Sex-biased gene expression is repeatedly masculinized in asexual females

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Abstract

Males and females feature strikingly different phenotypes, despite sharing most of their genome. A resolution of this apparent paradox is through differential gene expression, whereby genes are expressed at different levels in each sex. This resolution, however, is likely to be incomplete, leading to conflict between males and females over the optimal expression of genes. Here we test the hypothesis that gene expression in females is constrained from evolving to its optimum level due to sexually antagonistic selection on males, by examining changes in sex-biased gene expression in five obligate asexual species of stick insect, which do not produce males. We predicted that the transcriptome of asexual females would be feminized as asexual females do not experience any sexual conflict. Contrary to our prediction we find that asexual females feature masculinized gene expression, and hypothesise that this is due to shifts in female optimal gene expression levels following the suppression of sex.

Introduction

Genetic constraints between developmental stages, sexes, and castes arise as a result of a shared genome[1]. Species are able to mitigate these constraints by differentially expressing suites of genes in specific contexts to produce and maintain different phenotypes. This resolution, however, may be incomplete when regulatory control of gene expression is not sufficiently labile as to allow for optimal expression in each phenotypic context, leading to intralocus conflict[2].

This phenomenon has been most widely studied between the sexes, where strong sexual dimorphism is generally underlain by sex-biased gene expression[3]. Sex-biased gene expression is thought to have been largely driven by selection to resolve intralocus sexual conflict[4]. As such, contemporary sex-biased gene expression is expected to represent a combination of both resolved and partially un-resolved sexual conflict[5,6]. In the latter case, suboptimal gene expression levels are maintained by opposing selection in males and females, with the relative strengths of selection acting on each sex determining the difference between optimal and observed (suboptimal) expression levels.

Sexually antagonistic selection has the potential to constrain the optimal expression of large portions of a species' transcriptome and to thereby generate sub-optimal phenotypes in each sex. However, whether un-resolved conflict is pervasive can be difficult to investigate in natural populations, due to the relatively small (but numerous) effects of individual loci[5,7]. An ideal situation to address this question would be to examine how the transcriptome evolves following the cessation of sexual conflict. This is the case in asexually reproducing species when derived from a sexual ancestor. Because asexual species consist only of females, there is no sexual conflict and selection can optimize the female phenotype independently of any correlated effects in males. Despite the potential of this approach, previous studies have only used sexual species, examining how the transcriptome changes under experimentally altered levels of sexual selection[8–11].

The premise of these studies is that because sexual selection is typically stronger on males than females[12], a reduction in sexual selection (e.g. by enforcing monogamy) will disproportionately affect males, resulting in a shift in gene expression towards the female optimum. While this optimum is unknown, it is assumed that female-biased genes are generally beneficial for females, and male-biased genes for males[4,13], such that shifts towards female optima would generate a

58 feminization of gene expression (increased female-biased and decreased male-biased
59 expression). The empirical support for this hypothesis, however, remains mixed[8–11], and the most
60 recent study[9] further showed that shifts in sex-biased gene expression under altered sexual
61 selection varied among tissues and conditions. However, a potential constraint in these studies
62 is that even under reduced sexual selection, selection still acts on male phenotypes as fertile
63 males still need to be produced in each generation. Thus, many genes potentially subject to
64 sexually antagonistic selection therefore remain unaffected by reduced sexual selection, with
65 genes negatively affecting male viability or fertility being obvious examples. This constraint does
66 not apply to recently derived asexual species as *all* aspects of sexual conflict present in the sexual
67 species are absent in the asexual species.
68
69 Here we use *Timema* stick insects to examine how sex-biased genes change in expression
70 following a transition to asexuality. *Timema* comprise multiple independent transitions to
71 asexuality (Fig. 1)[14], allowing us to examine how idiosyncratic any shifts in sex-biased gene
72 expression are. Furthermore, male *Timema* have a single X and no Y chromosome (XX / X0 sex
73 determination)[15], avoiding any potential difficulties arising from sex-limited regions of the genome.
74 We first identify genes with sex-biased expression in five sexual *Timema* species by sequencing
75 the transcriptomes of three different tissue types in each sex. We then study the fate of these sex-
76 biased genes in close asexual relatives of each sexual species to test whether their expression
77 is consistently feminized. This allows us to test the importance of intralocus sexual conflict on
78 gene expression changes following a loss of sex. Contrary to our prediction, we find evidence for
79 an overall masculinization of sex-biased gene expression in asexual females, and hypothesise
80 that this is due to shifts in female trait optima levels following the suppression of sex.

Results

Asexuality repeatedly masculinizes gene expression

To examine changes in sex-biased gene expression in asexual females, we first identified orthologous genes in each of the five sexual-asexual sister species pairs using reciprocal best Blast hits. We then classified genes as being sex-biased by comparing male and female expression in each sexual species (FDR < 0.05, absolute fold-change > 2). Sex-biased genes were identified separately for each sexual species and each of the three tissue types (whole bodies, reproductive tract and leg tissue; see Methods). As expected, given their different roles and morphology in males and females, reproductive tracts featured large numbers of sex-biased genes in all species (2843-3845, corresponding to approximately 30% of each transcriptome; Supplementary Table 1). Legs and whole bodies had fewer genes with sex-biased gene expression overall, but with considerable variation among species (0.5-12%; Supplementary Table 1). Variation among species could be due to variation in sexually dimorphic physiology between species but is also likely driven (at least partially) by differences in between sample variance (Supplementary Table 2). Note that because sex-biased genes were identified separately for each sexual species this approach cannot be used to determine if sex-biased genes are the same across species. We therefore investigated if sex-biased genes are the same between species as a second step (see below). Although the genes may be different, we can examine if sex-biased genes in different species are involved in similar functions by comparing the GO terms of sex-biased genes across species. Sex-biased genes in each species and tissue type were significantly enriched for many functions (136-445 significant GO terms for male-biased genes, 138-726 for female-biased genes; Supplementary Data 1). Few GO terms overlapped between species (Supplementary Fig. 1) (though the overlap was greater than expected by chance (SuperExactTest, FDR < 0.05, Supplementary Data 2)), even when enriched GO terms were first clustered by parent or child terms (Supplementary Fig. 2).

We then examined whether sex-biased genes change in expression between sexual and asexual females. Surprisingly, we found that the transcriptomes of asexual females were strongly masculinized. The expression of female-biased genes was significantly reduced in all five independently evolved asexual species and in each tissue type (14 out of 15 instances, the exception being the whole-body comparison between *T. podura* and *T. genevievae*, which shows reduced female-biased gene expression, but not significantly (Wilcoxon test, FDR = 0.076), Fig.

115 2, Supplementary Table 3). By contrast, male-biased genes significantly increased in expression
116 in most tissue types of asexual females (10 out of 15 instances), although they also significantly
117 decreased in two instances (in *T. shepardi* reproductive tracts and *T. tahoe* legs) (Fig. 2,
118 Supplementary Table 3). We also examined if the amount of change in sex-biased gene
119 expression altered with asexual lineage age (measured as sex-asex species divergence time[18]).
120 While we found a relationship between sex-biased gene expression and asexual lineage age
121 (permutation ANCOVA, $P < 0.001$), it was small and inconsistent between tissue-types
122 (Supplementary Fig. 3, p-value of interaction term < 0.001).
123
124 In addition to sex-biased genes, one class of interesting genes is sex-limited genes (genes
125 expressed in only one of the two sexes). The expression of sex-limited genes depends on sex-
126 specific regulation in males and females. Sex-limited genes are therefore expected to be free
127 from sexual conflict over expression levels and may show different shifts in expression in asexual
128 females than sex-biased genes. In particular, we expect that there will be no overall change in
129 expression between sexual and asexual females, if relaxation of sexual conflict is the main driver
130 of changes in asexual females. Note that sex-limited genes were identified separately from sex-
131 biased genes to avoid inflating the dispersion of the model used to identify sex-biased genes (see
132 Methods). Overall, we find only a few sex-limited genes (0-50), with most of these in the
133 reproductive tracts (Supplementary Tables 4 and 5). Like female-biased genes, female-limited
134 genes also show a significant reduction in expression in asexual females in most cases (8 out of
135 the 9 instances with more than one female-limited gene) (Fig. 3, Supplementary Fig. 4,
136 Supplementary Table 4). Almost all male-limited genes show very little to no expression in asexual
137 females, and are expressed at much lower levels than found in males (Fig. 3, Supplementary Fig.
138 4, Supplementary Table 4), suggesting that few, if any, male-limited genes have been co-opted
139 for new functions in asexual females.
140
141 Our analyses show that gene expression in asexual females is generally masculinized. This effect
142 is particularly clear for female-biased genes which decrease in expression across five different
143 species and three different tissue types, showing the masculinization of sex-biased gene
144 expression in asexuals is very repeatable. Given this unexpected finding, we verified that our
145 results were not biased by the gene sets we chose to use, which excluded genes with very low
146 expression in asexual females, and genes without an ortholog between sexual and asexual sister
147 species (see Methods). Exclusion of these genes could bias our results if shifts in gene expression
148 disproportionately occur in these genes. To examine the impact of these factors we firstly

149 repeated our analyses without excluding genes with low expression in asexual females. Generally
150 excluded genes were few in number (1-6%) and more likely to be male-biased (Supplementary
151 Tables 6-7). Repeating our analyses with these genes included found that shifts in sex-biased
152 gene expression in asexuals remained qualitatively the same as when they were excluded
153 (Supplementary Fig. 5). Secondly, we mapped reads from all samples of a sexual-asexual
154 species pair to a single reference (the full transcriptome of either the sexual or the asexual
155 species). With this strategy there is no need to identify orthologs between sexual and asexual
156 species pairs. Repeating our analyses using the full sexual or asexual transcriptome, we found
157 very few sex-biased genes had no expression in asexual females (Supplementary Tables 8-11),
158 and we obtained qualitatively similar results as in the main analysis (Supplementary Figs 6 and
159 7). Taken together these analyses show that the masculinization of gene expression we observe
160 is not biased by our gene set selection.
161
162 ***Gene expression changes are independent of gene identity***
163
164 In the above analyses, each species-pair was treated separately, which allowed us to maximize
165 the number of genes used in comparing changes in sex-biased gene expression between sexual
166 and asexual females. In doing so we use five different reference gene sets (pairwise orthologs
167 between sexual and asexual sister species, see Methods), which prevents us from examining
168 whether repeated changes to the same sex-biased genes are responsible for the expression shifts
169 we observe in asexual females.
170
171 To answer this question we firstly repeated the above analyses using only genes with 1-to-1
172 orthology between all ten species (between 2886 and 3003 expressed genes depending on tissue
173 type, see Methods). Results based on this reduced gene set are qualitatively the same as using
174 the full gene set, i.e. an overall masculinization of sex-biased gene expression in asexual females
175 (Supplementary Fig. 8). As in the previous analyses the reproductive tract featured more sex-
176 biased genes (784-1071) than whole bodies and legs (43-375) (Supplementary Table 12). This
177 pattern is further illustrated by the fact that reproductive tract samples cluster first by sex and then
178 phylogeny, whereas it is the opposite for legs (Fig. 4). Whole-body samples show a more mixed
179 pattern with most samples clustering firstly by sex but with one species (*T. podura*, which has the
180 fewest sex-biased genes in this tissue type) clustering firstly by phylogeny. Despite the lower
181 power of this smaller gene set (compared to the full gene set), expression of female-biased genes
182 was significantly reduced in asexual females in 11 out of 15 instances. Male-biased gene

183 expression significantly increased in asexual females in 9 out of 15 instances (Supplementary
184 Fig. 8, Supplementary Table 13).
185
186 The overlap between sex-biased genes from different species is significantly greater than
187 expected by chance but rather small in size (Figs. 5A and 5B, Supplementary Data 3). Importantly
188 for our analyses, the small overlap between species means that the consistently masculinized
189 gene expression we observe in asexual females is largely independent of gene identity. This
190 finding is strengthened by an examination of the shifts in expression for genes sex-biased in 1, 2,
191 3, 4 or 5 sexual species, which show that the masculinization seen in asexual females is stronger
192 for genes that are sex-biased in fewer sexual species (Fig. 5C, Likelihood Ratio Test, FDR < 0.05
193 for male and female- biased genes in all tissues, Supplementary Table 14). These findings
194 suggest that the shifts in sex-biased gene expression we see are likely due to the property of
195 them being sex-biased, rather than them being involved in the same specific biological process.
196
197 ***Functional analysis of sex-biased genes***
198
199 A plausible explanation for decreased expression of female-biased genes in asexual females is
200 selection against traits used for sexual reproduction. In asexual *Timema*, several sexual traits are
201 known to be reduced, including the production of volatile and contact pheromones[16]. Here we
202 observe that female-biased genes are indeed enriched for terms linked to the production of sexual
203 phenotypes (e.g. pheromone biosynthetic process, reproductive behavior, etc, Supplementary
204 Data 1), however, to more specifically identify functions that may be affected by shifts in gene
205 expression, we examined the GO terms specifically enriched in female-biased genes with
206 decreased expression in asexual females.
207
208 Depending on species and tissue type, between 0 and 160 GO terms were significantly enriched,
209 with far fewer terms enriched in legs than in whole-bodies or reproductive tracts (Supplementary
210 Data 4), as expected given the smaller number of sex-biased genes in legs. There are no
211 consistently enriched GO terms between all species (Supplementary Fig. 9A), and although some
212 terms can be easily associated with reduction of sexual traits (e.g. olfactory behavior,
213 chemosensory behavior, detection of stimulus involved in sensory perception), the majority of
214 terms have no clear link to sexual trait reduction. Most enriched terms instead are related to
215 metabolic and developmental processes. This could potentially be a signature of a shift in energy
216 budget in asexual females which no longer have to produce costly sexual traits (Supplementary

217 Data 4). Male-biased genes that increased in expression were enriched for between 0 and 81
218 terms, and again, no terms were shared between all species, and very few between any pair of
219 species (Supplementary Data 5, Supplementary Fig. 9B).
220
221 The removal of sexual conflict is expected to cause the feminization of gene expression in asexual
222 females. Although overall the pattern of expression change we observe is opposite to this
223 prediction, it is possible that a feminization of gene expression still occurs for a small subset of
224 genes, but its effect is masked by the larger effect of masculinization. We specifically examine
225 the subset of sex-biased genes that follow the expected pattern of feminization, by looking at
226 processes enriched for female-biased genes that increase in expression and male-biased genes
227 that decrease in expression in asexual females. We would expect that genes showing
228 feminization would be enriched for processes associated with sexual conflict. Both male- and
229 female-biased genes showed an enrichment of many terms (between 0 and 360, and between 1
230 and 195, respectively), including some that could be associated with sexual conflict (e.g. sexual
231 reproduction, female mating behavior, etc). However, the majority of terms have no clear link to
232 sexual conflict, and again no terms were shared between all species (Supplementary Data 6,
233 Supplementary Data 7, Supplementary Fig. 10).
234
235 Taken together, the functional enrichment analyses suggest that the changes in gene expression
236 we observe are involved in a diverse set of processes in each of the species. This is in line with
237 what we observe from the gene expression analyses, which show that sex-biased genes have
238 little overlap between species, and that the largest shifts in gene expression are in genes that are
239 sex-biased in the fewest species. In addition to being different between species, the enriched GO
240 terms were not particularly informative for determining if they are involved in sexual trait decay or
241 sexual antagonism. This reflects the relative difficulty in obtaining functional annotations in
242 *Timema*, due to their evolutionary distance from a well characterised insect model system,
243 meaning that most functions are broad and difficult to attribute to specific roles in sexual traits or
244 sexual antagonism.
245
246 ***Sequence divergence of sex-biased genes in asexuals***
247
248 Sex-biased genes in sexual species often evolve rapidly, because of relaxed evolutionary
249 constraint[3] or due to strong sexual selection and/or sexual antagonism which drives positive
250 selection for amino-acid changes[17]. In asexual species, sex-biased genes are also expected to

evolve rapidly, but due to reduced purifying selection on redundant sexual traits underlain by sex-biased genes. Although interesting, identifying differences in evolutionary rates between gene classes in asexual species is difficult due to the overall elevated rates in asexual species (including in *Timema*[18]), and because genes are inherited as a single linkage group which reduces the power to detect differences in evolutionary rate between genes. Here we found evidence for an elevated rate of dN/dS in asexual species and in sex-biased genes (Supplementary Fig. 11, Supplementary Tables 15-17). We do not see any evidence for an interaction between sex-bias and reproductive mode (Supplementary Fig. 11, Supplementary Tables 15-17), indicating that the increase in dN/dS for sex-biased genes is similar in sexual and asexual species.

Discussion

Conflict over gene expression levels between males and females is thought to drive the evolution of sex-biased gene expression[4]. While sex-biased expression is expected to reduce the amount of intralocus sexual conflict, it is unlikely to be complete for many genes, meaning that some proportion of sex-biased genes are likely subject to sexually antagonistic selection[5,6]. Here we chose to investigate how sex-biased gene expression changes in asexual species which experience no sexual conflict. We predicted that transcriptomes of asexual females would be feminized as sex-biased genes in asexual females would no longer be constrained by countervailing selection pressures in males. Contrary to our prediction we found evidence for an overall masculinization of sex-biased gene expression in asexual females. This pattern of masculinization was very consistent across each of the five independently derived asexual species, and three tissue types we examined. In addition, masculinization was not driven by changes in expression of the same genes in each species, showing that it is the property of being sex-biased *per se* that is most likely to be responsible for the shifts in expression we observe.

Taken together, our results provide strong evidence for a masculinization of gene expression in asexual species. The strength of this finding does not mean there is no sexual conflict over optimal levels in sexual species, but rather that changes in asexual females driven by a release of conflict are negligible relative to changes driven by other mechanisms. The presence of such alternative mechanisms can best be illustrated by the fact that female-limited genes (that should experience no sexual conflict over gene expression level in sexual species), show a consistent masculinization similar to sex-biased genes. We suggest that this is because although reproducing asexually does remove the pressure of sexual conflict, it also removes the need for many of the sexual traits sexually dimorphic gene expression underlies. Consequently, while we expected gene expression in asexual females to be free to move to a female optimum, it is also likely that the optimal female phenotype is different for sexual and asexual females.

Female asexual *Timema* show reductions in several sexual traits including a reduced sperm storage organ, and reduced volatile and contact pheromone production[16]. Since sexually dimorphic traits are largely a product of sex-biased gene expression[3], a link between reduced female sexual traits and reduced female-biased gene expression is a plausible explanation for the decreased expression of female-biased genes we observe. It is less clear why we also see an accompanying increase of expression in male-biased genes in asexual females. We suggest

294 four, non-mutually exclusive, speculative explanations for this. Firstly, increased expression of
295 male-biased genes may arise as a result of sexual trait reduction in cases where high expression
296 of a gene in males acts to suppress the development of a trait, or when low expression in females
297 acts to enhance a female sexual trait. In such genes selection for sexual trait reduction in asexual
298 females would be expected to produce an increase in expression. A second potential explanation
299 is that in sexual species there are a number of products produced by males and then transferred
300 to females that are important for female fertility. For instance, in many insects, ovulation and
301 oviposition are stimulated by substances in the male ejaculate such as juvenile hormone,
302 prostaglandins, and myotropins[19,20]. Since these products are not provided by males in asexual
303 species, females may need to increase expression of the genes that produce these products to
304 compensate. While this explanation could explain some of the increased expression of male-
305 biased genes we observe in the reproductive tract, it is unlikely to be a general explanation for
306 the increased expression of male-biased genes across all species and tissue types. A third
307 potential explanation is that if males and females in sexual species have separate niches, a
308 transition to asexuality would allow asexual females to expand into the male niche. Differential
309 niche use is likely to, at least in part, be mediated by sex-biased gene expression, meaning that
310 asexual females would need to masculinize their gene expression in order to occupy the vacant
311 niche left by males. While differences in male and female niche use have not been extensively
312 studied in *Timema*, sexual dimorphism in mandible shape has been reported, implying that there
313 may be some differential use of niche-space in sexual *Timema* species[21]. Future work examining
314 sexual niche usage and gene expression is needed to evaluate this hypothesis. Finally, another
315 potential explanation for masculinization of gene expression in asexual *Timema* is the decay of
316 dosage compensation. *Timema* have an XX/X0 sex-determination system[15], meaning that in
317 sexual species the X chromosome is present as a single copy in males and as two copies in
318 females. *Timema* are likely to have evolved dosage compensation to equalise expression of X-
319 linked genes between the sexes. In asexual species selection for dosage compensation is absent,
320 which could lead to expression changes of X-linked genes. Changes in X-linked genes alone are
321 however unlikely to explain the masculinized gene expression of asexual females as
322 masculinization is also observed in the asexual species *T. monikensis* when only autosomal
323 transcripts are considered (Supplementary Fig. 12). While we cannot formally distinguish
324 between X-linked and autosomal transcripts in the other asexual species because the relevant
325 genomes are not available, these species would most likely also feature masculinization for
326 autosomal transcripts. Indeed, different sexual *Timema* species are characterized by quite

327 different sets of sex-biased genes (Fig. 5) yet there is no evidence of X-chromosome turnover in
328 *Timema*[15].
329
330 For the reasons detailed above, we believe that female trait reduction is the most likely
331 explanation for the majority of changes in sex-biased gene expression we observe, rather than
332 the cessation of sexual conflict. Similar to our findings, a recent study[9] found that experimentally
333 reduced sexual selection also produced an overall masculinization of gene expression in *D.*
334 *pseudoobscura*. However, Veltsos et al.[9] interpret their findings as a consequence of reduced
335 conflict, and attribute masculinization to the dynamic nature of sexually antagonistic selection
336 causing unpredictable changes in sex-biased gene expression. An alternative explanation,
337 however, is that the masculinization of gene expression in females seen in Veltsos et al.
338 corresponds to a reduction of sexual traits under reduced sexual selection, similar to our findings
339 in *Timema*. Previous studies have reported reduced sexual traits in females evolving under
340 reduced sexual selection in the *D. pseudoobscura* lines studied by Veltsos et al.[11,22]. As such,
341 both Veltsos et al. and our results highlight that the shifts in sex-biased gene expression we
342 observe in the absence, or under reduced levels, of sexual conflict may be in part due to a shift
343 in the optimal trait levels in females. Such shifts in female optima under different sexual selection
344 scenarios are important to consider as an explanation even for studies that observe the expected
345 feminization of sex-biased gene expression[8,10,11]. This is because reducing sexual selection can
346 also favour the increased expression of female sexual traits under some conditions. In these
347 situations, the feminization of sex-biased gene expression can be due to changes in sexual trait
348 optima rather than due to a reduction in the amount of intralocus sexual conflict. More generally,
349 optimal values for traits should be affected by the nature and level of sexual conflict present in a
350 population. Changes to optimal trait values under different selective scenarios are however
351 difficult to predict *a priori*[23], meaning future studies will require careful examination of optimal
352 phenotypes under different selective scenarios in order to correctly interpret any changes in sex-
353 biased gene expression.
354
355 In conclusion, we find that sex-biased gene expression is repeatedly masculinized following a
356 transition to asexuality, and suggest that this result is driven primarily by a reduction of female
357 sexual traits. While we observe similar patterns of masculinization across all five asexual species,
358 the genes involved were mostly different, reflecting the dynamic nature of sex-biased gene
359 expression. In line with this, the functional processes associated with expression change in each
360 species were also diverse. Finally, our study highlights the importance of considering explanations

361 other than intralocus sexual conflict for explaining shifts in sex-biased gene expression, since
362 differences in sexual conflict are also likely to be accompanied by changes in sexual trait optima
363 which may enhance or mask changes caused by a reduction or cessation of intralocus sexual
364 conflict.
365

366 # Methods

367

368 ***Samples***

369

370 Individuals for whole-body and tissue-specific samples were collected from the field as last instar
371 juveniles in spring 2013 and 2014, respectively (collection locations for all samples are given in
372 Supplementary Data 8). All individuals were raised in common garden conditions (23°C, 12h:12h,
373 60% humidity, fed with Ceanothus cuttings) until eight days following their final moult. Prior to
374 RNA extraction, individuals were fed with an artificial medium for two days to avoid RNA
375 contamination with gut content and then frozen at -80°C. For leg samples, three legs were used
376 from each individual (one foreleg, one midleg, and one hindleg). Reproductive tracts were
377 dissected to consist of ovaries, oviducts and spermatheca in females and testes and accessory
378 glands in males. Note the same individuals were used for leg and reproductive tract samples. To
379 ensure individuals were reproductively active at the time of sampling, all sexual individuals were
380 allowed to mate, and asexual and sexual females were observed to lay eggs. When analyses
381 were repeated using virgin sexual females, we obtained qualitatively similar results
382 (Supplementary Fig. 13). Note only whole-body samples were available for this comparison.
383 Ethical approvals or collection permits were not required for this research.

384

385 ***RNA extraction and sequencing***

386

387 We generated three biological replicates per species and tissue type from pooled individuals (1-
388 9 individuals per replicate, a total of 516 individuals, in 150 replicates in total (including the virgin
389 sexual females); see Supplementary Data 8). To extract RNA, samples were flash-frozen in liquid
390 nitrogen followed by addition of Trizol (Life Technologies) before being homogenized using
391 mechanical beads (Sigmund Lindner). Chloroform and ethanol were then added to the samples
392 and the aqueous layer transferred to RNeasy MinElute Columns (Qiagen). RNA extraction was
393 then completed using an RNeasy Mini Kit following the manufacturer's instructions. RNA quantity
394 and quality was measured using NanoDrop (Thermo Scientific) and Bioanalyzer (Agilent). Strand-
395 specific library preparation and single-end sequencing (100 bp, HiSeq2000) were performed at
396 the Lausanne Genomic Technologies Facility.

397

398 The 150 libraries produced a total of just under 5 billion single-end reads. 6 whole-body and 6
399 tissue-specific libraries produced significantly more reads than the average for the other samples.

400 To reduce any influence of this on downstream analyses, these libraries were sampled down to
401 approximately the average number of reads for whole-body or tissue-specific libraries respectively
402 using seqtk (https://github.com/lh3/seqtk Version: 1.2-r94).
403
404 ***Transcriptome references***
405
406 *De novo* reference transcriptome assemblies for each species were generated previously[18]. Our
407 expression analyses were conducted using two sets of orthologs. Firstly, we identified orthologs
408 between sexual and asexual sister species using reciprocal Blast as described in Parker et al.[24].
409 Secondly, we used the 3010 one-to-one orthologs present in all 10 *Timema* species as identified
410 by Bast et al.[18]. The identified ortholog sequences varied in length among different species. Since
411 length variation might influence estimates of gene expression, we aligned orthologous sequences
412 using PRANK (v.100802, default options)[25] and trimmed them using alignment_trimmer.py[26] to
413 remove overhanging gaps at the ends of the alignments. If an alignment contained a gap of
414 greater than 3 bases then sequence preceding or following the alignment gap (whichever was
415 shortest) was discarded. Any orthologous sequences that had a trimmed length of less than 300
416 bp were also discarded. Finally, before mapping, genes with significant Blast hits to rRNA
417 sequences were removed from the trimmed transcriptome references.
418
419 ***Read trimming and mapping***
420
421 Before mapping, adapter sequences were trimmed from raw reads with CutAdapt[27]. Reads were
422 then quality trimmed using Trimmomatic v 0.36[28], clipping leading or trailing bases with a phred
423 score of <10 from the read, before using a sliding window from the 5' end to clip the read if 4
424 consecutive bases had an average phred score of <20. Any reads with a sequence length of <80
425 after trimming were discarded. Reads from each libret were then mapped to the transcriptome
426 references using Kallisto (v. 0.43.1)[29] with the following options -l 210 -s 25 --bias --rf-stranded
427 for whole-body samples and -l 370 -s 25 --bias --rf-stranded for tissue-specific samples (the -l
428 option was different for whole-body and tissue-specific samples as the fragment length for these
429 libraries was different).
430
431 ***Differential expression analysis***
432

433 Expression analyses were performed using the Bioconductor package EdgeR (v. 3.18.1)[30] in R
434 (v. 3.4.1)[31]. Firstly, to identify sex-biased genes we compared male and female expression
435 separately for each tissue type in each sexual species. Genes with counts per million less than
436 0.5 in 2 or more libraries per sex were excluded from expression analyses. Normalization factors
437 for each library were computed using the TMM method. To estimate dispersion we then fit a
438 generalized linear model (GLM) with a negative binomial distribution with sex as an explanatory
439 variable and used a GLM likelihood ratio test to determine the significance of sex on gene
440 expression for each gene. P-values were then corrected for multiple tests using Benjamini and
441 Hochberg's algorithm[32]. Sex-biased genes were then defined as genes that showed a greater
442 than 2 fold difference in expression between males and females with an FDR < 0.05. Note all
443 genes not classified as sex-biased were classified as unbiased genes. We chose this threshold
444 in order to select a robust set of sex-biased genes, and to reduce the effect of sex-biased
445 allometry[33]. Note that analyses using just an FDR threshold to define sex-biased genes, or using
446 virgin sexual females to independently verify sex-biased genes in whole-body samples, produced
447 qualitatively similar results (Supplementary Tables 18-19, Supplementary Fig. 14).
448
449 Clustering of expression values ($\log_2$ CPM) was performed using Ward's hierarchical clustering
450 of Euclidean distances with the R package pvclust (v. 2.0.0)[34], with bootstrap resampling
451 (method.hclust="ward.D2", method.dist="euclidean", nboot=10000), and visualized using R
452 package pheatmap (v. 1.0.8)[35].
453
454 To quantify how sex-biased genes change in expression in asexual females we then compared
455 gene expression in sexual and asexual females separately for each species pair and each tissue
456 type. We also compared the change in expression in asexual females for male- and female-
457 biased genes to unbiased genes using a Wilcoxon test, corrected for multiple tests using
458 Benjamini and Hochberg's algorithm[32]. To determine if changes in sex-biased gene expression in
459 asexual females are larger for genes sex-biased in fewer species we fit a generalized linear mixed
460 model with the number of species a gene is sex-biased in as a fixed effect and gene ID as a
461 random effect in R. The significance of terms was determined using a Likelihood Ratio Test. A
462 separate model was fit for male- and female- biased genes in each tissue. P-values were
463 corrected for multiple tests using Benjamini and Hochberg's algorithm. We also examined gene
464 expression changes in the *T. cristinae* – *T. monikensis* species pair when X-linked transcripts
465 were excluded. X-linked transcripts were determined in these species by blasting (blastN)
466 transcripts to the *T. cristinae* reference genome, for which linkage groups have been assigned[41].

467 The gene expression analyses were then repeated on only those transcripts that had a significant
468 blast hit (e-value < 1 x 10^{-20}, query coverage > 60%) to a scaffold in an autosomal linkage group.
469
470 **Shifts in sex-biased genes and asexual lineage age**
471
472 The asexual species differ in age as estimated previously[2]. Since the age of asexuality varies we
473 tested if changes in sex-biased gene expression altered with asexual species age using a
474 permutation ANCOVA (number of permutations = 10,000) separately for male- and female-
475 biased genes with the following terms: asexual lineage age, tissue type and their interaction.
476
477 ***Analysis of sex-limited genes***
478
479 Sex-limited genes were classified as genes that had at least 2 FKPM (Fragments Per Kilobase
480 Million) in each replicate of one sex and 0 FKPM in each replicate of the other sex. FKPM values
481 were calculated using EdgeR. The expression levels of female-limited genes in sexual and
482 asexual females, and male-limited genes in sexual males and asexual females were compared
483 using a Wilcoxon test, corrected for multiple tests using Benjamini and Hochberg's algorithm[32].
484
485 ***Sequence evolution of sex-biased genes***
486
487 To test if sex-biased genes have a higher rate of divergence in asexuals, we examined if sex-
488 biased genes have elevated dN/dS ratios in asexuals. To do this we firstly fit a binomial glmm
489 (dN/dS values were transformed to fall into two categories: zero or non-zero), with reproductive
490 mode, sex-bias and their interaction as fixed effects and gene identity as a random effect.
491 Secondly, we firstly fit a glmm with a gamma distribution to the dN/dS values that were greater
492 than zero, with the same fixed and random effects as the binomial model. All glmms were fit using
493 the lme4 package (v. 1.1.14)[36] in R, and significance of terms was determined using a log-
494 likelihood ratio test. dN/dS values were calculated for each of the one-to-one orthologs using
495 codeml implemented in the PAML package[37] to generate maximum likelihood estimates of dN/dS
496 for each terminal branch in the phylogeny (using the "free model") as described in Bast et al.[18]
497
498 ***GO term analysis***
499

500 Genes were functionally annotated using Blast2GO (version 4.1.9)[38] as described in Parker et
501 al.[24] Briefly, sequences from each sexual species were compared with BlastX to either NCBI's nr-
502 arthropod or *Drosophila melanogaster* (drosoph) databases, to produce two sets of functional
503 annotations, one derived from all arthropods and one specifically from *Drosophila melanogaster*.
504 The *D. melanogaster* GO term annotation generated around four times more annotations per
505 sequence than NCBI's nr-arthropod database. We therefore conducted all subsequent analyses
506 using the GO terms derived from *D. melanogaster*, but note that results using the annotations
507 from all arthropods were qualitatively the same (see Supplementary Fig. 15).
508
509 To identify overrepresented GO terms we conducted gene set enrichment analyses (GSEA) using
510 the R package TopGO (v. 2.28.0)[39], using the elim algorithm to account for the GO topology. GO
511 terms were considered to be significantly enriched when $p < 0.05$. Repeating our GO term
512 analyses using the more liberal 'weight01' algorithm produced qualitatively the same results
513 (results not shown).
514
515 Since we defined sex-biased genes with both FDR and FC thresholds, we ranked sex-biased
516 genes for the GSEA to take both FDR and FC into account. To identify overrepresented GO terms
517 for female-biased genes, genes were ranked by FDR in four subsets: female-biased with FC > 2,
518 female-biased with FC < 2, male-biased with FC < 2, and male-biased with FC > 2. Female-biased
519 gene subsets were ranked so that small FDR values were ranked highly, male-biased gene
520 subsets were ranked so that small FDR values were ranked low in the list. The four lists were
521 then joined together in the order given above, and assigned a unique rank. This ranked list
522 produces a list where strongly female-biased genes are at the top, followed by weakly female-
523 biased genes, then weakly male-biased genes, and finally strongly male-biased genes at the
524 bottom. To identify overrepresented GO terms for male-biased genes the ranked list for female-
525 biased genes was simply inverted. Finally, to examine the GO terms overrepresented in sex-
526 biased genes which changed expression in asexuals, female- and male-biased genes were
527 ranked by fold-change between sexual and asexual females.
528
529 To determine if the overlap of sets of sex-biased genes or GO terms was greater than expected
530 by chance we used the SuperExactTest package (v. 0.99.4; [40]) in R which calculates the
531 probability of multi-set intersections. P-values were multiple test corrected using Benjamini and
532 Hochberg's algorithm implemented in R.
533

Data Availability

Raw reads have been deposited in SRA under accession codes SRR5748941-SRR5749000, for whole-body samples and SRR5786827-SRR5786961 for tissue-specific samples.

Code Availability

Scripts for the analyses in this paper are available at https://github.com/DarrenJParker/Timema_Sex_Biased_Gene_Exp, and will be archived at Zenodo after acceptance.

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Author Contributions

T.S. and D.J.P. designed the study. Z.D., K.J., J.B., and T.S. collected samples, performed dissections and molecular work. D.J.P. analyzed the data with input from J.B., T.S., and M.R.R.. D.J.P. and T.S. wrote the manuscript with input from all authors.

Competing Interests

The authors declare no competing interests.

Figures

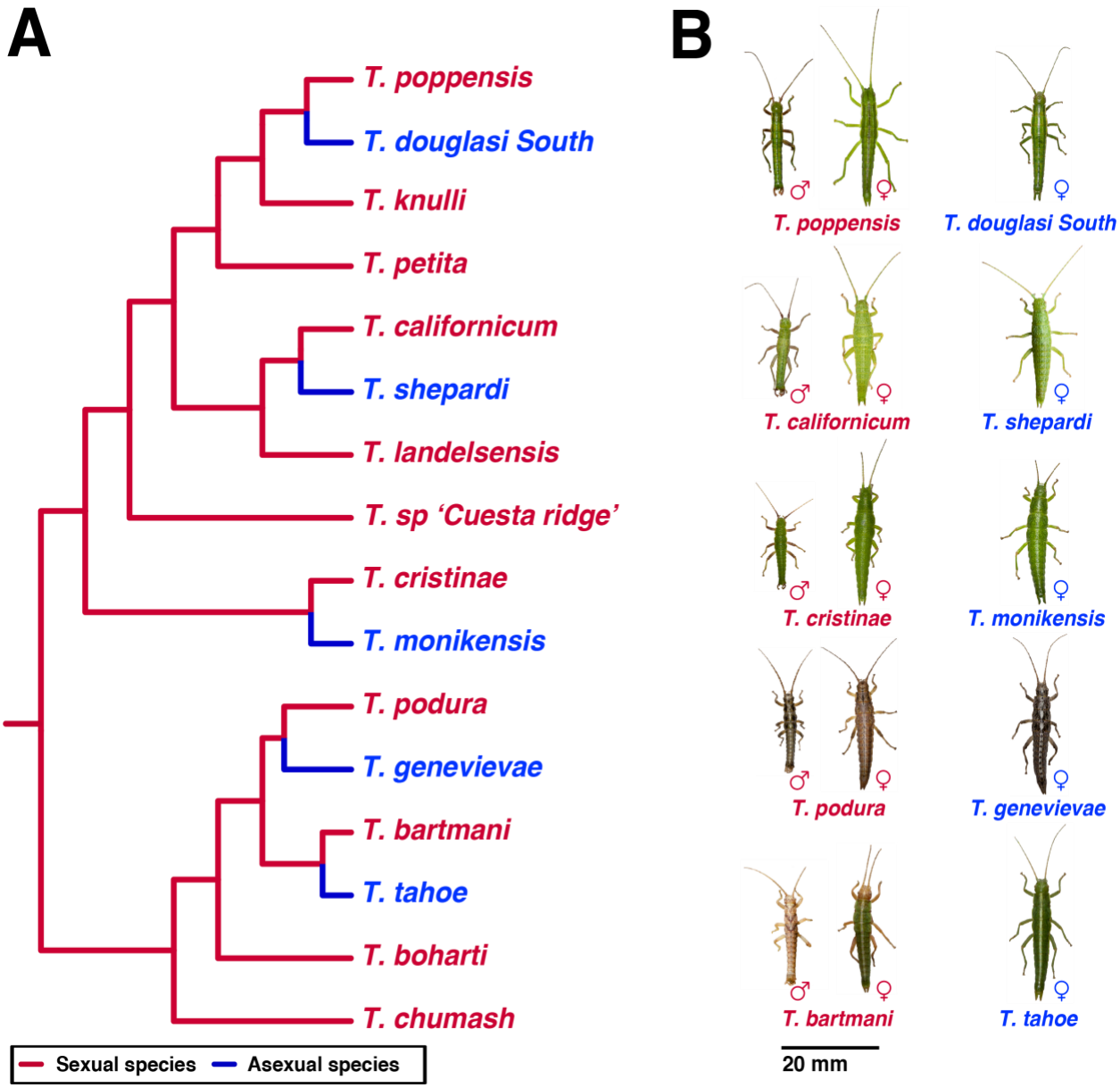

Figure 1 | *Timema* phylogeny and photographs. (A) Phylogeny of described *Timema* species (redrawn from Riesch et al.[41]) with asexual species added from Schwander et al.[14]). Sexually reproducing species are shown in red, independently derived asexual lineages in blue. Branches between sexual-asexual sister species indicate relative divergence time based on Jukes–Cantor corrected divergence from Bast et al.[18]. Note the oldest asexual lineage, *T. genevievae,* was previously estimated to be 1.5 My old.[14] (B) Photographs of the species used in this study scaled using median body lengths from their species descriptions.[42–46] Photographs

of *Timema* were kindly provided by Bart Zijlstra.

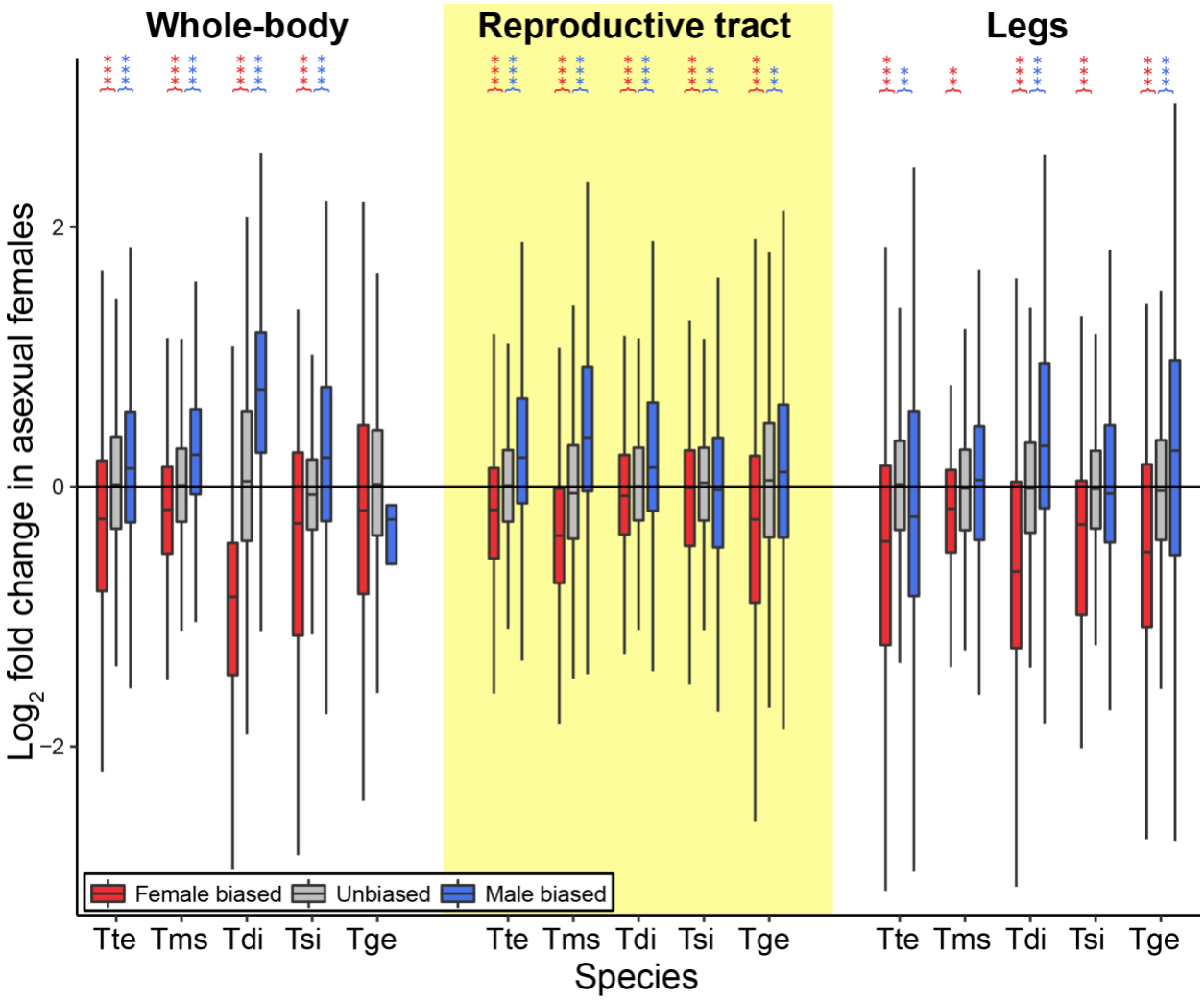

Figure 2 | Expression shifts in sex-biased genes in asexual females. Positive values on the y-axis indicate increased expression in asexual females. Asterisks indicate the significance level (FDR) of Wilcoxon tests comparing the change in expression in female-biased (red) and male-biased (blue) genes to unbiased genes (*** < 0.001, ** < 0.01, * < 0.05). Species names are abbreviated as follows: Tte = *T. tahoe*, Tms = *T. monikensis*, Tdi = *T. douglasi*, Tsi = *T. shepardi*, and Tge = *T. genevievae*. Boxes represent the interquartile range (25th and 75th percentiles) of the data with the line inside the box representing the median. Whiskers show the most extreme value in the data which is no more than 1.5 times the interquartile range from the box.

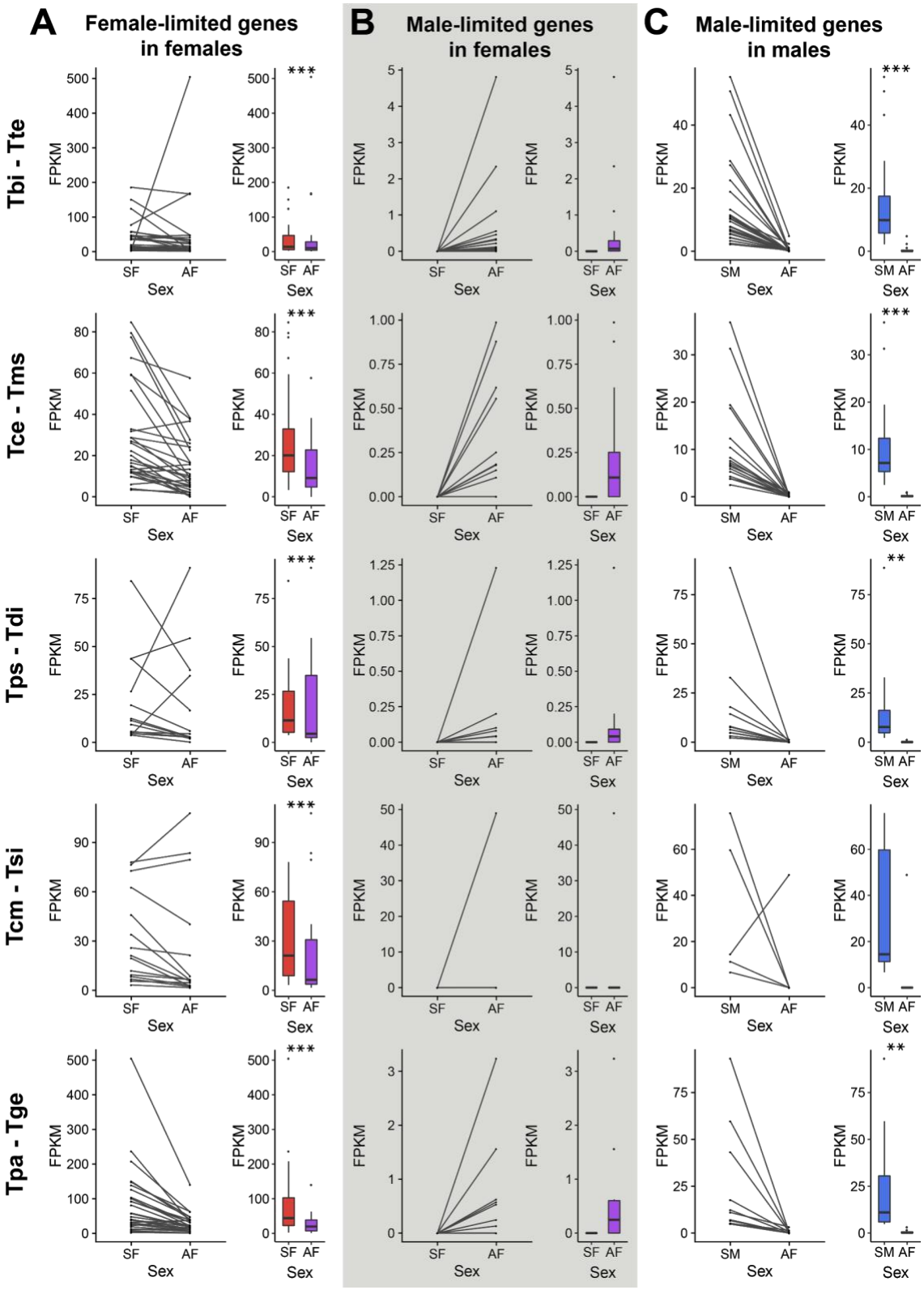

Figure 3 | Expression of sex-limited genes in the reproductive tract. A) Expression of female-limited genes in sexual females (SF, red) and asexual females (AF, purple), B)

Expression of male-limited genes in sexual females (SF, red) and asexual females (AF, purple), C) Expression of male-limited genes in sexual males (SM, blue) and asexual females (AF, purple). Asterisks indicate the significance level (FDR) of Wilcoxon tests (*** < 0.001, ** < 0.01, * < 0.05). Species names are given as abbreviations in the form sexual-species - asexual species at the left-hand side (Tbi = *T. bartmani*, Tce = *T. cristinae*, Tps = *T. poppensis*, Tcm = *T. californicum*, Tpa = *T. podura*, Tte = *T. tahoe*, Tms = *T. monikensis*, Tdi = *T. douglasi*, Tsi = *T. shepardi*, and Tge = *T. genevievae*). For the boxplots, boxes represent the interquartile range (25th and 75th percentiles) of the data with the line inside the box representing the median. Whiskers show the most extreme value in the data which is no more than 1.5 times the interquartile range from the box. Note this figure depicts only the results from the reproductive tract. For whole-bodies see Supplementary Figure 4. Legs were not plotted due to the small number of sex-limited genes in this tissue type (Supplementary Tables 4-5).

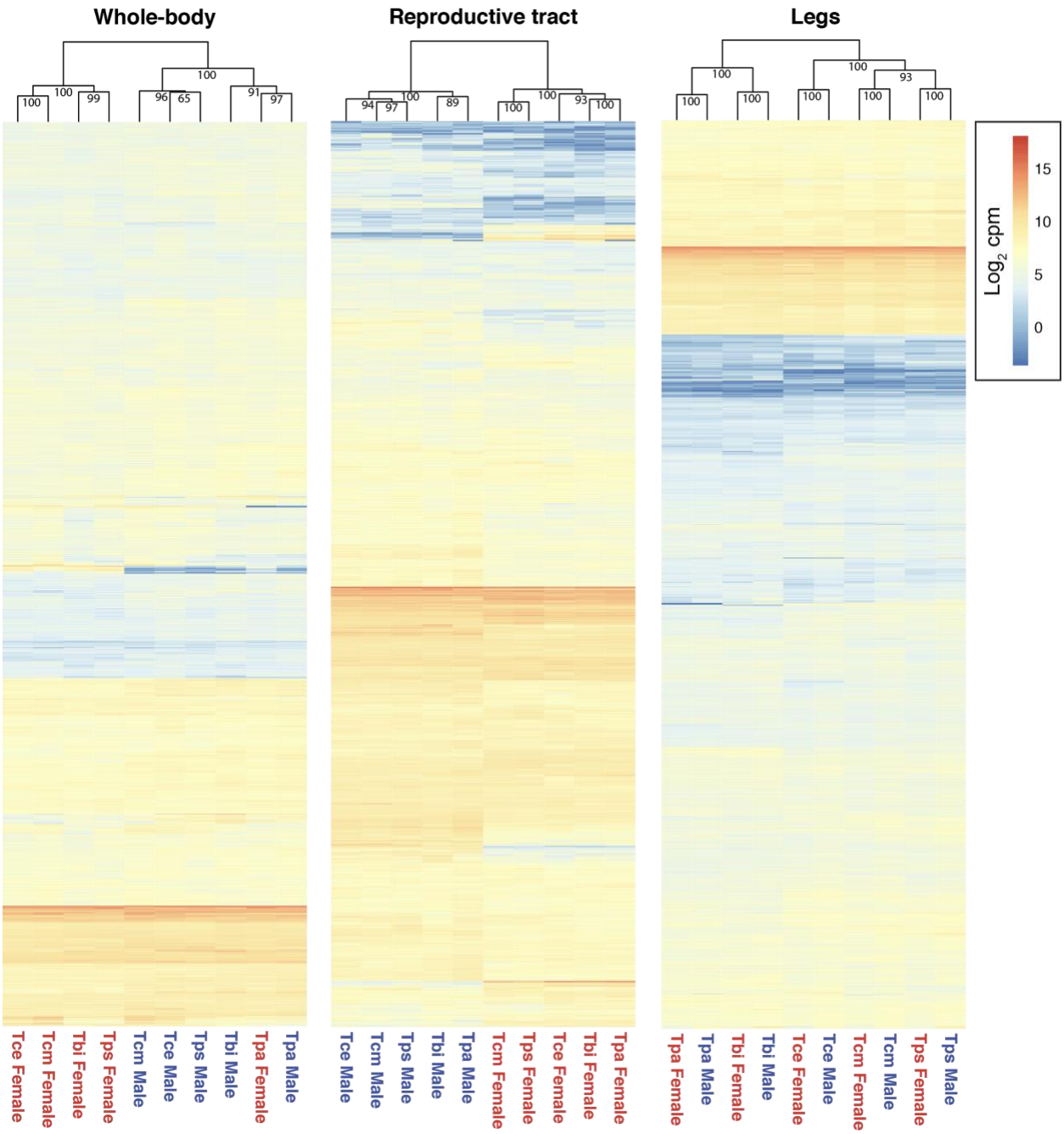

Figure 4 | Heatmaps and hierarchical clustering of gene expression. Gene expression (log_2 CPM) for whole-body, reproductive tract, and leg samples are plotted separately. Values on each node show the bootstrap support from 10,000 replicates. Species names are abbreviated as follows: Tbi = *T. bartmani*, Tce = *T. cristinae*, Tps = *T. poppensis*, Tcm = *T. californicum*, Tpa = *T. podura.*

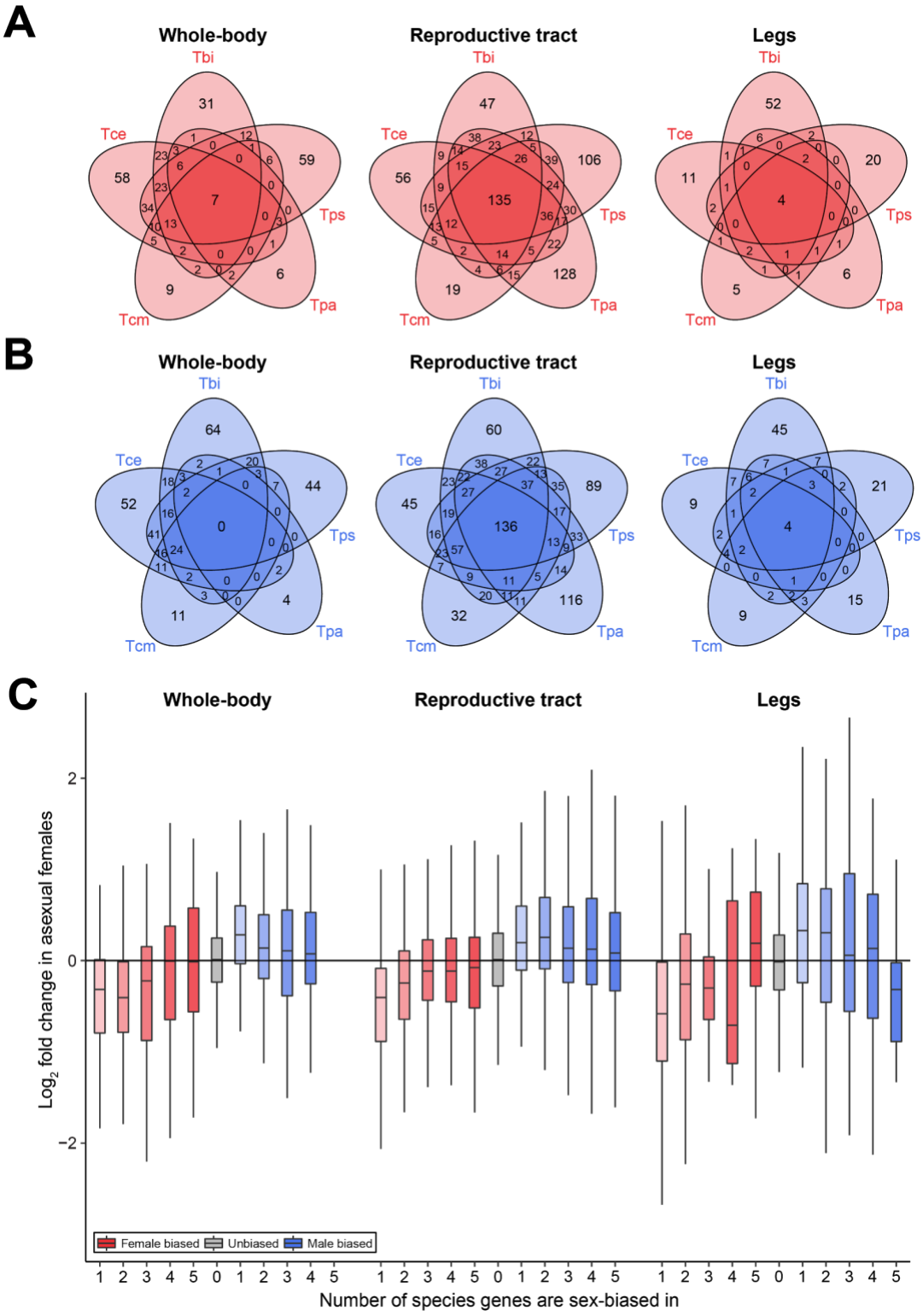

Figure 5 | Sex-biased genes across *Timema* species A) Venn-diagrams showing the overlap of female-biased genes B) Venn-diagrams showing the overlap of male-biased genes C) Boxplots showing the change in expression of female-biased (reds) and male-biased (blues) genes in

asexual females when a gene is female or male-biased in 1, 2, 3, 4 or 5 sexual species. Note for genes sex-biased in multiple species the plot includes fold-change values of that gene in each species it is sex-biased in. Boxes represent the interquartile range (25th and 75th percentiles) of the data with the line inside the box representing the median. Whiskers show the most extreme value in the data which is no more than 1.5 times the interquartile range from the box.

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