, one of the most consistently and strongly female-biased
306 genes across development was annotated as *vitellogenin receptor*. In insects,
307 *vitellogenin receptor* transfers yolk protein precursors into the oocytes and is
308 necessary for oocyte and early embryo development (Raikhel and Dhadialla 1992;
309 Cho and Raikhel 2001). The role of these processes in *Timema* hatchlings remain to
310 be investigated but *vitellogenin receptor* expression may indicate a very early onset of
311 oocyte development in *Timema*, consistent with studies in other stick insects (Taddei
312 *et al.* 1992). Sex-biased genes in juvenile and adult stages were not enriched for
313 processes obviously related to sexual traits. However, some processes such as
314 pigmentation and chemosensory/olfactory behavior could have a sex-related role.
315 Pigmentation is a sexually dimorphic trait in *Timema* (Sandoval 2008), while
316 chemosensory and olfactory behavior are important for mate recognition and mating

317 (Nosil *et al.* 2007; Schwander *et al.* 2013). We also checked the expression of two key
318 genes involved in insect sex-determination pathways: *transformer* and *doublesex*. In
319 holometabolous insects, *transformer* acts as a splicing regulator of *doublesex*, a sex
320 differentiation master switch gene (Geuverink and Beukeboom 2014). The *Doublesex-*
321 *Transformer* role in sex-differentiation is conserved among Diptera, Coleoptera and
322 Hymenoptera (Verhulst *et al.* 2010; Wexler *et al.* 2019), and likely play the same role
323 in sex differentiation in hemimetabolous insects (Zhuo *et al.* 2018; Wexler *et al.* 2019).
324 In *T. californicum,* we found that *doublesex* was expressed in all three developmental
325 stages, but its expression was too low to examine sex-bias. *Transformer* is not
326 annotated in the available *T. californicum* genome; a *Transformer-2 homolog* is
327 annotated but did not feature sex-bias at any of the three developmental stages. As
328 such, whether or not these classic sex-determining genes influence sexual
329 differentiation in *Timema* is unclear, and requires future studies.

330 We hypothesized that the dynamics of sex biased gene expression would be
331 strongly affected by hemimetabolous *vs* holometabolous development in insects, with
332 a gradual increase of sex-biased gene expression over development in the former and
333 an abrupt increase at the adult stage in the latter. Hemimetabolous insects such as *T.*
334 *californicum* have multiple nymphal stages that progressively resemble the adult
335 stage, together with a gradual increase in sexual dimorphism. On the other hand, a
336 holometabolous insect like *D. melanogaster* has more monomorphic larval stages and
337 pupae, with no resemblance to the sexually dimorphic adult stage. The change in
338 sexually dimorphic gene expression across development indeed mirrored these
339 different developmental patterns, with the proportion of sex-biased genes increasing
340 gradually in *T. californicum* as sexual dimorphism became more pronounced, whereas

341 sexually dimorphic gene expression increased abruptly at the adult stage in *D.*
342 *melanogaster*.

343 Although the change in sexually dimorphic gene expression across
344 development fits with our expectations, we were surprised to find a much higher
345 proportion of sex-biased genes in *D. melanogaster* than in *T. californicum* overall. This
346 difference is clearest at the adult stage when most of the expressed genes in *D.*
347 *melanogaster* were sex-biased (84%), compared to only 20% in *T. californicum*.
348 Similar to *Drosophila*, other holometabolous insects also featured sex-bias for more
349 than half of the genes expressed at the adult stage (Baker *et al.* 2011; Rago *et al.*
350 2020), while all studied hemimetabolous insects had a much smaller proportion of sex-
351 biased genes (<10%) (Pal and Vicoso 2015). Why there is such a difference in the
352 prevalence of sex-biased genes between species with different developmental
353 strategies is still an open question. One potential explanation is that the proportion of
354 sex-biased genes at the adult stage may be less constrained under holo- than
355 hemimetabolous development. For example, the complete reorganization of cells and
356 tissues during metamorphosis in holometabolous insects may allow for more drastic
357 phenotypic changes between the sexes, while incomplete metamorphosis constrains
358 the evolution of distinct sexual phenotypes. Data on additional species are required to
359 confirm that this difference is a general phenomenon and not simply due
360 idiosyncrasies of the few species yet investigated. Holometabolous insects studied are
361 often established lab-models, with high fecundity, and may thus be characterized by
362 comparatively large gonads, which would result in large proportions of sex-biased
363 genes in adults. By contrast, all hemimetabolous insects studied for sex-biased
364 expression are non-model species. However, the choice of model species is unlikely
365 to be the sole explanation for the different amount of sex bias in holo- and

366 hemimetabolous species. Indeed, in cricket, *Gryllus bimaculatus*, and *Timema* stick
367 insects, even the most sexually differentiated tissue (gonads) feature sex-bias of fewer
368 than 30% of genes (Parker *et al.* 2019; Whittle *et al.* 2020), suggesting that extensive
369 differences are not completely driven by large gonads.

370 Overall, our results describe the dynamics of sex-biased gene expression over
371 development in a hemimetabolous insect, and suggest profound differences in these
372 dynamics between holometabolous and hemimetabolous species. Generating
373 sexually dimorphic phenotypes is a developmental process, and we show that
374 dynamics of sex-biased gene expression mirror this development with sex-biased
375 genes being added gradually over development, and with the majority of genes sex-
376 biased at early stages remaining sex-biased in later stages.

377 Material and methods

378 Sample collection and preservation

379 *Timema californicum* eggs hatch in early winter. Upon hatching, insects moult several
380 times before reaching maturity in late spring (Sandoval 1994). Developmental stages
381 (especially adults and hatchlings) do not co-occur temporally, hence samples from
382 different stages cannot be collected simultaneously. Adults (4 males, 4 females) and
383 juveniles (4 males, 3 females) were collected in California (Saratoga County) in 2015
384 and 2016, respectively. They were fed with artificial medium for two days to prevent
385 contamination with plant cells from the gut, and subsequently frozen at -80°C.
386 Hatchlings were obtained from the eggs laid by field-collected adults in 2015.
387 Hatchlings were flash frozen and stored at -80 °C. Sexes cannot be distinguished
388 morphologically at the hatchling stage, thus single individuals were extracted to allow

389 for later identification of sex via genotyping (see below) and 5 male and 4 female
390 hatchlings were used.

391 **RNA extraction and sequencing**

392 Individuals (whole-bodies) from all developmental stages were mechanically
393 homogenized with beads (Sigmund Linder) in liquid nitrogen. We then added 900 ul
394 Trizol (Life Technologies), followed by 180 ul chloroform and 350 ul ethanol. The
395 aqueous layer was then transferred to RNeasy MinElute Columns (Qiagen). Upon
396 RNA extraction, samples were treated with DNase Turbo Kit (Life Tech) following the
397 manufacturer's protocol. Total RNA from the hatchlings was extracted with
398 MagMaxTM Express Robot (AB Applied Biosystems) using a MagMAXTM-96 Total
399 RNA Isolation Kit from Ambion (Life Technologies) following the manufacturer's
400 protocol, but without DNAse to be able to determine the sex of hatchlings via
401 genotyping. The quality and quantity of the extracted RNA was then measured using
402 a NanoDrop and Bioanalyzer. Library preparations (one for each individual) were done
403 using the Illumina TruSeq Stranded Total RNA kit, upon which samples were
404 sequenced together in six lanes. Paired-end sequencing with a read length of 100 bp
405 was done on a HiSeq2000 platform at the GTF (Genomic Technologies Facility,
406 Centre of Integrative Genomics, Lausanne, Switzerland).

407 **Hatchling sex identification**

408 To identify the sex of *T. californicum* hatchlings, we developed microsatellite markers
409 on the *T. californicum* X chromosome scaffolds reported by Parker et al. (in prep).
410 *Timema* have an XX/X0 sex determination system (Schwander and Crespi 2009)
411 meaning X-linked regions will be present in two copies in females and one copy in
412 males. Consequently, females can feature heterozygous genotypes for polymorphic

413 X-linked markers, while males are invariably hemizygous. Candidate microsatellite
414 markers were designed with msatcommander (v. 1.08, default options) (Faircloth
415 2008). A selection of four candidate markers were tested for polymorphism and sex-
416 linkage using 15 adult males and 15 adult females in two Multiplex PCR Kit reactions
417 (Qiagen; see Supplemental Table 1 for primer sequences and Supplemental Table 2
418 for PCR conditions). Microsatellite alleles were then determined with an ABI3100
419 machine (Applied Biosystem) and Genemapper v.4.1 (Applied Biosystems 2009).
420 Across microsatellite markers, all 15 tested females were heterozygous at minimum
421 two markers, whereas males were invariably hemizygous at all markers, confirming
422 the predictions for *Timema* X-linked markers (Supplemental table 2). We thus
423 genotyped the nine hatchlings at the four microsatellite markers to identify their sex,
424 and were able to identify four females and five males that were used for the
425 transcriptome study (Supplemental table 2).

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

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

437 ***Drosophila melanogaster* data**

438

439 *D. melanogaster* RNA-seq whole body data from Ingleby *et al.* (2016) was downloaded
440 from SRA (accession number SRP068235). To mirror the data available for *Timema*
441 and to facilitate comparisons, four replicates per sex and three developmental stages
442 from different hemiclonal lines were used. A complete list of samples is provided in
443 the supplementary materials (Supplemental Table 3). Although reads were deposited
444 as paired-end, we found that most of them were unpaired, thus we used only the
445 forward reads for analysis. Reads were trimmed, mapped to the reference genome
446 (FlyBase, r6.23) (Gramates *et al.* 2017) and counted using the methods described
447 above.

448 **Differential gene expression analysis**

449 Differential expression between the sexes was performed using edgeR v.3.16.5
450 (Robinson *et al.* 2009; McCarthy *et al.* 2012) in RStudio v.1.2.5019 (Team 2019). This
451 analysis fits a generalized linear model following a negative binomial distribution to the
452 expression data and calculates a p-value associated with the hypothesis that gene
453 counts differ between experimental groups. EdgeR implements a normalization
454 method (trimmed mean of M values, TMM) to account for composition bias. We
455 analyzed data separately for each developmental stage. We filtered out genes with
456 low counts; we required a gene to be expressed in a majority of male or female libraries
457 dependent on the number of replicates per sex (i.e. a minimum three libraries when
458 replicate number is >= 4, two libraries when replicate number is = 3), with expression
459 level > 0.5 CPM (counts per million). To test for differential gene expression between
460 the sexes, we used a generalized linear model with quasi-likelihood F- test, with

461 contrasts for each stage between females and males (Chen *et al.* 2016). To correct
462 for multiple testing, we applied the Benjamini-Hochberg method (Benjamini and
463 Hochberg 1995) with an alpha of 5%. Genes with significantly higher expression in
464 males were considered as male-biased, genes with significantly higher expression in
465 females as female-biased, and the genes without significant differential expression
466 between sexes as un-biased. To identify genes showing a significant sex by
467 developmental stage interaction we used a similar GLM modelling approach but with
468 all stages included. We used SuperExactTest v.0.99.4 (Wang *et al.* 2015) to test if the
469 overlap of sex-biased genes between the three developmental stages was greater
470 than what is expected by chance. To visualize the overlap of sex-biased genes
471 between developmental stages we used VennDiagram v.1.6.20 (Chen and Boutros
472 2011). We visualized the $\log_2$FC sex-biased gene expression across developmental
473 stages, using pheatmap v.1.0.12 (Kolde 2018)

474 **Stage specific gene expression**

475 Median gene expression (CPM) values were calculated for each developmental stage
476 and sex. These values were used to calculate Tau, an index of specificity of gene
477 expression over development (Yanai *et al.* 2004; Liu and Robinson-Rechavi 2018)
478 Values of Tau range from zero (broadly expressed over development) to one (gene
479 expressed in only one stage). To visualize the results we made boxplots using ggplot2
480 v. 3.3.2 (Wickham 2016).

481

482 **Divergence rates**

483 To calculate sequence divergence rates, we used the 7098 one-to-one orthologs
484 between *T. californicum* and *T. poppensis* from Jaron *et al.* (2020). Coding sequences
485 were codon-aligned using prank v.170427 (Loytynoja 2014). For each of the orthologs,

486 the number of nonsynonymous substitutions per sites (d_N), the number of synonymous
487 substitutions per site (d_S) and their ratio (d_N/d_S) was calculated using PAML v4.9 (Yang
488 2007). To test for differences in sequence divergence rates (d_N/d_S) between different
489 gene categories (female-biased, male-biased and un-biased), we used Wilcoxon tests
490 (ggpubr v.0.2.5, in R) with p-values adjusted for multiple comparisons using the
491 Benjamini-Hochberg method (Haynes 2013). Note that some of the *T. californicum*
492 sex-biased genes had no identified ortholog in *T. poppensis*, thus we do not have
493 sequence evolution rates for all sex-biased genes. Sequence divergence rates were
494 obtained for 14 out of 26 SBG at the hatchling stage, 323/568 at the juvenile stage,
495 and 1286/2485 at the adult stage. To account for the influence of positive selection we
496 repeated our analyses after removing genes with evidence for positive selection in the
497 *T. californicum* lineage (identified in Jaron *et al.* (2020)).
498
499 Furthermore, we used a partial correlation between the strength of the sex bias
500 (log_2FC) and the sequence divergence rate (d_N/d_S) on sex-biased genes only, while
501 controlling for the average expression level (CPM) of the gene in all libraries (ppcor
502 v.01 (Kim 2015), in R) in order to determine if genes with higher log_2FC have faster
503 sequence divergence rates.
504
505 **Functional analysis of sex-biased genes**
506 We performed Gene Ontology (GO) enrichment analysis using TopGO v.2.26.0 on
507 sex-biased genes obtained with EdgeR (see above) at each developmental stage. We
508 used a functional annotation derived from the blasting sequences to *Drosophila*
509 *melanogaster* database. Only GO terms with minimum ten annotated genes were
510 used. Enrichment of terms was determined using a weighted Kolmogorov-Smirnov-

511 like statistical test, equivalent to the gene set enrichment analysis (GSEA method).
512 We applied the “elim” algorithm, which considers the Gene Ontology hierarchy, i.e. it
513 first assesses the most specific GO terms, and then more general ones (Alexa *et al.*
514 2006). Our analysis focused on gene sets in the Biological Processes (BP), GO
515 category. We considered terms as significant when $p < 0.05$. Enriched GO terms were
516 then semantically clustered using ReviGO (Supek *et al.* 2011) to aid interpretation.

517

518 **Data availability**

519 *Timema californicum* raw reads have been deposited in SRA under PRJNA678950
520 bioproject, with accession codes SRR13084978-SRR13085001 (Supplemental table
521 9). *Timema californicum* mapped read counts are provided in Supplemental table 13
522 and fold changes in Supplemental table 14. *Drosophila melanogaster* mapped read
523 counts are provided in Supplemental table 15.

524

525 **Code availability**

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

528

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532

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