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

Jelisaveta Djordjevic[1], Zoé Dumas[1], Marc Robinson-Rechavi[1,2],
Tanja Schwander[1,a], Darren James Parker[1,2,a]

[1] Department of Ecology and Evolution, University of Lausanne, Lausanne, Switzerland
[2] Swiss Institute of Bioinformatics, Lausanne, Switzerland
[a] equal contribution

Correspondence should be addressed to jelisaveta.djordjevic@unil.ch or DarrenJames.Parker@unil.ch

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. Sex-biased genes identified at early developmental stages were generally consistently male- or female- biased at later stages, suggesting their importance in sexual differentiation. We then 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,* sex-biased gene expression increases abruptly at the adult stage when morphological sexual dimorphism is manifested. This supports the prediction that sex-biased gene expression mirrors phenotypic sexual dimorphism. Our study details for the first time the dynamics of sex-biased gene expression over development in a hemimetabolous insect and suggests that these dynamics differ extensively between holometabolous and hemimetabolous species.

Introduction

Males and females often have divergent evolutionary interests, resulting in sex-specific selection pressures and ultimately the evolution of sexually dimorphic phenotypes (Khila et al., 2012; Lande, 1980). 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 (Chauhan et al., 2016; Pointer et al., 2013). 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 if 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 morphology.

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

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

71 Here, we studied the dynamics of sex-biased gene expression across development in
72 a hemimetabolous insect, *Timema californicum.* These are sexually dimorphic walking-stick
73 insects, found in California (Vickery and Sandoval, 2001). Males are smaller than females, with
74 different coloration, and have conspicuous cerci used for holding on to the female during
75 copulation. We selected three developmental stages (Figure 1a): the hatchling stage where
76 sexes are phenotypically identical, the third juvenile stage with minor phenotypic differences,
77 and the adult stage, with pronounced sexual dimorphism. We investigated whether sex-
78 biased genes are recruited in a stage-specific manner, and if the magnitude of sex-bias
79 increases with development. We further explored sequence evolution rates of sex-biased
80 genes in pre-adult stages, to determine if they show elevated rates as commonly observed

81 for sex-biased genes at adult stages in many species (Ellegren and Parsch, 2007; Grath and
82 Parsch, 2012; Perry et al., 2014).

83 We then compared the dynamics of sex-biased gene expression over development in
84 the hemimetabolous stick insect to those of the holometabolous fly *Drosophila melanogaster*
85 by reanalyzing publicly available RNA-seq data from 3rd. instar larvae, pupal and adult stages,
86 using the same pipeline as for *T. californicum.* In *D. melanogaster*, both larval and pupal stages
87 have little sexual dimorphism, while the adult stage has extensive sexual dimorphism (Figure
88 1b). We hypothesized that sex biased gene expression follows the establishment of
89 morphological sexual dimorphism, with a gradual increase in sex-biased gene expression over
90 development in *T. californicum*, and an abrupt increase at the adult stage in *D. melanogaster*.
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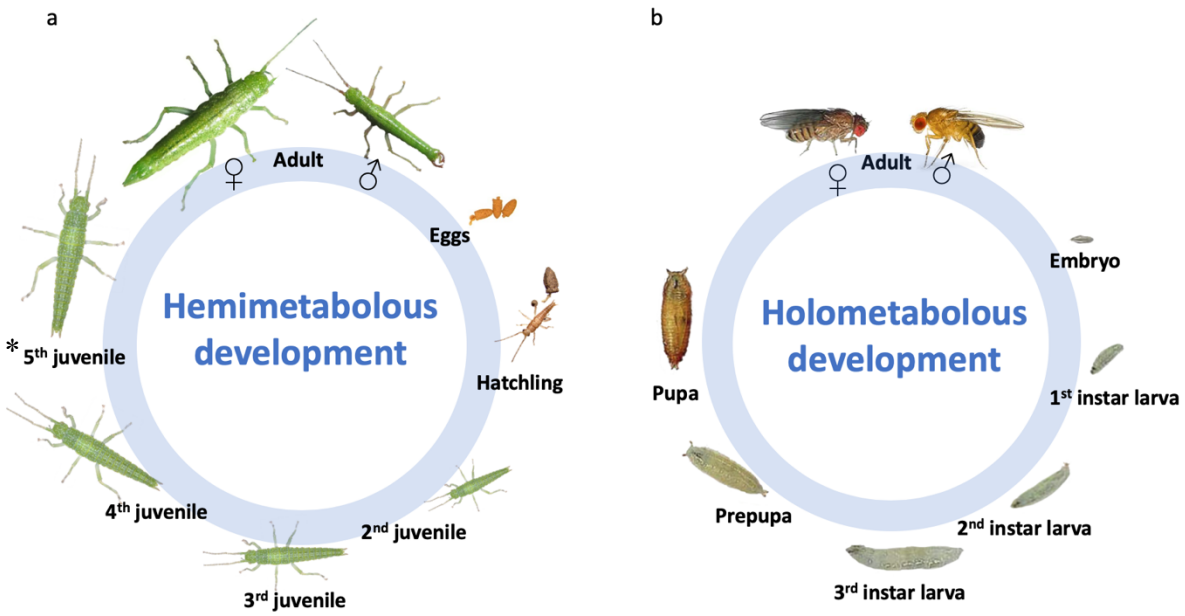

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94 Figure 1. Life cycles of (a) the stick insect *T. californicum* and (b) the fly *D. melanogaster.*
95 *represents a stage present only in females, as *Timema* males have one moult less than
96 females. Hatchling and egg photographs of *Timema* were kindly provided by Bart Zijlstra

97 (http://www.bartzijlstra.com), juvenile and adult stages by Jelisaveta Djordjevic. Life cycle of
98 *D. melanogaster* modified from (Weigmann et al., 2003)
99

100 Material and methods

101 Sample collection and preservation

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

113 RNA extraction and sequencing

114 Individuals (whole-bodies) from all developmental stages were mechanically homogenized
115 with beads (Sigmund Linder) in liquid nitrogen. We then added 900 ul Trizol (Life
116 Technologies), followed by 180 ul chloroform and 350 ul ethanol. The aqueous layer was then
117 transferred to RNeasy MinElute Columns (Qiagen). Upon RNA extraction, samples were
118 treated with DNase Turbo Kit (Life Tech) following the manufacturer's protocol. Total RNA

119 from the hatchlings was extracted with MagMaxTM Express Robot (AB Applied Biosystems)
120 using a MagMAXTM-96 Total RNA Isolation Kit from Ambion (Life Technologies) following the
121 manufacturer's protocol, but without DNAse to be able to determine the sex of hatchlings via
122 genotyping. The quality and quantity of the extracted RNA was then measured using a
123 NanoDrop and Bioanalyzer. Library preparations (one for each individual) were done using
124 the Illumina TruSeq Stranded Total RNA kit, upon which samples were sequenced together in
125 six lanes. Paired-end sequencing with a read length of 100 bp was done on a HiSeq2000
126 platform at the GTF (Genomic Technologies Facility, Centre of Integrative Genomics,
127 Lausanne, Switzerland).

128 **Hatchling sex identification**

129 To identify the sex of *T. californicum* hatchlings, we developed microsatellite markers on the
130 *T. californicum* X chromosome scaffolds reported by Parker et al. (in prep). *Timema* have an
131 XX/X0 sex determination system (Schwander and Crespi, 2009) meaning X-linked regions will
132 be present in two copies in females and one copy in males. Consequently, females can feature
133 heterozygous genotypes for polymorphic X-linked markers, while males are invariably
134 hemizygous. Candidate microsatellite markers were designed with msatcommander (v. 1.08,
135 default options) (Faircloth, 2008). A selection of four candidate markers were tested for
136 polymorphism and sex-linkage using 15 adult males and 15 adult females in two Multiplex
137 PCR Kit reactions (Qiagen; see Supplemental Table 1. for primer sequences and Supplemental
138 table 2. for PCR conditions). Microsatellite alleles were then determined with an ABI3100
139 machine (Applied Biosystem) and Genemapper v.4.1 (Applied Biosystems 2009). Across
140 microsatellite markers, all 15 tested females were heterozygous at minimum two markers,
141 whereas males were invariably hemizygous at all markers, confirming the predictions for

142 *Timema* X-linked markers (Supplemental table 2). We thus genotyped the nine hatchlings at
143 the four microsatellite markers to identify their sex, and were able to identify four females
144 and five males that were used for the transcriptome study (Supplemental table 2).

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

146 The quality of the reads was checked using FastQC v.0.11.2 (Andrews, 2014). We used
147 Cutadapt v. 2.3 with Python v. 3.5.2 (Martin, 2011) to remove adapter sequences. Low quality
148 bases at both ends of the reads were trimmed when below a Phred score of 10. Bases with
149 an average Phred score below 20 in a 4bp sliding window were trimmed. Finally, reads with a
150 length below 80 bp were removed Trimmomatic v. 0.36 (Bolger et al., 2014). Trimmed reads
151 were then mapped to the reference *T. californicum* genome from (Jaron et al., 2020) using
152 STAR v. 2.6.0c (Dobin et al., 2013). HTSeq v.0.9.1 (Anders et al., 2014) was used to count the
153 number of reads uniquely mapped to each gene, with the following options (htseq-count -f
154 bam -r name -s reverse -t gene -i ID -m union --nonunique none).
155

156 ***Drosophila melanogaster* data**

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

165 **Differential gene expression analysis**

166 Differential expression between the sexes was performed using edgeR v.3.16.5 (McCarthy et
167 al., 2012; Robinson et al., 2009) in RStudio v.1.2.5019 (Team, 2019). This analysis fits a
168 generalized linear model following a negative binomial distribution to the expression data
169 and calculates a p-value associated with the hypothesis that gene counts differ between
170 experimental groups. EdgeR implements a normalization method (trimmed mean of M
171 values, TMM) to account for composition bias. We analyzed data separately for each
172 developmental stage. We filtered out genes with low counts; we required a gene to be
173 expressed in n-1 libraries of males or females (where n is number of replicates per stage),
174 with the expression level > 0.5 CPM (counts per million). To test for differential gene
175 expression between the sexes, we used a generalized linear model with quasi-likelihood F-
176 test, with contrasts for each stage between females and males (Chen et al., 2016). To correct
177 for multiple testing, we applied the Benjamini- Hochberg method with an alpha of 5%. Genes
178 with significantly higher expression in males were considered as male- biased, genes with
179 significantly higher expression in females as female- biased, and the genes without significant
180 differential expression between sexes as un-biased. To identify genes showing a significant
181 sex by developmental stage interaction we used a similar GLM modelling approach but with
182 all stages included. We used SuperExactTest v.0.99.4 (Wang et al., 2015) to test if the overlap
183 of sex-biased genes between the three developmental stages was greater than what is
184 expected by chance. To visualize the overlap of sex-biased genes between developmental
185 stages we used VennDiagram v.1.6.20 (Chen and Boutros, 2011). We visualized the $\log_2$FC
186 sex-biased gene expression across developmental stages, using the pheatmap: Pretty
187 Heatmaps v.1.0.12 (Kolde, 2018)

188 **Stage specific gene expression**

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

194

195 **Divergence rates**

196 To calculate sequence divergence rates, we used the 7098 one-to-one orthologs between *T.*
197 *californicum* and *T. poppensis* from (Jaron et al., 2020). Coding sequences were codon-aligned
198 using prank v.170427 (Loytynoja, 2014). For each of the orthologs, the number of
199 nonsynonymous substitutions per sites (d_N), the number of synonymous substitutions per site
200 (d_S) and their ratio (d_N/d_S) was calculated using PAML v4.9 (Yang, 2007). To test for differences
201 in sequence divergence rates (d_N/d_S) between different gene categories (female-biased,
202 male-biased and un-biased), we used Wilcoxon tests (ggpubr v.0.2.5, in R) with p-values
203 adjusted for multiple comparisons using the Benjamini-Hochberg method (Haynes, 2013).
204 Note that some of the *T. californicum* sex-biased genes had no identified ortholog in *T.*
205 *poppensis*, thus we do not have sequence evolution rates for all sex-biased genes. Sequence
206 divergence rates were obtained for 14 out of 26 SBG at the hatchling stage, 323/568 at the
207 juvenile stage, and 1286/2485 at the adult stage. To account for the influence of positive
208 selection we repeated our analyses after removing genes with evidence for positive selection
209 in the *T. californicum* lineage (identified in (Jaron et al., 2020)).

210

211 Furthermore, we used a partial correlation between the strength of the sex bias (log_2FC) and
212 the sequence divergence rate (d_N/d_S) on sex-biased genes only, while controlling for the
213 average expression level (CPM) of the gene in all libraries (ppcor v.01 (Kim, 2015), in R) in
214 order to determine if genes with higher log_2FC have faster sequence divergence rates.
215

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

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

227 **Results**

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

229 Sex-biased gene expression gradually increased over the three developmental stages, with
230 0.2% (26) of the expressed genes sex-biased at the hatchling stage, 4.7% (568) at the juvenile,
231 and 20.3% (2485) at the adult stage (Figure 2). The hatchling stage had more female than
232 male biased genes ($x^2_{(1)}$ =12.46, $p<0.05$), while the juvenile and adult stages had more male

233 biased genes ($x^2_{juvenile\ (1)}$ =457.92, $x^2_{adult\ (1)}$ = 77.554, p<0.05) (Supplemental Table 4). The
234 majority (85% and 91%) of sex biased genes at the two preadult stages had strong sex bias
235 (≥2 log_2FC, P_{adj}<0.05) in contrast to the adult stage where sex bias was slight (57% of the sex-
236 biased genes had <2 log_2FC, P_{adj}<0.05) (Figure 2). The intensity of sex-bias varied greatly over
237 development with 1137 genes showing a significant sex by development interaction
238 (P_{adj}<0.05).

239 To characterize the dynamics of sex-biased expression over development, we then classified
240 all 2671 genes sex-biased at one or more developmental stages into 13 categories dependent
241 on their expression patterns over development (Supplemental Figure 1). This classification
242 revealed that sex-biased genes are added gradually over development, with genes sex-biased
243 at earlier stages generally remaining sex-biased in the same direction at later stages
244 (Supplemental Figure 2). Only a single gene shifted from male-biased to female-biased
245 expression. Out of 26 sex-biased genes at the hatchling stage, 34% remained sex-biased
246 throughout development, while out of 567 sex-biased genes at the juvenile stage, 67% stayed
247 sex-biased at the adult stage. This classification is conservative, as it depends on the sex-
248 biased gene expression detection threshold.

249 Sex-biased genes at the hatchling stage are particularly interesting because they reveal sexual
250 differentiation prior to visible morphological differences. We therefore looked for functional
251 annotations of the 26 sex-biased genes at the hatchling stage in the *T. californicum* reference
252 genome from (Jaron et al., 2020). However, only 16 out of the 26 sex-biased genes had
253 functional annotations and only one (*vitellogenin receptor*) had a clear link to sexual
254 differentiation (Figure 3b). While key genes known to play a role in insect sex-determination
255 and differentiation pathways (*doublesex*, *transformer-2* and *sex-lethal*) were expressed at the

256 hatchling stage, *doublesex* had very low expression, preventing analysis of sex-bias, and
257 *transformer-2 and sex-lethal* did not feature sex-biased expression.

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

259 Genes sex-biased at the hatchling stage were enriched for GO-terms related to
260 developmental processes (e.g., "regulation of cell development", "regulation of cell
261 proliferation", "cuticle development"), and some to female specific functions such as
262 oogenesis ("oocyte construction", "oocyte development") (Supplemental figure 8a, and
263 Supplemental table 10). At the juvenile stage, sex-biased genes were enriched for GO-terms
264 related to metabolic processes, in particular to catabolism (e.g., "lipid catabolic processes",
265 "cellular catabolic processes", "regulation of catabolic processes") (Supplemental figure 8b,
266 and Supplemental table 11). At the adult stage, sex-biased genes were enriched for GO-terms
267 related to diverse metabolic and physiological processes with no clear association to sexual
268 differentiation (Supplemental figure 8c, and Supplemental table 12); however, several terms
269 were related to chemosensory and olfactory behavior, which may play a role in mate
270 detection. Furthermore, adult stage, sex-biased genes were enriched for pigmentation (e.g.,
271 "developmental pigmentation", "eye pigmentation"), which is a sexually dimorphic trait in
272 *Timema californicum*.

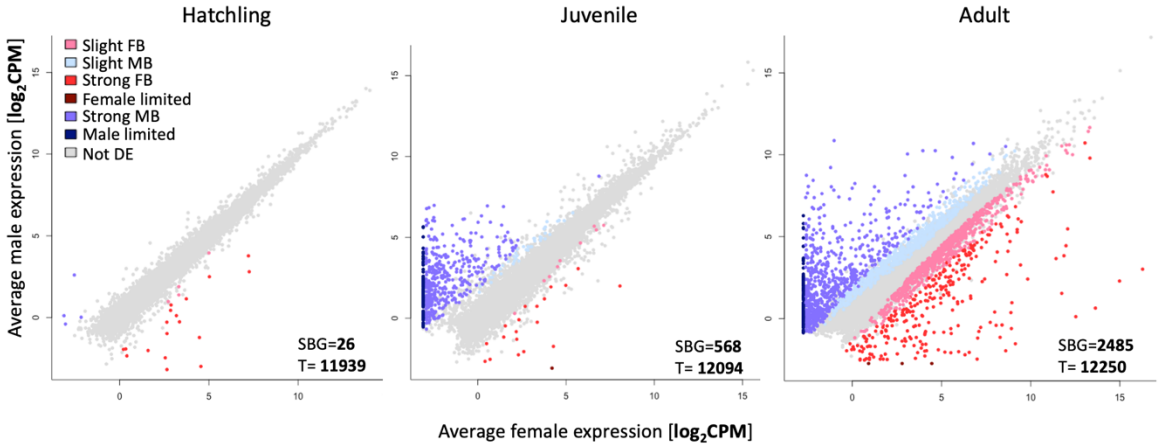
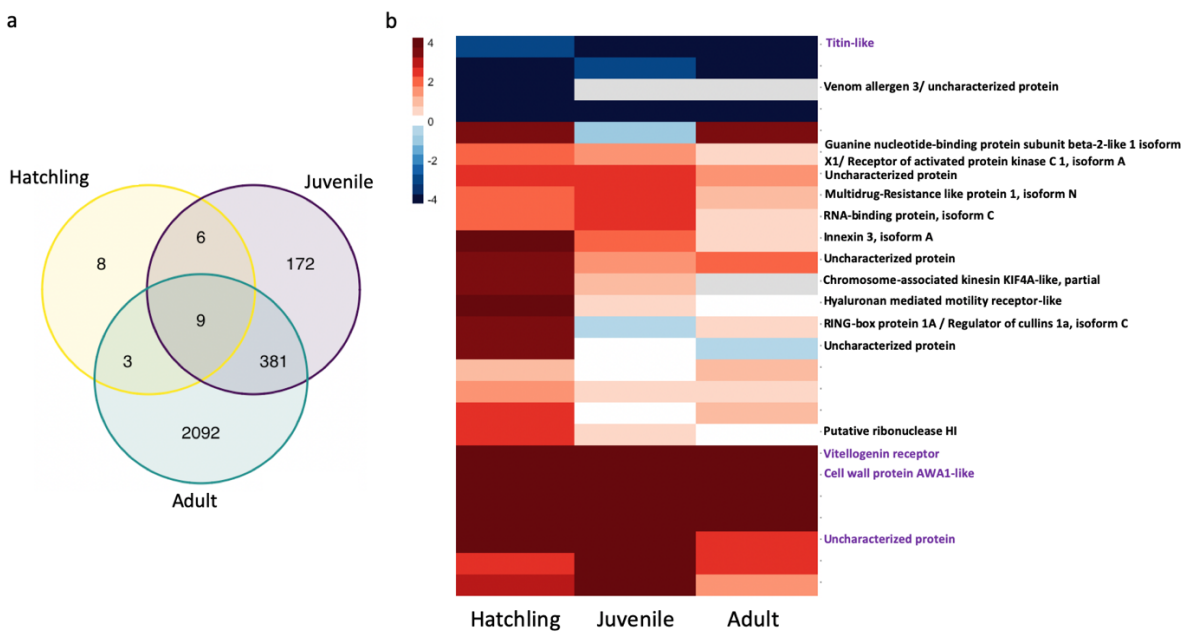


Figure 2 | Gene expression (log_2CPM) in *T. californicum* males and females at the hatchling (left), juvenile (middle) and adult stage (right). The number of differentially expressed genes (SBG) is shown at the bottom right corner of each plot, as well as the total number of expressed genes at each stage (T). Genes are classified based on their sex-bias into seven categories: "slight FB"- female bias (<2 log_2FC), "strong FB"- female bias (≥2 log_2FC), "female limited"- with no expression in males, "slight MB"- male bias (<2 log_2FC), "strong MB"- male bias (≥2 log_2FC), "male limited"- no expression in females, "Not DE"- not differentially expressed genes.

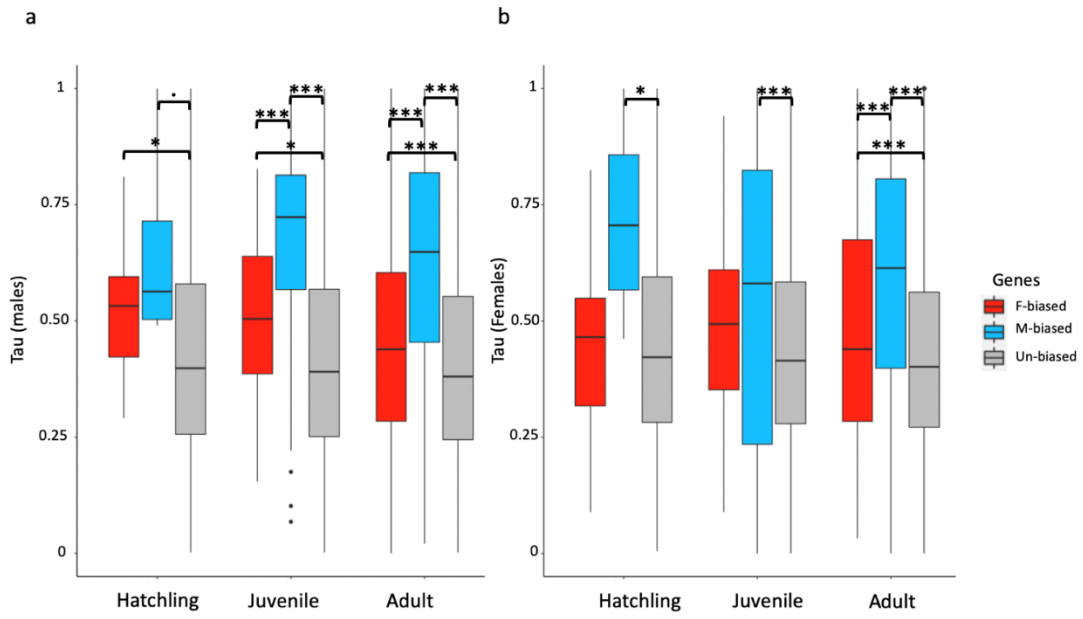


283 **Figure 3 | Sex-biased genes shared across development**. (**a**) Venn-diagram of sex-biased
284 genes in three developmental stages: hatchling (yellow), juvenile (purple), and adult (green).
285 The number of genes shared between all three stages was greater than expected by chance
286 (observed overlap= 9, expected overlap= 0.007, Exact test of multi set interactions: p-value <
287 0.0001). (**b**) A heatmap showing the 26 sex-biased genes at the hatchling stage with their
288 annotations and their expression in three developmental stages. Genes in red are female-
289 biased, blue are male-biased. Saturation of the colors increases with the $\log_2$FC. Genes
290 without a label have no annotation, annotation in purple is for genes that were sex-biased in
291 all three stages

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

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

298



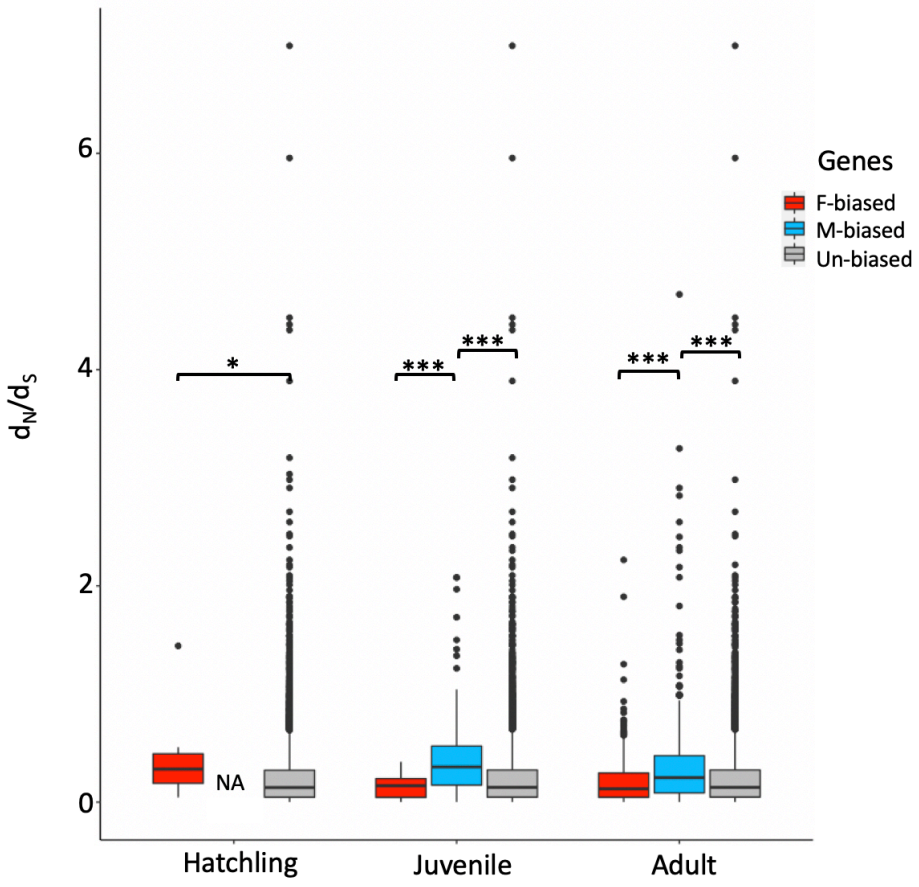
299 **Figure 4 |** Tau index of gene expression at three developmental stages in males (a) and
300 females (b). Tau range from zero (similarly expressed over development) to one (gene
301 expressed in only one stage). Three gene categories are depicted with different colors;
302 female-biased in red,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

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

307 Male-biased genes have faster rates of sequence evolution (dN/dS) compared to both un-
308 biased and female-biased genes, at juvenile and adult stages (Figure 5). At the hatchling stage,
309 female-biased genes evolve faster than the un-biased genes, but not at the other two
310 developmental stages. Elevated rates of sequence evolution are likely the result of reduced
311 purifying selection, but stronger positive selection may also contribute to fast sequence
312 evolution in some genes. To test this possibility, we repeated our analysis after excluding 19

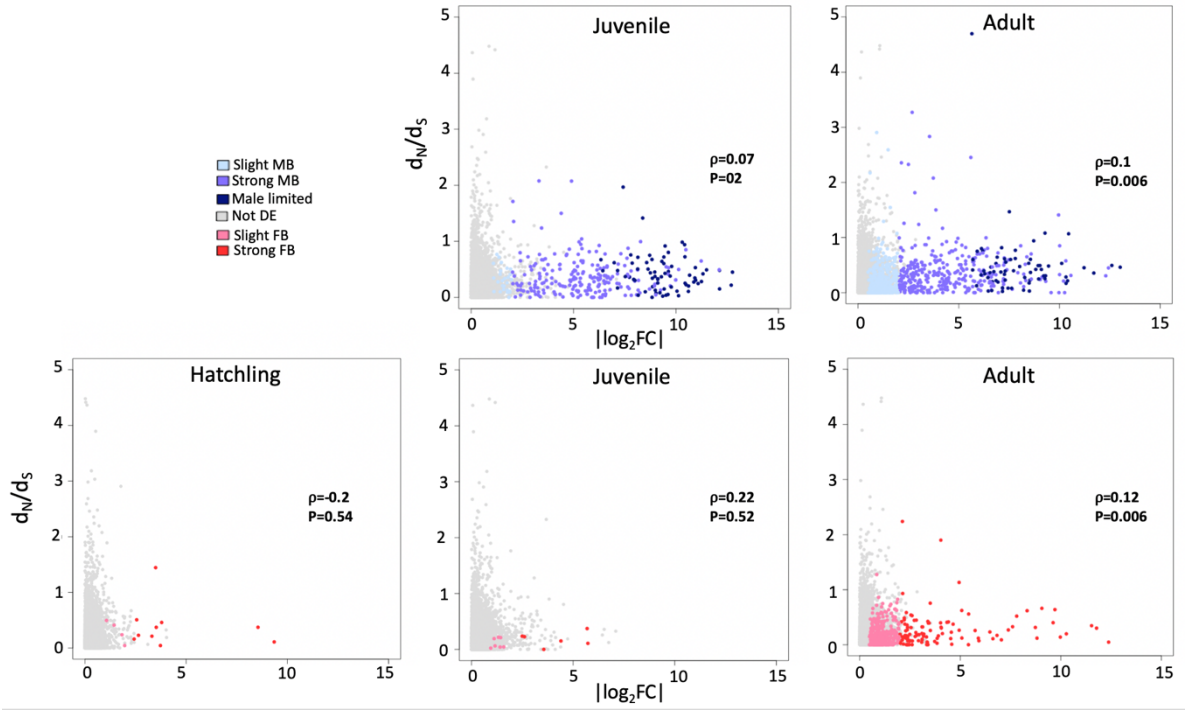
313 (11 F- biased and 8 M-biased) genes for which a previous study (Jaron et al., 2020) detected
314 signatures of positive selection. Excluding these 19 genes did not change our results
315 (Supplemental Table 7a). More generally, we did not find an enrichment of positively selected
316 genes among those with sex-biased expression (Fisher's exact test; p-value=0.39, odds ratio:
317 1.1) (Supplemental Table 7b).

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



323

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



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

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

335 In order to compare the dynamics of sex-biased gene expression over development between
336 hemimetabolous and holometabolous insects, we reanalyzed publicly available data from *D.*

337 *melanogaster* from (Ingleby et al., 2016), using the same pipeline as for *T. californicum.* This
338 experiment was chosen for comparison as it has a similar design, i.e. whole-body RNA-seq
339 data from several developmental stages. In addition, although *D.* melanogaster from this
340 experiment were lab-reared, they had a similar between sample variance as *T. californicum*
341 (biological coefficient of variation at the adult stage: *D. melanogaster* = 0.266, *T. californicum*
342 = 0.348).

343 In contrast to the gradual increase in the amount of sex-biased expression over development
344 in *T. californicum,* sex-biased gene expression increased abruptly at the adult stage in *D.*
345 *melanogaster* (Figure 8), from 22% to 85% of sex-biased genes (Figure 7). Overall, at each
346 developmental stage, there were more male than female-biased genes (Supplemental table
347 2). The majority (85% and 63%) of sex-biased genes at the two preadult stages had strong sex
348 bias ($\geq$2 $\log_2$FC, P_{adj} <0.05) in contrast to the adult stage where weaker sex-bias was more
349 common (51% of the sex-biased genes had <2 $\log_2$FC, P_{adj} <0.05) (Supplemental table 2). Out
350 of 2113 sex-biased genes at the larval stage, 58% remained sex-biased throughout
351 development, and 93% of the pupal sex-biased genes remained sex-biased in adults (Figure
352 7b).

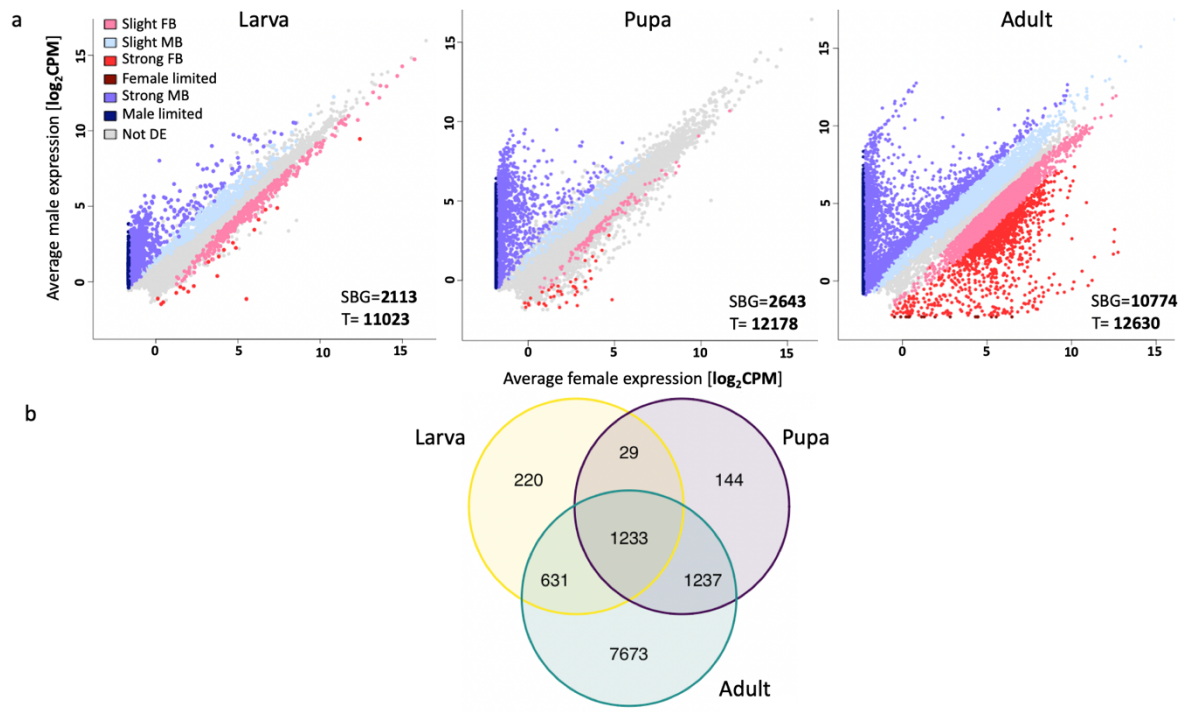

Figure 7 | (a) Gene expression (log_2CPM) in males and females at the larval (left), pupal (middle) and adult stage (right). The number of differentially expressed genes (SBG) is shown at bottom right corner of each plot, as well as the total number of expressed genes at each stage (T). Genes are classified based on their sex-bias into seven categories: "slight FB"- female bias (<2 log_2FC), "strong FB"- female bias (≥2 log_2FC), "female limited"- with no expression in males, "slight MB"- male bias (<2 log_2FC), "strong MB"- male bias (≥2 log_2FC), "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= 1233, expected overlap=195.2), Exact test of multi set interactions: p-value < 0.0001).

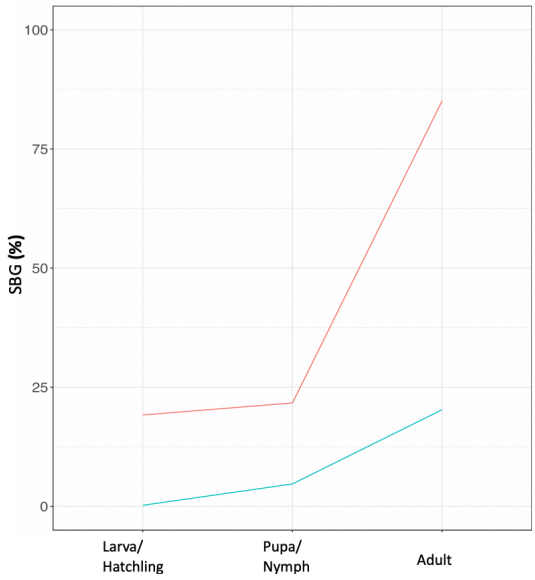

Figure 8 | Percentage of sex-biased genes (SBG) in *T. californicum* (blue) and *D. melanogaster* (red) at three developmental stages.

Discussion

In the stick insect *T. californicum,* gene expression differs between males and females before morphologically detectable sexual dimorphism. Over 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 over animal development. For example, in humans or mosquitos, some stages have bursts of sex-biased gene expression, suggesting that specific developmental stages contribute discretely to sexual differentiation, and that sexual phenotypes are not gradually established (Magnusson et al., 2011; Shi et al., 2016).

379 Although only 26 genes were sex-biased in *T. californicum* at the hatchling stage, they
380 all had a strong sex-bias and nine of them stayed sex-biased at juvenile and adult stages. As
381 such these 26 genes likely represent key genes involved in the sexual differentiation of
382 *Timema*. Most of the sex-biased genes at the hatchling stage were female-biased. This might
383 suggest that the process of building a female phenotype begins earlier in development. Later
384 developmental stages had more male-biased than female- biased genes. A similar shift from
385 female- to male-bias in gene expression during development was found in *Nasonia* jewel
386 wasps, where little male-biased expression was detected until the pupal stage, during the
387 activation of spermatogenesis. Similar to *Timema*, the shift was interpreted as a different
388 developmental timing of the two sexes (Rago et al., 2020). In *Timema*, male-biased genes
389 were also expressed in a relatively stage-specific manner, in contrast to a more constant
390 expression of female-biased genes. This further supports the idea that *Timema* individuals
391 first develop along the female trajectory (or the female phenotype being the "default" in
392 *Timema*) and may indicate greater pleiotropy of female-biased genes. Additional support for
393 this interpretation comes from the lower sequence divergence rates of female-biased genes
394 than male-biased genes, implicating stronger selective constraints on female- than male-
395 biased genes. In addition, we observed that genes with greater sex-bias have faster sequence
396 divergence rates. This is largely due to relaxed purifying selection, with positive selection
397 contributing little to the accelerated evolutionary rate of sex-biased genes. These findings are
398 consistent with the idea that sex-biased genes evolve from genes with few evolutionary
399 constraints and that are relatively "dispensable" (Catalan et al., 2018; Mank and Ellegren,
400 2009).

401 Sex-biased genes in *Timema* hatchlings were enriched for processes related to
402 oogenesis. Moreover, one of the most consistently and strongly female-biased genes across
403 development was annotated as *vitellogenin receptor*. In adult flies, *vitellogenin receptor*
404 transfers yolk protein precursors into the oocytes and is necessary for the development of
405 the oocytes and later embryo (Liu et al., 2016). The role of these processes in *Timema*
406 hatchlings remain to be investigated. Sex-biased genes in juvenile and adult stages were not
407 enriched for processes obviously related to sex- specific functions. However, some processes
408 such as pigmentation and chemosensory/olfactory behavior could have a sex-related role.
409 Pigmentation is a sexually dimorphic trait in *Timema* (Sandoval, 2008), while chemosensory
410 and olfactory behavior are important for mate recognition and mating (Parker et al., 2019b;
411 Schwander et al., 2013). We additionally looked for the expression of two key genes involved
412 in insect sex-determination pathways: *transformer* and *doublesex*. In holometabolous insects,
413 *transformer* acts as a splicing regulator of *doublesex*, a sex differentiation master switch gene
414 (Geuverink and Beukeboom, 2014). The *Doublesex- Transformer* role in sex-differentiation is
415 conserved among Diptera, Coleoptera and Hymenoptera (Verhulst et al., 2010; Wexler et al.,
416 2019). Emerging studies suggest that *doublesex* and *transformer* likely play the same role in
417 sex differentiation in hemimetabolous insects (Wexler et al., 2019; Zhuo et al., 2018). In *T.*
418 *californicum,* we found that *doublesex* was expressed in all three developmental stages, but
419 too lowly for analyzing sex-bias. *Transformer* is not annotated in the available *T. californicum*
420 genome; a *Transformer-2 homolog* is annotated but did not feature sex-bias at any of the
421 three developmental stages. As such, whether or not these classic sex-determining genes
422 influence sexual differentiation in *Timema* is unclear, requiring future studies.

423 We hypothesized that the dynamics of sex biased gene expression would be strongly
424 affected by hemimetabolous vs holometabolous development in insects, with a gradual
425 increase of sex-biased gene expression over development in the former and an abrupt
426 increase at the adult stage in the latter. Hemimetabolous insects such as *T. californicum* have
427 multiple nymphal stages that progressively resemble the adult stage, together with a gradual
428 increase in sexual dimorphism. On the other hand, a holometabolous insect like *D.*
429 *melanogaster* has more monomorphic larval stages and pupae, with no resemblance to the
430 sexually dimorphic adult stage. The change in sexually dimorphic gene expression across
431 development mirrored these different developmental patterns, with the proportion of sex-
432 biased genes increasing gradually in *T. californicum* as sexual dimorphism became more
433 pronounced, whereas sexually dimorphic gene expression increased abruptly at the adult
434 stage in *D. melanogaster*.

435 Although the change in sexually dimorphic gene expression across development fit
436 with our expectations, we were surprised to find a much higher proportion of sex-biased
437 genes in *D. melanogaster* than in *T. californicum* overall. This difference is clearest at the adult
438 stage when most of the expressed genes in *D. melanogaster* were sex-biased (85%),
439 compared to only 20% in *T. californicum*. Similar to *Drosophila*, other holometabolous insects
440 also featured sex-bias for more than half of the genes expressed at the adult stage (Dean A
441 Baker et al., 2011; Rago et al., 2020), while all studied hemimetabolous insects had a much
442 smaller proportion of sex-biased genes (<10%) (Pal and Vicoso, 2015). Why there is such a
443 difference in the prevalence of sex-biased genes between species with different
444 developmental strategies is still an open question. One potential explanation is that the
445 proportion of sex-biased genes at the adult stage may be less constrained under holo- than

446 hemimetabolous development. For example, the complete reorganization of cells and tissues
447 during metamorphosis in holometabolous insects may allow for more drastic phenotypic
448 changes between the sexes, while incomplete metamorphosis constrains the evolution of
449 distinct sexual phenotypes. Data on additional species are required to confirm that this
450 difference is not simply due to the species investigated, but is indeed general.
451 Holometabolous insects studied are often established lab-models, with high fecundity, and
452 may thus be characterized by comparatively large gonads, which would result in large
453 proportions of sex-biased genes in adults. By contrast, all hemimetabolous insects
454 characterized for sex-biased expression are non-model species. However, the choice of model
455 species is unlikely to be the sole explanation for the different amount of sex bias in holo- and
456 hemimetabolous species. Indeed, in crickets and *Timema* stick insects, even the most sexually
457 differentiated tissue (gonads) feature sex-bias of fewer than 30% of genes (Parker et al.,
458 2019a; Whittle et al., 2020), suggesting that extensive differences are not completely driven
459 by large gonads.

460 Overall, our results describe the dynamics of sex- biased gene expression over
461 development in a hemimetabolous insect, and suggest profound differences of these
462 dynamics between holometabolous and hemimetabolous species. We showed that the
463 extent of sex-biased gene expression follows the development of sexual dimorphism.
464 Generating sexually dimorphic phenotypes is a developmental process, and sex-biased genes
465 are added gradually over development, with the majority of early sex-biased genes
466 consistently sex-biased throughout development.

467

468 **Data availability**

469 Raw reads have been deposited in SRA under PRJNA678950 bioproject, with accession codes
470 SRR13084978- SRR13085001 (Supplemental table 9). Mapped read counts are provided in
471 Supplemental table 13 and fold changes in Supplemental table 14.

472

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475

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