

Absence of a faster-X effect in beetles (*Tribolium*, Coleoptera)

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1 **Abstract**

2 **Background.** The faster-X effect, namely the rapid evolution of protein-coding genes on the X-chromosome,
3 has been reported in numerous metazoans. However, the prevalence of this phenomenon across metazoans and
4 its potential causes remain largely unresolved. Analysis of sex-biased genes may elucidate its possible
5 mechanisms: a more pronounced faster-X effect in male-biased genes than in female-biased or unbiased genes,
6 suggests fixation of recessive beneficial mutations rather than genetic drift. Further, theory predicts that the
7 faster-X effect should be promoted by X-chromosome dosage compensation, but this topic remains rarely
8 empirically examined.

9
0 **Results.** Here, we asked whether we could detect a faster-X effect in genes of the beetle *Tribolium castaneum*
1 (and *T. freemani* orthologs), which has X/Y sex-determination and heterogametic males. Our comparison of
2 protein sequence divergence (dN/dS) on the X-chromosome versus autosomes indicated the complete absence
3 of a faster-X effect. Further, analyses of sex-biased gene expression revealed that the X-chromosome was
4 strongly enriched for ovary-biased genes, which evolved under exceptionally high constraint. An evaluation of
5 male X-chromosome dosage compensation in the gonads and in non-gonadal somatic tissues showed an
6 extreme lack of compensation in the testis. This under-expression of the X chromosome in males may limit the
7 phenotypic effect, and therefore likelihood of fixation, of recessive beneficial X-linked mutations in genes
8 transcribed in male gonads.

9
0 **Conclusions.** We show that these beetles display a rare unequivocal example of the absence of a faster-X effect
1 in a metazoan. We propose two potential causes for this, namely high constraint on X-linked ovary-biased
2 genes, and an extreme lack of dosage compensation of genes transcribed in the testis.

3
4 **Keywords:** *Tribolium castaneum*, faster-X, sex-biased expression, dosage compensation, dN/dS
5

26 **Background**

27 The “faster-X” effect, that is, the rapid evolution of protein-coding genes on the X
28 chromosome, has been widely reported in a range of metazoan systems with sex chromosomes
29 [1, 2]. Higher rates of protein divergence of genes on the hemizygous X-chromosome (faster-X,
30 or faster-Z in W/Z systems) than on autosomes has been observed in organisms including
31 primates [3, 4], humans [4], rodents [5], birds [6, 7], moths [8], aphids [9], and very recently in
32 spiders [10]. In other organisms, however, a faster-X effect is more ambiguous. For example,
33 signals of this effect have sometimes, but not always, been observed in studies of fruit flies [2,
34 11, 12], and variable results on the presence or strength of the faster-X effect have been reported
35 in butterflies [13, 14].

36 With regards to the mechanisms that might account for the faster-X effect, it has been
37 proposed that X-linked genes might evolve faster in protein sequence than those on autosomes
38 due to efficient fixation of recessive beneficial mutations in the hemizygous state, a notion that
39 has found empirical support in some animal taxa [1, 2, 4, 5, 11, 15]. An alternative mechanism is
40 that the effect results largely from fixation of recessive, mildly deleterious mutations via genetic
41 drift. Studies of birds and aphids support this mechanism, which has been suggested to be
42 facilitated by the lowered effective population size of the X chromosome [1, 7, 9, 16].

43 The study of sex-biased gene expression, that is, those genes preferentially upregulated in
44 one sex, has helped to decipher the forces shaping the molecular evolutionary rates on the X-
45 chromosome versus autosomes [17-20], and thus to better understand the faster-X effect [7, 8,
46 12-16]. For instance, under a model wherein the faster-X effect is caused by rapid fixation of
47 beneficial mutations in the hemizygous state, in organisms where males are the heterogametic
48 sex, this effect is predicted to be strongest in male-biased genes, and relatively lower in female-
49 biased and unbiased genes [16]. This prediction is based on the hypothesis that X-linked
50 recessive beneficial mutations should largely exert their fitness effects in males, as their
51 hemizygous state would preclude the possibility of non-mutant alleles masking the phenotypic
52 effect of such recessive mutations [7, 15, 16]. Empirical support from this model comes from a
53 study of *Drosophila*, in which assessment of protein divergence (dN/dS) of genes on the X-
54 chromosome and autosomes, revealed a faster-X effect for all three classes of sex biased genes
55 (male-biased, female-based and unbiased). In this study, protein sequence divergence was
56 highest in male-biased genes and lowest in female-biased genes, empirically supporting a model

57 of fixation of beneficial mutations on the X chromosome [15, 16]. In chickens, which have WZ
58 sex chromosomes and female heterogamy, elevated dN/dS has been reported across all studied
59 genes on the Z-chromosome, consistent with the faster-X (or faster-Z in this case) effect [21].
60 However, the prediction of higher dN/dS for female-biased genes on the Z-chromosome was not
61 met in this study, suggesting that the faster-Z in these birds was not due to fixation of recessive
62 beneficial mutations, and rather might be attributable to fixation of neutral or slightly deleterious
63 mutations via genetic drift [7, 16]. Recently, similar results were reported for the W/Z
64 chromosomes of *Heliconius* butterflies [14]. At present however, the study of the faster-X effect,
65 including the role of sex-biased gene expression, remains limited to just a few model organisms,
66 and the putative underlying mechanisms appear to be variable.

67 The faster-X effect may be expected to be most strongly observed in organisms with
68 complete dosage compensation, wherein expression levels of X-linked genes are upregulated in
69 the heterogametic sex, such that the X to autosome ratio (X:A) is one or close to one [1, 2, 22,
70 23]. Under this hypothesis, in organisms with incomplete X-chromosome dosage compensation,
71 such that $X:A < 1$, X-linked recessive beneficial mutations would have relatively low expression
72 levels, and thus putatively weak phenotypic effects (or selection coefficients[1]), in the
73 hemizygous sex. This could make beneficial mutations exposed on the single male X-
74 chromosome unlikely to be fixed any more frequently than they would be if they were
75 autosomal, possibly resulting in evolutionary rates of X-linked genes that are not significantly
76 faster than those of autosomal genes [1, 7]. Support for the notion that dosage compensation may
77 impact the rates of X- (or Z-) linked gene evolution is provided by the observation that certain
78 butterflies display incomplete dosage compensation [24]; this correlates with, and thus may
79 contribute towards, the observed lack of an elevated faster-Z effect in female-biased genes in
80 those organisms [14, 24]. Similar patterns have been described in birds [7]. At present however,
81 the relationship between faster-X effect and dosage compensation remains rarely empirically
82 evaluated.

83 An important factor to consider in the study of dosage compensation, is that this
84 phenomenon may vary among tissues within an organism. For instance, studies in *Drosophila*
85 have shown that complete dosage compensation of X-linked genes is observed in male somatic
86 tissues, but not in the testis [25-27]. In the context of these findings in *Drosophila*, it appears
87 possible that the degree of gonadal and somatic dosage compensation could in theory [1]

88 influence its observed faster-X effect [2, 11, 12]. Thus, given that dosage compensation may
89 vary among tissues, and particularly in the gonads, specific study is warranted of the faster-X
90 effect and its association to gonadal and non-gonadal dosage compensation in animal models.

91 A model insect genus that offers new opportunities to study the faster-X effect is the
92 beetle system *Tribolium* (Coleoptera). Coleoptera is the largest insect order, with recent
93 estimates of over 1.5 million species, thus comprising approximately 40% of all arthropod
94 species [28]. The rust red flour beetle *T. castaneum* is a well-established model system for
95 genetics and for the evolution of developmental mechanisms [29-33], and has extensive genomic
96 resources available for research [34-36]. In addition, its less well-studied sister species *T.*
97 *freemani*, from which it diverged approximately 12-47 Mya, comprises a suitable system for
98 comparative genomic study [37]. To date, however, to our knowledge the primary genome-wide
99 sex-biased expression research in *Tribolium* that includes X-chromosome analyses consists of a
100 foundational study based on whole male versus whole female contrasts and microarray data in *T.*
101 *castaneum* [38]. That assessment made several significant findings, including that the female-
102 biased genes were highly overrepresented on the X-chromosome [38], which was proposed to be
103 explained by a mechanism of overexpression of X-linked genes in females as an imperfect
104 response to male dosage compensation [38]. In addition, the study's authors reported that X-
105 linked genes with male-biased expression were comparatively uncommon, a trend also observed
106 in other organisms such as *Drosophila* [38]. In addition to that assessment, other transcriptome
107 studies in *Tribolium* include a recent study using RNA-seq in *T. castaneum* that examined
108 differential expression among somatic, germ line, and embryonic tissues [39]. The study reported
109 identification of potentially useful transcripts and genes for generating genetic constructs for the
110 investigation of development and pest control in this species [39]. A separate investigation of
111 codon and amino acid usage was also conducted across the *T. castaneum* genome with respect to
112 gene expression [35]. None of these studies, however, assessed evidence for or against the faster-
113 X effect in *Tribolium*. Moreover, to our knowledge, there have been no between-species analyses
114 of protein sequence divergence (dN/dS), and its potential relationship to sex-biased gene
115 expression and dosage compensation. Thus, we considered that original analyses addressing
116 these important topics in this beetle model could provide valuable insights into the breadth of the
117 faster-X phenomenon across animals, and help decipher its underlying mechanisms.

118 Here, we describe a rigorous assessment of the faster-X effect in *T. castaneum*, including
119 evaluation of its relationship to sex-biased gene expression and dosage compensation, using
120 newly generated RNA-seq data from gonads and gonadectomized (GT-) males and females. Our
121 assessment of dN/dS in 7,751 *T. castaneum* genes with high confidence orthologs in its sister
122 taxon *T. freemani* reveals the complete absence of a faster-X effect in this taxon. Instead, we find
123 a slower rate of evolution of X-linked as compared to autosomal genes. Further, we show that the
124 faster-X effect is not found at any level for male-biased, female-biased or unbiased genes
125 identified from the gonads and from non-gonadal somatic tissues. We demonstrate that the slow-
126 X effect in this taxon is largely due to the slow sequence evolution of ovary-biased genes located
127 on the X-chromosome, which are more common, and are more highly constrained, than those on
128 autosomes. Moreover, with respect to dosage compensation, we report that GT-males exhibit
129 high X-chromosome dosage compensation with an X/A ratio near one. However, an extreme
130 absence of dosage compensation is evident for hemizygous X-linked genes expressed in the
131 testis. We suggest that this may give rise to weak phenotypic effects of such genes [1],
132 potentially limiting fixation of recessive beneficial mutations when transcribed in male gonads,
133 thereby impeding a faster-X effect. Our results thus provide additional empirical support [7, 14]
134 for a notion that has previously been proposed theoretically [1, 2, 7, 22]. Taken together, we
135 propose that the unusual absence of a faster-X effect in these beetles may be influenced by two
136 major phenomena: (1) the accumulation of highly constrained ovary-biased genes on the X-
137 chromosome, and (2) the lack of dosage compensation in the male gonads, which may act to
138 minimize fixation of recessive beneficial mutations of genes transcribed in these tissues.

139

140 **Results**

141 The complete list of previously annotated [36] protein-coding genes in our main target
142 taxon *T. castaneum* were downloaded for study (N=16,434 genes, Ensembl Metazoa
143 (<http://metazoa.ensembl.org>). Using the full CDS list (longest CDS per gene) we identified 7,751
144 high confidence orthologs in its sister species *T. freemani* for our study of protein sequence
145 evolution (dN/dS, see Methods; note that expression results for all 16,434 *T. castaneum* genes
146 are described throughout when appropriate). The use of closely related sister species is a
147 common approach to study the protein sequence divergence of sex-biased genes in metazoan
148 models (*cf.* [6, 9, 15, 40-42]). Values of dN/dS <1, =1, and >1 suggest that purifying, neutral

149 and positive selection respectively are likely to predominantly shape the evolution of protein
150 coding genes [43]. However, even when $dN/dS < 1$ (as is typical in gene-wide analysis),
151 relatively elevated values suggest reduced constraint, which could be due to relaxed selection
152 and/or adaptive evolution. Under the faster-X effect, dN/dS is predicted to be elevated for
153 protein-coding genes on the X-chromosome as compared to those on autosomes [7, 15, 16, 21].

154 We first assessed whether this beetle system exhibited a faster-X effect. Box plots of
155 dN/dS of genes located on the X-chromosome versus autosomes are shown in Fig. 1, showing no
156 tendency for higher dN/dS in genes on the X chromosome. In fact, we observed the opposite:
157 dN/dS was statistically significantly lower for X-linked genes than for autosomal genes in this
158 taxon (MWU-test $P=0.002$). From a total of 432 studied X-linked genes and 7,319 autosomal
159 genes distributed across nine autosomes, the median dN/dS values were 0.686 and 0.906
160 respectively (Fig. 1A), yielding a ratio of dN/dS values for the X-chromosome to autosomes
161 across all genes ($X/A_{dN/dS}$ (all genes)) of 0.76 (Fig. 1B). Thus, the $X/A_{dN/dS}$ (all genes) value is
162 considerably below 1, a result opposite to the >1 value expected under a faster-X effect [4, 14-
163 16, 21]. Further, the mean dN/dS on the X-chromosome was about half (ratio of 0.54) that
164 observed on autosomes (Fig. 1B). Thus, these results indicate the absence of a faster-X effect in
165 this taxon, differing from that observed in most other metazoan systems studied to date.
166 Together, these data show a slower-X pattern in *Tribolium*.

167

168 **Assessment of sex-biased genes on the X-chromosome versus autosomes**

169 Sex-biased gene expression has primarily been used to help discern the potential causes
170 of the faster-X (or -Z) after it has been detected in an organism [7, 12-16]. Having observed no
171 evidence of a faster-X effect for this beetle taxon, we asked if sex-differences in gene expression
172 could help suggest mechanisms that might explain the absence of this effect (Fig. 1). We also
173 wished to determine whether a faster-X effect was present for specific categories of sex-biased
174 genes, including male-biased, female-biased and unbiased genes. For this assessment, we
175 generated new large-scale RNA-seq datasets for adult male testes and ovaries, and for the
176 gonadectomized bodies of adult males and females (hereafter referred to as GT-males and GT-
177 females respectively, or non-gonadal somatic tissues) (Table S1). We mapped reads to annotated
178 *T. castaneum* genes (See Methods), and identified sex-biased genes for the gonads (testis versus
179 ovary) and for the GT-soma (GT-males versus GT-females) as those with a two-fold and

180 statistically significant difference ($P < 0.05$) in expression using Deseq2 [44]. We found that
181 25.8% of all genes in the genome ($N = 16,434$) had gonad-biased expression ($N = 4,242$), and 9.6%
182 of genes ($N = 1,573$) had biased expression in the GT-soma (shown in Fig. S1). The N values of
183 sex-biased genes for those genes with orthologs ($N = 7,751$) are shown in Fig. S2 ($N = 2,341$
184 (30.2%) and 836 (10.7%) for gonads and GT-soma respectively). We then assessed the sex-
185 biased expression status of X-linked and autosomal genes with respect to dN/dS.

186 The proportion of genes on the X-chromosome and on each of the nine autosomes that
187 had sex-biased or unbiased expression is shown in Fig. 2A, which includes all genes for which
188 we had calculated dN/dS values ($N = 7,751$) (see Fig. S3 for all annotated *T. castaneum* genes,
189 which yielded similar patterns according to sex-biased expression status). We found that a
190 disproportionately large fraction of genes on the X chromosome were ovary-biased: 53.9% of the
191 X-linked genes under study were ovary-biased ($N = 233$ of the 432 X-linked genes for which we
192 assessed dN/dS) (Fig. 2A), while only 16.3% of autosomal genes showed ovary-biased
193 expression ($N = 1,192$ of 7,319 genes pooled across autosomes, χ^2 with Yate's correction
194 $P < 0.0001$). In contrast, relatively few testis-biased, GT-male biased or GT-female biased genes
195 were located on the X chromosome (each of these gene expression categories constituted $\leq 5.5\%$
196 of the X-linked genes under study in Fig. 2AB). These chromosomal distributions of the different
197 sex-bias expression categories for this set of 7,751 genes with high confidence orthologs between
198 *T. castaneum* and *T. freemani* (Fig. 2AB) largely parallels that observed for all *T. castaneum*
199 genes in the genome (Fig. S3AB) and agrees with the aforementioned prior report for all *T.*
200 *castaneum* genes [38]. That study compared whole males versus whole females, and showed that
201 the X-chromosome contained a high abundance of female-biased genes and very few male-
202 biased genes. Our results extends these results to explicitly show that ovary-biased genes (Fig.
203 2A), rather than genes with female-biased expression in somatic tissues (Fig. 2B), are highly
204 concentrated on the X-chromosome, and that X-linked testis-biased genes, GT-male-biased, and
205 GT-female-biased genes are each relatively rarely observed on the X-chromosome.

206

207 ***The absence of a faster-X effect is largely caused by slow-evolving X-linked ovary-biased*** 208 ***genes***

209 Having identified that ovary-biased genes were highly overrepresented on the X
210 chromosome relative to autosomes (Fig. 2A), we asked if this might contribute to the observed

211 slower-X effect. We compared dN/dS values for these ovary-biased genes on X-chromosomes to
212 those values for autosomal ovary-biased genes (Fig. 2CE; N values in Table S2, Fig. S2). We
213 found that the dN/dS values of X-linked ovary-biased genes were statistically significantly lower
214 than dN/dS values for autosomal ovary-biased genes (MWU-test $P < 0.001$, Fig. 2C). Thus, the
215 faster-X effect is absent in ovary-biased genes. Further, the ratio of the median dN/dS values
216 when calculated using only the subset of X-linked ovary-biased genes versus those on
217 autosomes, $X/A_{dN/dS}(\text{ovary-biased})$, was 0.74 (Fig. 2E), also suggesting higher selective constraint on
218 ovary-biased genes on the X-chromosome than autosomes. Moreover, ovary-biased genes on the
219 X-chromosome had lower dN/dS than unbiased genes on the X-chromosome and on autosomes
220 (MWU-tests $P < 0.001$) and markedly lower dN/dS values than testis-biased genes on the
221 autosomes (two-fold lower, 0.060 versus 0.120 median dN/dS values, MWU-test $P < 0.001$; note
222 there were too few X-linked testis-biased genes for reliable testing). Together, given the high
223 frequency of genes located on the X-chromosome that were ovary-biased (Fig. 2A), these
224 findings indicate that constrained evolution of ovary-biased genes contributes to the global
225 absence of a faster-X effect in this organism (Fig. 1).

226 For the genes with GT-soma-biased expression, there were only 24 genes with GT-
227 female biased expression on the X-chromosome (as compared to 233 with ovary-biased
228 expression on the X-chromosome). Nonetheless, as we had observed for ovary-biased genes, this
229 small number of GT-female biased genes also had statistically significantly lower dN/dS values
230 than the GT-female biased genes on autosomes (MWU-test $P = 0.031$, Fig. 2D), and the $X/A_{dN/dS}$
231 ($_{GT-female}$) value when calculated for this subset of genes was also low, at 0.45 (Fig. 2F). Thus, it
232 appears that there has also been high purifying selection on X-linked GT-female biased genes in
233 this taxon. Upon close examination however, and as shown in Table S2, 17 of the 24 (70.8%) X-
234 linked GT-female biased genes also had ovary-biased expression, suggesting that the observed
235 effect could be due to purifying selection on ovarian expression rather than somatic expression.
236 Nonetheless, the seven genes with GT-female biased but not ovary-biased expression yielded a
237 $X/A_{dN/dS}(\text{GT-female})$ ratio of 0.32, indicating that X-linked GT-female-biased genes are under
238 higher constraint than those on autosomes, regardless of their ovary-biased expression status.
239 Thus, we find no evidence of a faster-X effect for any female-biased genes, regardless of gonadal
240 or somatic expression, and in fact these genes likely contribute to the slow evolution of the X-
241 chromosome.

242 We next assessed whether the faster-X effect was observable for male-biased genes
243 (testis- or GT-male-biased), which would be expected to exhibit a pronounced faster-X effect
244 under a hypothesis of rapid fixation of beneficial recessive mutations in the heterogametic sex [7,
245 16]. We found that very few testis-biased genes or GT-male-biased genes were located on the X
246 chromosome (N=9 and N=12 for testis-biased and GT-male-biased X-linked genes with high
247 confidence interspecies orthologs), and that neither group of male-biased genes showed even
248 mild evidence of a faster-X effect. The median dN/dS value was lower for these genes on the X
249 chromosome than on autosomes for both categories of genes (Fig. 2CD). The $X/A_{dN/dS}$ (testis-biased)
250 ratio was 0.71 for testis-biased genes, and the $X/A_{dN/dS}$ (GT-male biased) ratio was 0.52 for GT-male
251 biased genes (Fig. 2EF), markedly below 1 in both cases. No overlap was observed between the
252 testis-biased and GT-male biased gene sets (Table S2), and thus the low dN/dS effects were
253 independently observed in each group. For stringency, we examined and noted that three of the
254 GT-male-biased genes were also ovary-biased, but exclusion of those genes from the analysis
255 still yielded an $X/A_{dN/dS}$ (GT-male biased) ratio of 0.59, and thus the low dN/dS effect is directly linked
256 to the GT-male-biased expression. In sum, while the small number of X-linked testis-biased and
257 GT-male-biased genes precludes rigorous statistical testing of those genes, the patterns observed
258 for these genes are inconsistent with a faster-X effect in male-biased genes, whether gonad- or
259 soma-biased.

260 We next asked whether there was evidence for the faster-X effect in the gonadally
261 unbiased genes. Given that such genes were common on all chromosomes (Fig. 2A, Table S2),
262 which provides the potential for high statistical power, and that they by definition they exclude
263 the highly constrained X-linked ovary-biased genes and the testis-biased genes described above
264 (Fig. 2C), we predicted that if there were even a mild tendency for a faster-X effect in this taxon,
265 it would be readily apparent in this group of genes. However, we found no significant difference
266 in dN/dS values between X-linked and autosomal gonadally unbiased genes (MWU-test $P > 0.05$
267 Fig. 2C). Rather, we observed an $X/A_{dN/dS}$ (gonadally unbiased) ratio of 1.04, indicating highly similar
268 dN/dS between these two groups (Fig. 2E). In this regard, we conclude that the faster-X is fully
269 absent in gonadally unbiased genes.

270 Finally, we assessed the GT-unbiased genes, and found evidence for greater constraint on
271 the sequence evolution of these genes on the X chromosome as compared to autosomes ($X/A_{dN/dS}$
272 (GT-unbiased) = 0.78, MWU-test $P < 0.05$, Fig. 2DF). As expected, however, given that a majority of

273 X-linked genes under study were ovary-biased (Fig. 2A, Table S2), and that most genes
274 expressed in the GT-soma are not sex-biased (Fig. 2B), many of the X-linked GT-unbiased genes
275 (N=396) were also ovary-biased (N=213). Excluding these genes, so that we could consider only
276 those 183 GT-unbiased genes that were not ovary-biased, we found no differences in dN/dS
277 values for these genes between the X-chromosome and autosomes (MWU-test $P > 0.05$). In fact,
278 the $X/A_{dN/dS}$ (GT-unbiased) ratio for these GT- and ovary-unbiased genes was 1.04, identical to that
279 observed for gonadally unbiased genes (Fig. 2EF). Thus, the GT-somatically unbiased genes,
280 whether they were co-biased in the ovaries or not, exhibited no signals of a faster-X effect.

281 Taken together, the collective results in Fig. 2 show that the slower-X effect observed
282 here in *Tribolium* is largely explained by highly constrained evolution of the abundant X-linked
283 ovary-biased genes, with some minor contributions from the relatively smaller number of testis-
284 biased, GT-male biased, and GT-female-biased genes (Fig. 2C-F). Crucially, the faster-X effect
285 was not even observed in either gonadally-unbiased or GT-soma-unbiased genes, which each
286 yielded an effective $X/A_{dN/dS}$ ratio of 1.04. This latter finding cannot be explained by constrained
287 evolution of X-linked sex-biased genes, suggesting that other factors likely also contribute
288 towards the absence of the faster-X in this taxon (see the below section “*Absence of dosage*
289 *compensation in the T. castaneum testis*”).

290

291 **Why do X-linked ovary-biased genes evolve slowly?**

292 We wished to further consider why the X-linked ovary-biased genes evolved extremely
293 slowly (Fig. 2CE). The exceptionally low dN/dS values observed for ovary-biased genes on the
294 X chromosome (Fig. 2CE) as compared to autosomes suggests that they could be essential genes
295 subjected to high purifying selection, and their ovary-biased expression suggests that they may
296 be involved in female reproduction and thus fitness. To examine this, we determined the
297 predicted GO functions (see Methods: GO functions determined in DAVID [45]) of the ovary-
298 biased genes located on the X-chromosome (Fig. 2A). Indeed, in agreement with this hypothesis,
299 we found that ovary-biased genes on the X chromosome were enriched for genes involved in
300 ovarian follicle development and *wnt* signalling (Table 1), which is crucial for ovarian
301 development and function in multiple animals (see [46-55] for examples). X-linked ovary-biased
302 genes also included those with predicted roles in female meiosis and oocyte function (Table 1).
303 These essential ovarian roles were not among the top functional categories observed for ovary-

304 biased genes on autosomes (Table 1). Given these results, we suggest that high purifying
305 selection on ovary-biased genes on the X chromosome is likely at least partly due to the
306 important female reproductive roles of some of these genes. Moreover, their high concentration
307 on the X-chromosome may suggest a history of preferential translocation of essential female
308 reproductive genes to the X-chromosome.

309 We next considered whether expression breadth could explain the slow evolution of X-
310 linked ovary-biased genes. It has been proposed that greater expression breadth across tissues,
311 which reflects pleiotropic functionality, constrains dN/dS. For example, the rapid evolution of
312 male-biased than female-biased genes observed in various organisms, as was also found here for
313 testis versus ovaries (MWU-test $P < 0.001$ of all testis- versus all ovary-biased genes, Fig. 2C)
314 may result from low pleiotropy [17, 40, 56, 57]. Indeed, we found herein that expression breadth
315 across the four studied tissue-types was lower for testis-biased than for ovary-biased genes.
316 Specifically, only 25.5% of testis-biased genes (pooled for X-linked and autosomal) were
317 expressed in all four tissue types (at >1 FKPM) while 72.8% of ovary-biased genes were
318 transcribed in all four tissues. In this regard, ovary-biased genes as a group exhibit higher
319 pleiotropy, suggesting potential roles across various tissues that may contribute to their slower
320 evolution relative to testis-biased genes (Fig. 2C). In turn, the accumulation of ovary-biased
321 genes on the X-chromosome would act to constrain evolution of this chromosome.

322 Nonetheless, it is worth noting that broad expression breadth (expressed in in all four
323 tissues) was observed for the majority of ovary-biased genes independently of chromosomal
324 location (78.9% of X-linked ovary-biased genes and 71.6% of autosomal ovary-biased genes
325 were expressed in all tissues). Thus, the specific finding of a lower dN/dS values of X-linked
326 ovary-biased genes (compared to their counterparts on autosomes, Fig. 2C) cannot be fully
327 explained by high pleiotropy. We therefore propose that the slower evolution of ovary-biased
328 genes on the X-chromosome than those on autosomes is likely at least partly due to their
329 preferential involvement in essential ovary functions (Table 1).

330

331 **Absence of dosage compensation in the *T. castaneum* testis**

332 In X/Y sex determination systems, it has been posited that mechanisms should exist to
333 ensure that the expression levels of genes on the X-chromosome (X) and autosomes (A) would
334 be approximately equivalent in both males (with hemizygous X) and females (homozygous X),

335 such that the ratio of expression of X/A in each sex should equal one [38, 58]. In turn, it may be
336 expected that $X_{\text{male}}/X_{\text{female}} = A_{\text{male}}/A_{\text{female}} = 1$ [38]. Measurements of gene expression levels in a
337 number of animals have shown that mechanisms for acquiring elevated expression on the single
338 male X-chromosome, or dosage compensation, are highly variable and that full dosage
339 compensation is sometimes, but not always, achieved [38, 58-60]. In one prior study of gene
340 expression using microarrays of whole males versus whole females in *T. castaneum* [38], it was
341 reported that males exhibited full X-chromosome dosage compensation, with $X_{\text{male}}/A_{\text{male}} = 1.0$
342 and that females exhibited overexpression of the X chromosome, with $X_{\text{female}}/A_{\text{female}} = 1.5$,
343 thereby yielding $X_{\text{male}}/X_{\text{female}}=0.79$ and $A_{\text{male}}/A_{\text{female}}=1$. Those results were interpreted as
344 evidence that the genes on the X-chromosome exhibited complete dosage compensation in males
345 (meaning that expression of the hemizygous X linked genes was equalized to expression of
346 autosomal genes in males), and were overexpressed in females as an imperfect response to
347 dosage compensation [38]. However, a recent study that examined published RNA-seq data for
348 somatic glandular tissues in *T. castaneum* did not find evidence for hypertranscription of the X-
349 chromosome in females [60]. Given that dosage compensation can vary among tissues in insects,
350 particularly its absence in the testis of *Drosophila* [25-27], and that complete dosage
351 compensation has been theorized to promote the faster-X effect by fixation of recessive
352 beneficial mutations in hemizygous males [1, 2, 22], we next aimed to assess dosage
353 compensation separately for genes expressed in the gonads and those expressed in the GT-
354 somatic tissues for *T. castaneum*. As the sex organs play central roles in reproduction, recessive
355 beneficial mutations in genes are apt to have their greatest fitness consequences (and thus be
356 fixed) in the hemizygous male gonad (rather than male soma), and thus we predicted that if a
357 lack of dosage compensation were found in the testis, this might significantly contribute towards
358 the absent faster-X in this organism.

359 In Fig. 3, we show the median expression level (FPKM) for genes on the X-chromosome
360 and each of the nine *T. castaneum* autosomes for the gonads (A) and for the GT-soma (B) using
361 all genes that had high-confidence *T. freemani* orthologs (N=7,751; results for all *T. castaneum*
362 genes are in Fig. S4, showing similar patterns). We report that expression levels in ovaries (Ov)
363 were largely similar across the nine autosomes (median 14.7 FPKM across nine autosomal
364 medians) and were relatively elevated on the X-chromosome (18.8 FPKM, MWU-test P=0.023
365 of the X-chromosome versus autosomes, Fig. 3A; note that X/A is measured using multiple

366 decimal places), yielding X_{OV}/A_{OV} of 1.26 and is consistent with overexpression of X-linked
367 genes in the ovary. For the testis (Ts), however, while expression was also largely similar across
368 all nine autosomes (median 7.9 FPKM across nine autosomal medians), a strikingly lower
369 expression level was observed for the X-chromosome (3.2 FPKM, Fig. 3A), giving an X_{Ts}/A_{Ts}
370 value of 0.41. Thus, there is 2.5-fold lower expression of X-linked testis genes than of autosomal
371 testis genes (MWU-test $P < 0.001$, Fig 3A; see also Fig. S4A where the value was also < 0.5),
372 inconsistent with hypertranscription of the single X chromosome in males, at least for the testis-
373 expressed genes. This complete absence of dosage compensation in the *T. castaneum* testis is
374 even beyond that reported for the testis of *Drosophila*, which had an 0.65 value for this
375 parameter [25]. Further, the low value potentially not only suggests an absence of
376 hyperexpression on the X-chromosome in the hemizygous state (to balance autosomes), but
377 could also be consistent with an active mechanism of suppression of X-linked expression [25, 27,
378 61] in this beetle.

379 Moreover, we found that testis expression was lower than ovary expression across all
380 nine autosomes, such that A_{Ts}/A_{Ov} was equal to 0.53 (MWU-test $P < 0.001$ of autosomal testis to
381 ovary expression), differing from the equal male/female expression typically expected on
382 autosomes [25, 38]. This effect was even more pronounced for the X-chromosome, where
383 X_{Ts}/X_{Ov} had a value of 0.17 (Fig. 3A, MWU-test $P < 0.001$ for X-chromosome testis expression
384 versus ovary expression), indicating that even after taking into account the lower expression
385 level observed on all autosomes for testis genes versus ovary genes (median 1.9 fold), testis
386 genes exhibited a marked drop (5.9-fold) in expression on the X-chromosome. In this regard,
387 both X_{Ts}/A_{Ts} and X_{Ts}/X_{Ov} (Fig. 3A) suggest a complete absence of dosage compensation in this
388 beetle.

389 Considering the GT-soma, we observed nearly perfect dosage compensation on the X-
390 chromosome for GT-males, both with respect to GT-female expression levels, such that X_{GT-}
391 $_{female}/X_{GT-male} = 0.93$ (median of 3.02 and 3.25 FPKM respectively MWU-test $P = 0.74$), and with
392 respect to autosomal GT-male expression levels, with $X_{GT-male}/A_{GT-male} = 0.91$ (MWU-test
393 $P = 0.11$). Thus, unlike genes expressed in the testis, genes expressed in the non-gonadal tissues of
394 males (GT-males) exhibited high dosage compensation (Fig. 3B, Fig. S4B). The median GT-
395 male expression across all nine autosomes was consistently higher than the median expression in
396 GT-females, yielding $A_{GT-male}/A_{GT-female}$ of 1.27 (MWU-test $P < 0.001$), a trend opposite to the

397 higher expression level observed for ovary genes relative to testis genes (Fig. 3AB). Nonetheless,
398 GT-female genes on the X-chromosome were expressed at higher levels than such genes on
399 autosomes, yielding $X_{GT-female}/A_{GT-female}=1.26$ (MWU-test $P=0.064$), and thus contributing to the
400 observed highly similar expression levels between GT-females and GT-males on the X-
401 chromosome. In sum, the GT-males show evidence of nearly complete dosage compensation,
402 differing markedly from its complete absence in the testis. Additional study of more individual
403 somatic tissues (e.g., brain, hindgut), similar to that in other recent studies [25, 60], will be
404 needed to assess whether the variation in GT-female expression among autosomes is observed in
405 various somatic tissue types in *T. castaneum*.

406 Taken together, the results presented here in Fig. 3 and Fig. S4 show a complete lack of
407 dosage compensation in the testis. Given that the faster-X effect may be anticipated to be
408 strongest in taxon groups with complete dosage compensation, due to elevated phenotypic
409 protein product and effects of beneficial recessive mutations in males [1, 14, 22], the under-
410 transcription in the *Tribolium* testis could contribute to the absence of the faster-X we report here
411 (Fig. 1, Fig. 2). Further, this effect may transcend all sex-biased expression categories. For
412 instance, all X-linked testis-biased genes, 98.7% ovary-biased genes, and 92.3% of gonadally
413 unbiased genes were expressed in the testis, and thus low dosage compensation may affect all
414 groups of genes due to under-expression (relative to autosomal genes) on the single X-
415 chromosome in males. Finally, it should be noted that the absence of X-chromosome dosage
416 compensation found in the testis, combined with a relatively modest elevation in ovary
417 expression (Fig. 3A), are consistent with the concentration of ovary-biased genes on the X
418 chromosome in this organism (Fig. 2AB, Fig.S3AB).

419

420 **Discussion**

421

422 **Absence of a faster-X effect and sex-biased genes**

423 Our results show that the absence of a faster-X effect, and tendency for a slower-X, in
424 *Tribolium* (Fig. 1), is explained in part by strong purifying selection on the highly abundant X-
425 linked ovary-biased genes (Fig. 2ACE). Accordingly, we hypothesize that many X-linked ovary-
426 biased genes play essential roles in this organism, as indicated by their low dN/dS values (Fig.
427 2CE), GO functional analysis (Table 1) and high cross-tissue pleiotropy (72.8% expressed in all

428 four tissues). We further hypothesize that ovary-biased genes may have been preferentially
429 translocated to the X-chromosome over the history of this beetle taxon. This type of localization,
430 or translocation, phenomenon is also supported by findings from other systems (e.g. mice,
431 humans, flies) showing that genes involved in sex and sexual dimorphism, and particularly those
432 showing female-biased expression, have been preferentially localized to the X-chromosome [62-
433 65]. The preferential transfer of female genes (as compared to male-biased genes) to the X-
434 chromosome may be facilitated by the unique selection pressures experienced by these
435 chromosomes, especially the fact that two-thirds of X-chromosomes in population are carried by
436 females and only one third in males, which may make the concentration of female functional
437 genes on the X-chromosome an innate benefit to females [18, 61, 65].

438 For the testis-biased and GT-male biased genes, of which there were very few on the X-
439 chromosome (Fig. 2AB, Fig. S3AB), the low dN/dS observed on the X-chromosome
440 ($X/A_{dN/dS}=0.71$ and 0.52 respectively) is also discordant with a faster-X effect in those genes.
441 The constraint on X-linked male-biased genes could be readily explained by the immediate
442 exposure of any mildly deleterious recessive mutations to purifying selection in the hemizygous
443 state in testis and GT-males (and not on autosomes). Thus, it is possible that a much different
444 mechanism could cause the slow evolution of those genes, than that operating on ovary-biased
445 genes. In other words, it may be surmised that if most protein sequence evolution is due to
446 fixation of weakly deleterious alleles in beetles, then the X-chromosome would be expected to
447 evolve slowly due to rapid purging of these mutations as a result of hemizygous exposure, as
448 compared to autosomes. This phenomenon was theorized to occur for some organisms under the
449 original faster-X hypothesis [1] and has been proposed to contribute to the relatively mild faster-
450 X effect observed in *Drosophila* (by countering the accelerated evolutionary rates on the X due
451 to rapid fixation of recessive beneficial mutations) [22]. In Satyrine butterflies with W/Z
452 systems, for example, it was suggested that slow evolution of Z-linked female-biased genes
453 occurred due to high purifying selection on the Z-chromosome in the hemizygous state [13].
454 Thus, the same phenomenon may occur for male-biased genes in *Tribolium*.

455 Importantly, the unbiased genes in the gonads, and in the GT-somatic tissues, showed no
456 tendency for a faster-X effect, with $X/A_{dN/dS}(\text{unbiased})=1.04$ for each tissue type (after exclusion of
457 ovary-biased genes from the latter dataset, see Results, Fig. 2C-F). Thus, the lack of faster-X
458 effect in those genes cannot be explained by higher selective constraint on the X-chromosome

459 than autosomes. Albeit, we do not exclude some purging of recessive mutations in males could
460 occur in these genes, similar as proposed possible for male-biased genes above, however this
461 would be expected to slow the X [1], which was not observed for unbiased genes. The result
462 indicates that another mechanism likely contributes to the absence of a faster-X effect in these
463 beetles, which our data strongly suggest involves the lack of dosage compensation in the male
464 gonads.

465

466 **Lack of dosage compensation**

467 Our finding of a complete absence of dosage compensation combined with the absence of
468 the faster-X effect is highly suggestive that fixation of beneficial sequence mutations on the X-
469 chromosome may have been uncommon or absent in this taxon due to under-expression in males
470 [1, 22]. Additional findings of an absent faster-X effect in a broader range of organisms, and that
471 include assessments of the degree of dosage compensation, will help further resolve the role of
472 this putative mechanism. Our results suggest the effect may extend beyond the passive absence
473 of dosage compensation (or hyperexpression of the X-chromosome), and potentially reflects an
474 active mechanism of X-chromosome silencing. Recent reports from *Drosophila* have also shown
475 that dosage compensation is absent for the testis [25], and that this insect exhibits active
476 suppression of X-linked expression in males [25, 61]. Consistently, it was found that the transfer
477 of X-linked testis-expressed genes to the autosomes resulted in marked upregulation in *D.*
478 *melanogaster* [61], suggesting an active mechanism of suppression of expression on the X-
479 chromosome in testis. While the mechanism for X-linked active suppression of expression is
480 unknown, it could reflect male meiotic sex chromosome inactivation (MSCI). Empirical support
481 for MSCI has been observed for *D. melanogaster* [27, 66], and a strong effect has been found in
482 range of other animal systems including mammals [67] and *Caenorhabditis elegans* [68]. Further
483 study will be needed to ascertain whether the absence of dosage compensation in the testes for *T.*
484 *castaneum* involves lack of upregulation on the X-chromosome and/or also includes an active
485 process involving X-chromosome suppression or silencing.

486 We emphasize that the separation of gonads and GT-somatic tissues herein for expression
487 analyses was essential for revealing the absence of dosage compensation in *T. castaneum*. A
488 prior report of dosage compensation in *T. castaneum* using microarray expression data from
489 whole males and females [38] showed much different results for male dosage compensation than

490 those observed in the present study. In that assessment, males were reported to exhibit complete
491 dosage compensation on the single X-chromosome as compared to autosomes, while females
492 were reported as exhibiting overexpression of X-chromosomes (relative both to X-linked
493 transcription in males and to female expression on autosomes) [38]. The latter was interpreted as
494 an imperfect (female) response to male dosage compensation [38]. In contrast, our tissue-specific
495 expression data allow us to explicitly show the absence of dosage compensation in the male
496 testis, and nearly perfect dosage compensation for X-linked GT-male expression with respect to
497 the autosomes and with respect to X-linked expression in GT-females. Thus, our separation of
498 gonadal from somatic expression data was essential for the detection of key differences in dosage
499 compensation in this taxon, and was particularly crucial to discerning the absence of dosage
500 compensation in the testis (Fig. 3A, Fig. S4A), that had been obscured previously in the
501 examination of whole males and females.

502 Finally, while we propose that the absence of the faster-X effect herein is best explained
503 by constrained evolution of the abundant X-linked ovary-biased genes and lack of dosage
504 compensation in the testis, we do not exclude a role of standing genetic variation. For instance,
505 large populations tend to contain more polymorphic loci, which can accelerate autosome
506 evolution if adaptation occurs via standing genetic variation rather than *de novo* mutations [2,
507 69]. This phenomenon could possibly occur in beetles, and thus we do not exclude this factor in
508 partly contributing towards the absence of a faster-X effect in this taxon.

509

510 **Conclusions and Future Directions**

511 We have shown the complete absence of the faster-X effect in a *Tribolium* system, which
512 our data strongly suggest is largely explained by constrained evolution of X-linked ovary-biased
513 genes and the extreme absence of dosage compensation in the testis of this taxon. Future studies
514 should aim to study additional genomes of more *Tribolium* species, which would allow tests of
515 positive selection in protein sequences on the X-chromosome and autosomes [43]. In addition,
516 studies using population-level data from *T. castaneum* will allow tests of polymorphism versus
517 divergence, which comprises an alternate method to test adaptive evolution expected under the
518 faster-X effect [13, 14]. A further understanding of dosage compensation in this taxon may be
519 achieved by attainment of transcriptional data from a wide range of individual somatic tissue
520 types in *T. castaneum*, similar to analyses recently conducted in *Drosophila* [25]. Such multi-

521 tissue expression data will also allow further assessments of cross-tissue pleiotropy of sex-biased
522 genes [40, 57, 70] and may help further disentangle its role in constraining evolution of ovary-
523 biased genes (Fig. 2).

524 Moreover, experimental research of MSCI in *T. castaneum*, as has been conducted in
525 other organisms [66, 67, 71], will help reveal whether the lack of dosage compensation observed
526 in the testis is due to transcriptional silencing in the male meiotic cells. In addition, studies using
527 X-linked genes inserted into the autosomes, and vice-versa [25, 61, 68] may help discern the
528 dynamics of dosage compensation in *T. castaneum*. Finally, studies of the faster-X effect,
529 including analyses of sex-biased genes and dosage compensation, should be extended to include
530 more understudied organisms, to help reveal the breadth of this phenomenon in metazoans and to
531 better understand its underlying mechanisms.

532

533 **Methods**

534 **Biological Samples and RNA-seq**

535 *T. castaneum* and *T. freemani* specimens were provided by the Brown lab at Kansas State
536 University (strain IDs; <https://www.k-state.edu/biology/people/tenure/brown/>). Samples were
537 grown under standard laboratory conditions until adulthood as previously described [29].
538 Additionally, to ensure that all adult animals for both species remained unmated until the time of
539 tissue dissection, all animals were separated as late stage larvae into individual vials containing
540 flour and allowed to pupate into adults. Tissue dissections were then performed on unmated
541 adults within a week after they emerged from the molt. For *T. castaneum* a total of 150 animals
542 per sex per biological replicate were sampled, whereas for *T. freemani*, a total of 50 males and
543 females were collected per sample. For each sample of males and of females, the gonadal and
544 nongonadal tissues were separated and placed into two separate vials containing TRIzol reagent
545 (Ambion Life Technologies, catalog number 15596-018) on dry ice. Technical details on tissue
546 collection, PCR, and RNA-seq are provided in Additional File 1, Text File S1.

547 For males, the isolated reproductive tissues included the testes, accessory glands
548 (mesadenia, ectadenia), and directly attached tissues (vesicular seminalis, vas deferens and
549 ejaculatory duct) whilst for females, gonad samples included the ovaries and their linked tissues
550 (spermathecal gland, common oviduct, spermathecae, and vagina). For simplicity, we refer to the
551 male and female reproductive organs and tissues collectively as “testis” and “ovary” or the sex-

552 neutral “gonads” herein, with the understanding that they include the abovementioned
553 reproductive tissues directly linked to the respective gonads. All remaining non-gonadal tissues
554 of the adult body are referred to as the gonadectomized (GT-) soma, or GT-males and GT-
555 females. For the sister species *T. freemani*, four RNA-seq samples, one per tissue-type, testes,
556 ovaries, GT-males and GT-females, were obtained and used for refining the CDS list for this
557 species (see Methods) that was employed to assess protein divergence (dN/dS).

558

559 **CDS per Species and Defining Orthologs**

560 The annotated CDS of our main target species *T. castaneum* (v.5.2) were downloaded
561 from Ensembl Metazoa (<http://metazoa.ensembl.org>) and are also available at BeetleBase [34,
562 36]). The full CDS per gene (longest CDS per gene) was used for the study of sex-biased gene
563 expression.

564 For the genome of *T. freemani*, which we used as a reference to determine dN/dS, CDS
565 have not yet been annotated and thus were extracted from available scaffolds. The scaffold
566 assembly was downloaded from BeetleBase (version 4, <http://www.Beetlebase.org>, [34]). Details
567 on extracting the CDS for *T. freemani* are provided in Additional File 1, Text File S1.

568 In the final CDS list for *T. castaneum* and for *T. freemani*, only those CDS having a start
569 codon, not having unknown or ambiguous nucleotides or internal stop codons, and ≥ 33 amino
570 acids were retained for study. The total number of CDS after filtering was 16,434 for *T.*
571 *castaneum*, marginally more than the 16,404 gene models first defined for this species [36], and
572 was 12,628 for the sister species *T. freemani*. The average GC content of the *T. castaneum*
573 protein-coding genes was 46.1% ($\pm 5 \times 10^{-4}$), which is above the 33% reported for the global
574 genome encompassing all coding and noncoding DNA as has been noted previously for this
575 taxon [35, 36].

576

577 **Identification of Sex-Biased Genes**

578 The RNA-seq reads (76bp) per sample were trimmed of adapters and poor-quality bases
579 using the program BBduk available from the Joint Genome Institute ([https://jgi.doe.gov/data-
580 and-tools/bbtools/](https://jgi.doe.gov/data-and-tools/bbtools/)) and run as a plug-in in Geneious v11.0.3 using default parameters.

581 Gene expression level per gene was determined by mapping each RNA-seq dataset per
582 tissue to the full CDS list for each species using Geneious Read Mapper, a program based our

583 comparisons and other analyses provides similar read match performance as other common read-
584 mappers such as Bowtie [72] or Bbmap (<https://jgi.doe.gov/>; data not shown). Read counts per
585 CDS were converted to FPKM for each gene. Expression level was compared separately for the
586 gonads and for the GT-soma. Expression was compared between the testes and ovaries, and
587 between GT-males and GT-females by using Deseq2 to obtain P-values [44] and the average
588 FPKM of the replicates per tissue type (Table S1). Any gene having at least a two-fold difference
589 in average expression and a statistically significant P-value ($P < 0.05$) as well as a FPKM of at
590 least one in one tissue type was identified as sex-biased [17, 73]. All other genes with nonzero
591 expression in gonadal and in nongonadal contrasts were defined as unbiased.

592

593 **Ortholog Identification and Sequence Divergence**

594 For dN/dS analysis, orthologs between *T. castaneum* and *T. freemani* were identified
595 using reciprocal BLASTX of the full CDS list between species in the program BLAST+ v2.7.1
596 (<https://blast.ncbi.nlm.nih.gov>). Only genes having the same best match in both forward and
597 reverse contrasts and an e-value $< 10^{-6}$ were defined as orthologs. In the rare cases when two
598 CDS had the same e-value, the one with the highest bit score was taken as the best match. For
599 additional stringency of genes used for the study of dN/dS, only those genes that were reciprocal
600 BLASTX best matches and where both dN and dS values of alignments (≥ 33 amino acids) had
601 values < 1.5 , and thus were unsaturated in substitution (see below paragraph), were defined as
602 orthologs between *T. castaneum* and *T. freemani* (and used for dN/dS analyses). Thus, the
603 alignments and dN/dS measures herein are conservative.

604 Orthologous gene sequences in *T. freemani* and *T. castaneum* were aligned by codon
605 using MUSCLE set to default parameters (except the gap penalty which was set at -1.9) in the
606 program Mega-CC v7 [74]. Alignments were then filtered to remove gaps. It has been found that
607 removal of highly divergent segments from alignments, despite some loss of sequence regions,
608 improves measures of protein divergence; thus, highly divergent segments were removed using
609 the program Gblocks v. 0.91b set at default parameters [75, 76]. Each gene alignment was then
610 run in yn00 of PAML, which accounts for codon usage biases [43], to measure dN, dS, and
611 dN/dS [43].

612 We note that the percentage of ovary-biased genes on the X-chromosome in our 7,751 *T.*
613 *castaneum* genes with orthologs in *T. freemani* was 53.9%, while for all 16,434 *T. castaneum*

614 genes it was 42.2%. This likely reflects the fact that we studied genes that had high confidence
615 orthologs between species, which are apt to be more frequently identified for ovary-biased genes
616 due to their slowed evolution (Fig. 2C), an effect that was pronounced on the X-chromosome
617 (Fig. 2C). Ovary-biased genes on autosomes had largely similar percentages on autosomes in the
618 studied genes for dN/dS analyses and all genes (Fig. 2A, Fig. S3A). In this regard, our dN/dS
619 analyses inherently include those genes with conserved between-species orthologs.

620

621 **X-Chromosomes Versus Autosomes**

622 Chromosomal locations of genes are available in the annotation for *T. castaneum*
623 (<http://metazoa.ensembl.org>, also available at BeetleBase [34, 36]). We note that the Y-
624 chromosome of *T. castaneum* is small (<5MB), highly degenerate, contains few if any protein-
625 coding genes, and is not included in the genetic linkage map; accordingly it was not studied [29,
626 36, 38, 77].

627

628 **Gene Ontology**

629 Gene ontology (GO) was assessed using DAVID software [45]. For this, we identified
630 orthologs to *T. castaneum* in the reference insect model *D. melanogaster* (CDS v6.24 available
631 from www.flybase.org [78]) using BLASTX (<https://blast.ncbi.nlm.nih.gov>) to identify the best
632 match (lowest e-value with cut off of $e < 10^{-6}$). *D. melanogaster* gene identifiers, which are
633 accepted as input into DAVID, were used to obtain GO functions for *T. castaneum* genes. Single
634 direction BLASTX with *T. castaneum* CDS as the query to the *D. melanogaster* database was
635 used for this assessment (unlike for the reciprocal BLASTX between *Tribolium* species), as we
636 considered reciprocal BLASTX to be overly stringent between these divergent insects (which are
637 from different orders) for functional analysis.

638

639 **Availability of Data**

640 The CDS v. 5.2 for *T. castaneum* are available at Ensembl Metazoa
641 (<http://metazoa.ensembl.org>). Scaffolds for *T. freemani* are available at BeetleBase [34, 36].
642 RNA-seq data and SRA Biosample identifiers for all 12 samples from *T. castaneum* and *T.*
643 *freemani* described in Table S1 are available at the SRA database under Bioproject accession
644 number PRJNA564136.

645

646 **Declarations**

647 *Ethics approval and consent to participate:* N/A

648 *Consent for publication* N/A

649 *Availability of data and material:* Available at the SRA database under Bioproject accession
650 number PRJNA564136. See also Table S1 for SRA Biosample identifiers for each sample
651 studied herein.

652 *Competing interests:* None

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654 *Authors' contributions:* CAW, AK, and CGE designed the study, CAW conducted data analyses,
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660 and the Bauer core sequencing facility at Harvard for generating RNA-seq data.

661

662 **Additional Files:**

663 Additional File 1: contains the supplemental tables, figures and SI Text including detailed
664 methods.

665

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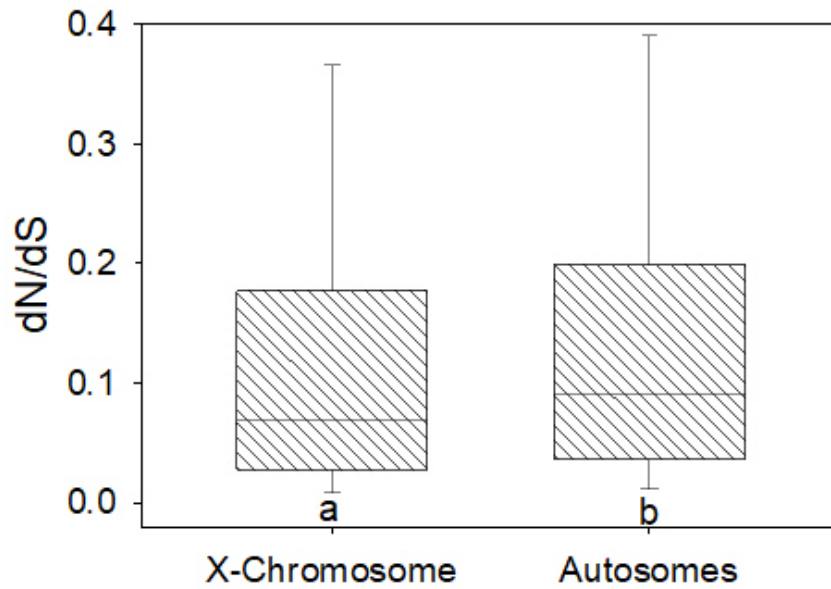
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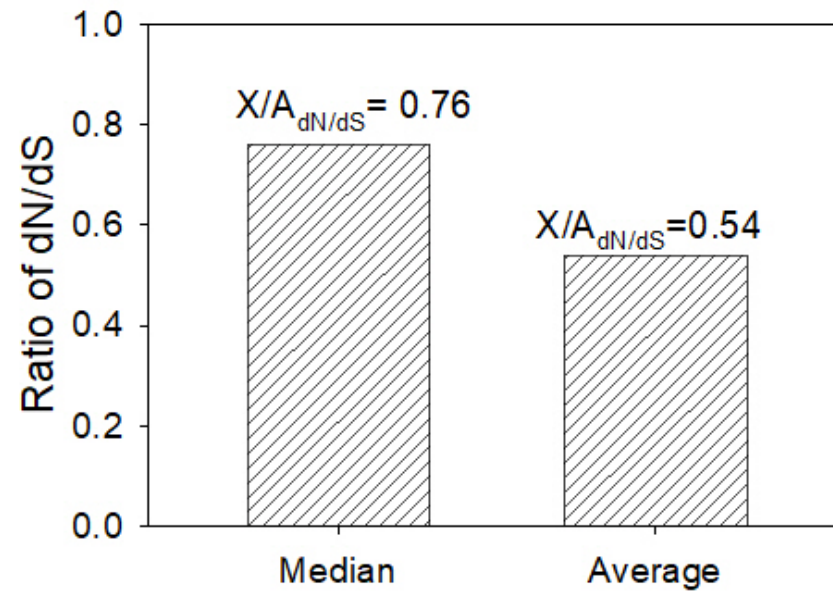
Table 1. Gene ontology (GO) clustering of ovary-biased genes located on the X chromosomes and on autosomes. The top clusters with the greatest enrichment scores are shown per category. *P*-values are from a modified Fisher's test, wherein lower values indicate greater enrichment. Data is from DAVID software [45] using those genes with *D. melanogaster* orthologs.

Ovary-Biased Genes on X Chromosome		Ovary-Biased Genes on Autosomes^a	
<u>Cluster 1: Enrichment Score 3.09</u>	P-value	<u>Cluster 1: Enrichment Score 3.56</u>	P-value
Wnt signaling pathway	4.20E-06	Metal-binding	6.00E-05
Segmentation polarity protein	8.20E-05	Zinc ion binding	5.50E-04
Regulation of Wnt signaling pathway	1.60E-04	Zinc-finger	6.60E-04
Segment polarity determination	1.30E-03		
Ovarian follicle cell development	6.70E-03	<u>Cluster 2: Enrichment Score 2.81</u>	
Somatic stem cell population maintenance	2.50E-02	Pleckstrin homology-like domain, signalling	1.80E-04
Heart development	3.90E-02	Pleckstrin homology domain, signalling	4.90E-04
<u>Cluster 2: Enrichment Score 2.92</u>		<u>Cluster 3: Enrichment Score: 2.78</u>	
ATP-binding	2.00E-04	SH2 domain, oncoproteins, signalling	1.40E-04
Nucleotide-binding	3.70E-04	SH3 domain, intracellular or membrane-associated proteins	2.10E-04
Nucleotide phosphate-binding region:ATP	1.60E-03		
Protein kinase, ATP binding site	7.70E-03		

^a Data was pooled for all nine autosomes and also includes genes yet unmapped in the genome.

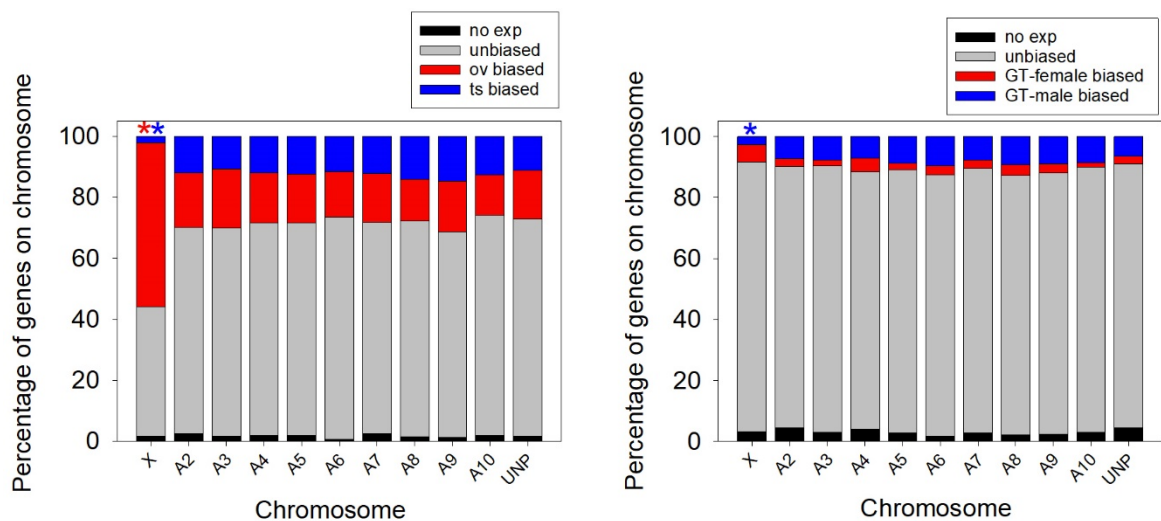


A. Box plot of dN/dS for X-chromosome and autosomes



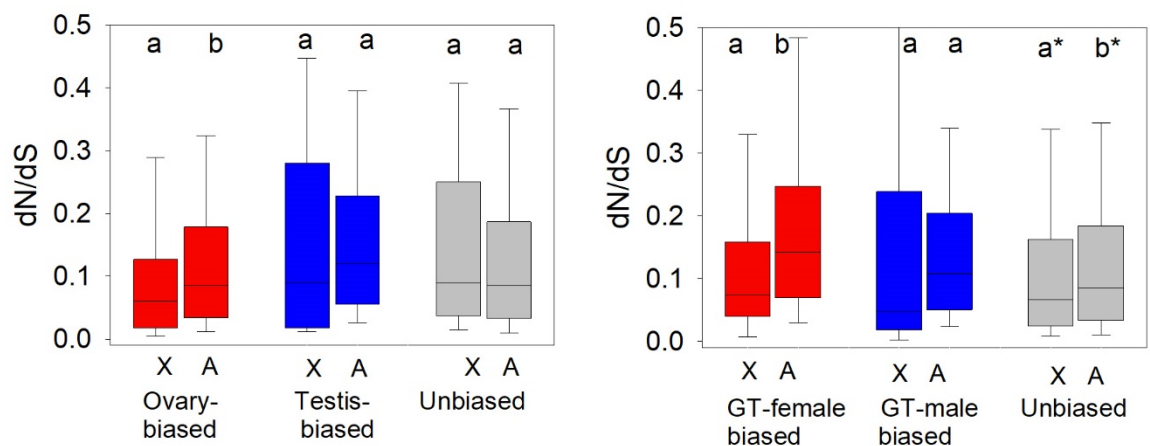
B. Ratio of dN/dS of the X-chromosome to autosomes

Fig. 1. The dN/dS of genes located on the X-chromosome versus autosomes. A) Box plots of dN/dS showing the median, upper and lower quartiles, and 95/5th percentiles; B) the ratio of dN/dS for the X-chromosome versus the autosomes using the median and mean values per group. Different letters under bars in panel A indicate a statistically significant difference using MWU-tests.



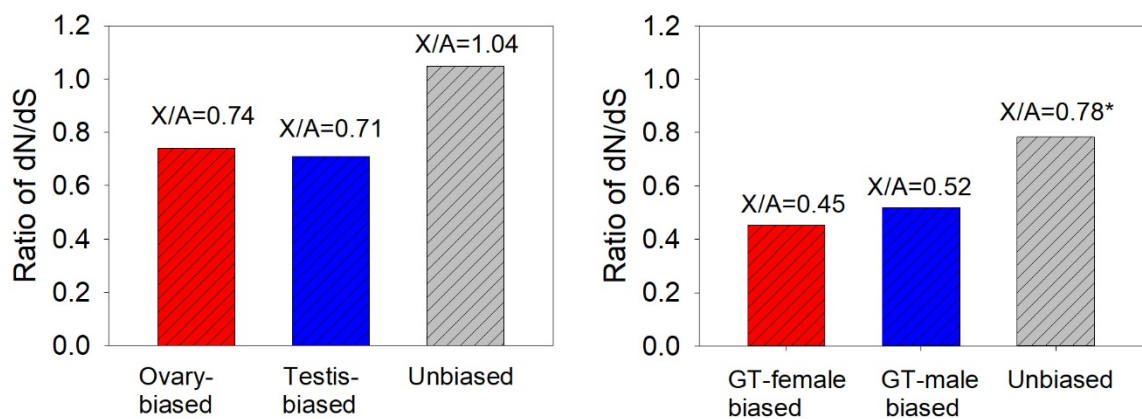
bioRxiv preprint doi: <https://doi.org/10.1101/754903>; this version posted September 9, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

A. Frequency of gonadally-biased genes on the X-chromosome and autosomes **B. Frequency of GT-soma-biased genes on the X-chromosome and autosomes**



C. dN/dS of gonadally-biased genes (X= X-chromosome, A= autosomes)

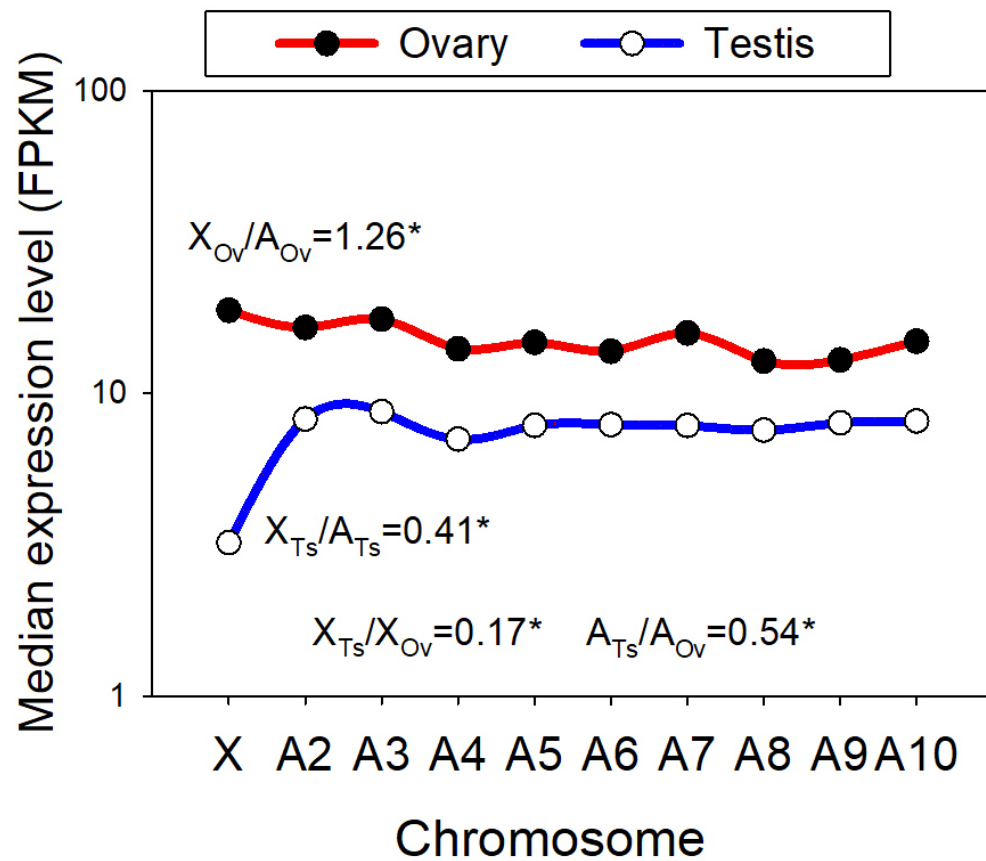
D. dN/dS of GT-soma biased genes (X= X-chromosome, A= autosomes)



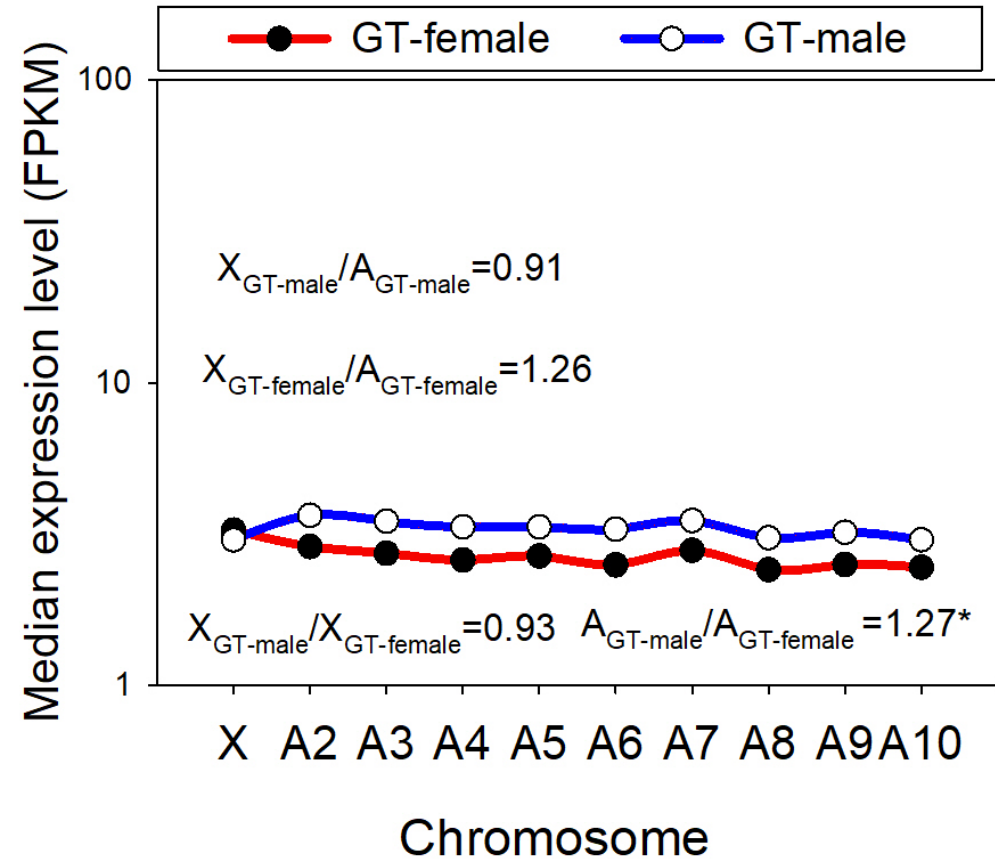
E. Ratio of dN/dS of autosomes to the X-chromosome for gonadally biased genes

F. Ratio of dN/dS of autosomes to the X-chromosome for GT-soma biased genes

Fig. 2. Assessment of the faster-X effect with respect to sex-biased genes in *T. castaneum*. A) The frequency of gonadally sex-biased genes on the X chromosome and nine autosomes for the 7,751 genes under study; B) the frequency for GT-soma sex-biased genes; C) the dN/dS of ovary-biased, testis-biased and unbiased genes on the X-chromosome and autosomes; D) the dN/dS of GT-male biased, GT-female biased, and GT-unbiased genes on the X-chromosome and autosomes; E) the ratio of the median dN/dS of the X chromosome to the autosomes ($X/A_{dN/dS}$) for all three categories of sex-biased expression for the gonads; and F) for the GT-soma. In A, the red and blue asterisks indicate more ovary-biased and fewer testis-biased (or GT-male biased in B) genes were located on the X-chromosomes than on pooled autosomes (χ^2 -P with Yate's correction $P < 0.05$ for each contrast). Different lowercase letters on top of each pair of bars in C and D indicate MWU-test $P < 0.05$. In C-F, unmapped genes were included with autosomal genes and their inclusion in or exclusion from the analysis yielded similar results. Unbiased genes included those with no detectable expression. *Note that differences in X-linked and autosomal unbiased genes in panel D and F are explained by ovary-biased genes (Table S2) as outlined in the main text. After removal of ovary-biased genes $X/A_{dN/dS} = 1.04$ for GT-unbiased genes.



A. Median expression in gonads



B. Median expression in GT-soma

Fig. 3. Median expression in the male and female tissues on each of the ten chromosomes in *T. castaneum* for all genes under study A) Gonads; B) GT-soma. For panel A, the ratio of median expression on the X chromosome (X) and autosomes (A) for testis-biased genes and for ovary-biased genes are shown (X_{Ts}/A_{Ts} and X_{Ov}/A_{Ov}). Also shown are X_{Ts}/X_{Ov} and A_{Ts}/A_{Ov} . Panel B contains the equivalent results for the GT-soma. *Indicates a statistically significant difference between the two groups contained in each ratio using MWU-tests.

ADDITIONAL FILE 1

Absence of a faster-X effect in beetles (*Tribolium*, Coleoptera)

Carrie A. Whittle, Arpita Kulkarni, Cassandra G. Extavour

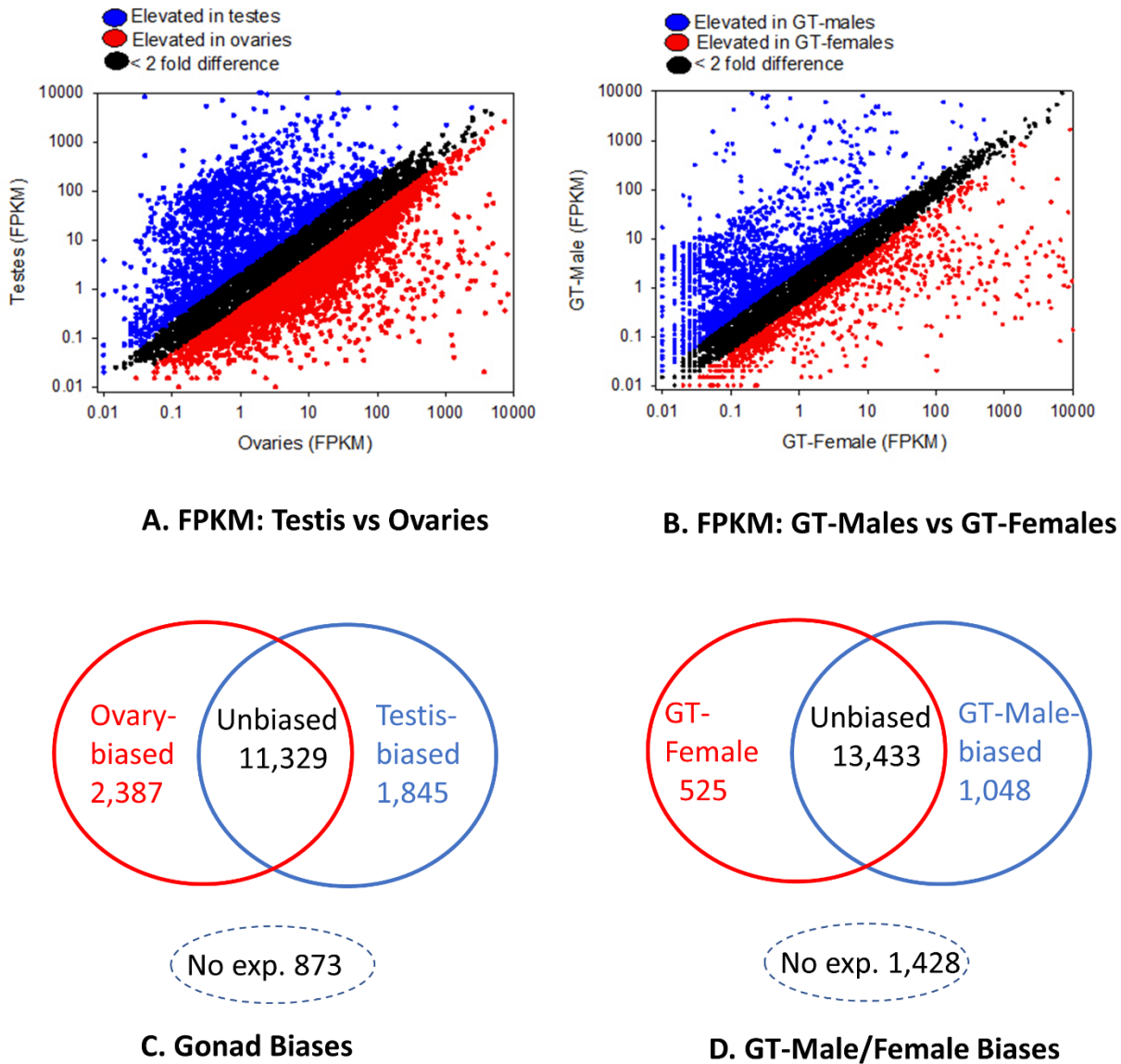


Fig. S1. Sex-biased expression in 16,434 genes of *T. castaneum*. A) Expression level (FPKM) in the testes versus ovaries; B) expression level (FPKM) in GT-males versus GT-females; C) Venn diagram of sex-biased expression the gonads and; D) Venn diagram of sex-biased gene expression in the GT-soma. In A and B, all genes are shown and those with two-fold or greater difference in expression in the male and female tissues are in blue and red respectively (those statistically significant in C and D). Genes with no expression in both tissues were excluded in A and B and are shown in C and D (No exp.).

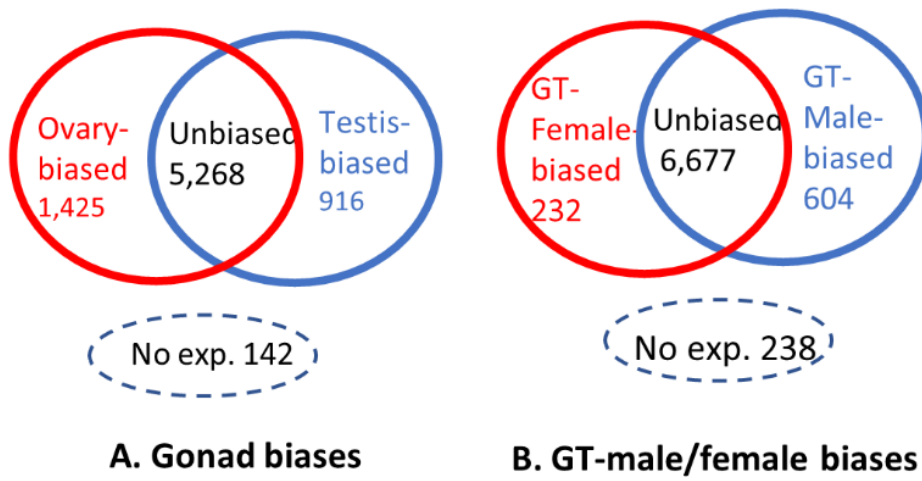


Fig. S2. A Venn diagram showing the number of sex-biased and unbiased genes in the A) gonads; B) GT-soma.

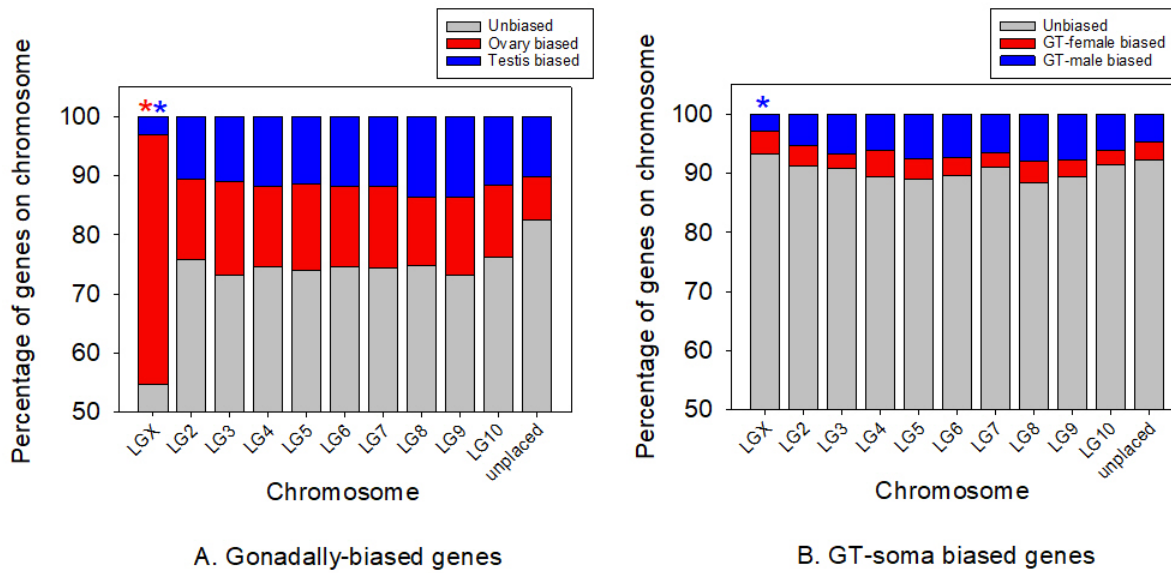
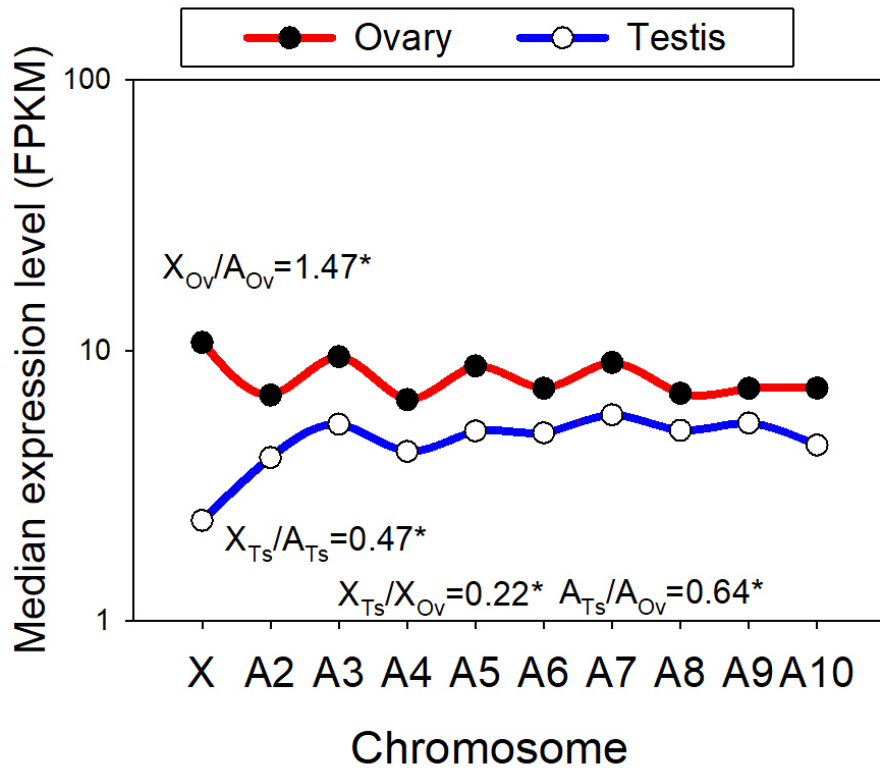
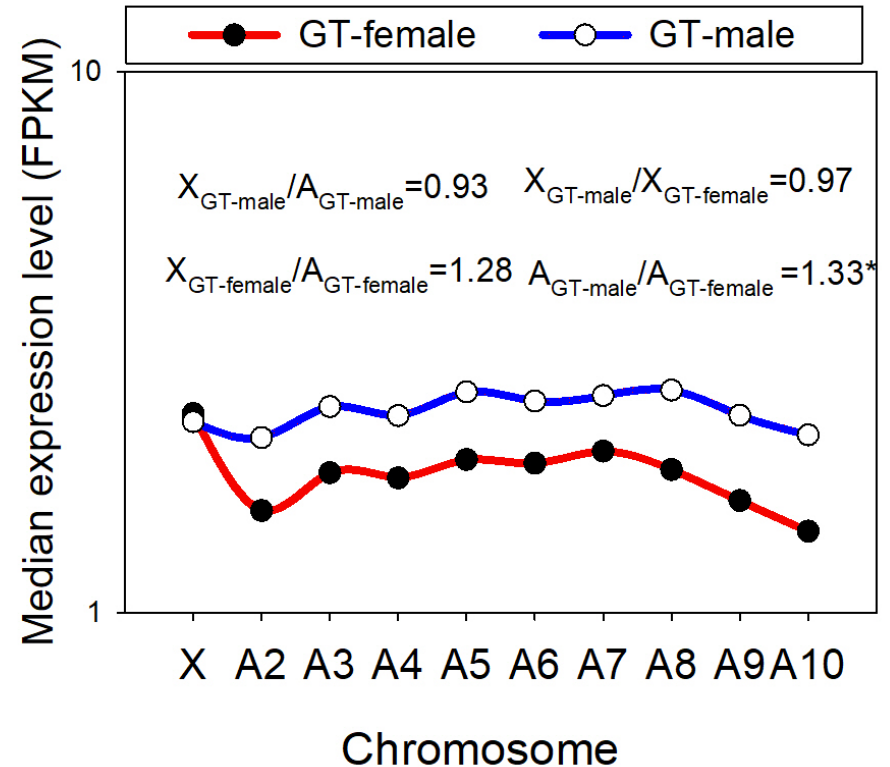


Fig. S3. The frequency of sex-biased genes and unbiased genes on the X chromosome and autosome when all 16,434 genes of *T. castenum* are included in assessment. A) Gonadally biased genes; B) GT-soma biased genes. In A, the red and blue asterisks indicate more ovary-biased and fewer testis- (or fewer GT-male biased in B) biased genes respectively on the X-chromosomes than on pooled autosomes (Chi²-P with Yate's correction P<0.05 for each contrast).



A. Median expression in gonads
(all 16,434 genes)



B. Median expression in GT-soma
(all 16,434 genes)

Fig. S4. Median expression in the male and female tissues on each of the ten chromosomes in *T. castaneum* using all 16,434 genes in the genome. A) Gonads; B) GT-soma. For panel A, the ratio of median expression on the X chromosome (X) and autosomes (A) for testis-biased genes and for ovary-biased genes are shown (X_{TS}/A_{TS} and X_{OV}/A_{OV}). Also shown are X_{TS}/X_{OV} and A_{TS}/A_{OV} . Panel B contains the equivalent results for the GT-soma. Unmapped genes on chromosomes were excluded. *Indicates a statistically significant difference between the two groups contained in each ratio using MWU-tests.

Table S1. RNA-seq data used in present study before and after adapter and quality trimming with BBDuk (<https://jgi.doe.gov/data-and-tools/bbtools/>). The Short Read Archive (SRA) Biosample identifiers are also shown (<https://www.ncbi.nlm.nih.gov/sra>).

Species Sample ^a	No. of Reads		SRA Biosample ID
	Before trimming	After trimming	
<i>Tribolium castaneum</i>			
Testes sample 1	18,006,255	17,995,655	SAMN12702873
Ovary sample 1	39,140,493	39,122,050	SAMN12702874
GT-male sample 1	25,630,261	25,609,723	SAMN12702875
GT-female sample 1	41,513,717	41,472,348	SAMN12702876
Testes sample 2	24,795,583	24,787,238	SAMN12702877
Ovary sample 2	22,306,622	22,286,961	SAMN12702878
GT-male sample 2	62,781,001	62,712,242	SAMN12702879
GT-female 2	52,275,340	52,211,149	SAMN12702880
<i>Tribolium freemani</i>			
Testes sample 1	32,222,092	32,203,312	SAMN12702881
Ovary sample 1	40,395,198	40,368,671	SAMN12702882
GT-male sample 1	32,933,858	32,926,509	SAMN12702883
GT-female sample 1	33,163,960	33,147,066	SAMN12702884

^a Reads were obtained from for two RNA-seq runs of each biological sample.

Table S2. A. The number of studied genes (N=7,751) with sex-biased expression in the gonads and GT-soma. **B.** The degree of overlap in sex-biased status on the X-linked genes between the gonads and GT-soma are also shown.

A

	N values gonads					N values GT-soma			
	Ovary-biased	Testis-biased	Unbiased ^a	Total		GT-female biased	GT-male biased	GT-unbiased ^a	Total
X-linked	233	9	190	432	X-linked	24	12	396	432
Autosomes	1192	907	5220	7319	Autosomes	208	592	6519	7319
				7751					7751

^aIncludes sexually unbiased genes and those with no expression in Fig. S2.

B

Overlap on the X-Chromosome	N overlap in sex-biased status on the X-chromosome
Ovary-biased and GT-female biased	17
Testis-biased and GT-male biased	0
Ovary-biased and GT-unbiased	213
Testis-biased and GT-unbiased	7
GT-male biased and ovary-biased	3

Text File S1. Additional Methods

Lab procedures

The gonads and other elements of the reproductive system were dissected as a single unit in ice cold 1x Phosphate Buffer Saline (PBS) and transferred immediately into TRIzol in a vial kept on dry ice. The reproductive tissues of males included the testes, accessory glands (mesadenia, ectadenia), vesicular seminalis, vas deferens and ejaculatory duct. The reproductive tissues for females included the ovaries, spermathecal gland, common oviduct, spermathecae, and vagina. All remaining nongonadal tissues of the adult body were collected and defined as GT-males and GT-females. Two biological samples per tissue type (testis, ovary, GT-males, GT-females) were collected for RNA-seq for our main target species for study, *T. castaneum* (eight total samples) while one sample per tissue type was obtained for *T. freemani* (four samples). A total of twelve samples were thus obtained for RNA-seq as shown in Table S1.

The testes, ovaries, GT-males and GT-females were stored in separate vials at -80°C until RNA extraction. RNA-isolation was performed according to the Ambion Life Technologies TRIzol Reagent Protocol, following which the RNA was used for RNA library preparation. Polyadenylated mRNAs were selected from total RNA samples using oligo-dT-conjugated magnetic beads on an Apollo324 automated workstation (PrepX PolyA mRNA isolation kit, Takara Bio USA). Entire poly-adenylated RNA samples were immediately converted into stranded Illumina sequencing libraries using 200 base pair (bp) fragmentation and sequential adapter addition on an Apollo324 automated workstation following manufacturer's specifications (PrepX RNA-seq for Illumina Library kit, Takara Bio USA). Libraries were enriched and indexed using 14 cycles of amplification (LongAmp Taq 2x MasterMix, New England BioLabs Inc.) with PCR primers that included a 6bp index sequence to allow for multiplexing (custom oligo order from Integrated DNA Technologies). Excess PCR reagents were removed using magnetic bead-based cleanup on an Apollo324 automated workstation (PCR Clean DX beads, Aline Biosciences). Resulting libraries were assessed using a 2200 TapeStation (Agilent Technologies) and quantified by QPCR (Kapa Biosystems). Libraries were pooled and sequenced on two Illumina NextSeq 500 high output flow cells using single end, 75bp reads.

Extracting CDS from *T. freemani*

To extract gene sequences from scaffolds in this species we used Web Augustus version 3.3.1 (<http://bioinf.uni-greifswald.de/webaugustus/> [79]) that was trained to the *T. castaneum* genome and set at default parameters with the option to identify full length genes. The Augustus-generated CDS list for *T. freemani* was then assessed in ORF predictor, using its downloadable Perl script [80] to identify the highest quality reading frame per sequence. In ORF predictor, we employed the option to include the best-hit (lowest e-value) BLASTX alignment (conducted in BLAST+ v2.7.1, <https://blast.ncbi.nlm.nih.gov>) of *T. freemani* CDS versus the reference *T. castaneum* protein database to define reading frames, an approach which yielded 12,432 uninterrupted sequences of full or partial CDS for *T. freemani*. For further stringency in curating the *T. freemani* CDS list, we pooled the identified CDS with all 138,645,558 *T. freemani* RNA-seq reads (trimmed reads, Table S1) across all four tissue types (testis, ovaries, male carcass, female carcass) and mapped all sequences to the known and annotated CDS from *T. castaneum* using Geneious (v11.0.3), which generated consensus CDS. We then extracted CDS wherein all bases had a minimum of 10X coverage, and these were trimmed to the *T. castaneum* reference CDS. In those *T. freemani* CDS (obtained after ORF predictor) wherein the CDS was improved in quality (contained no unknown or ambiguous nucleotides) or in its length and/or the terminal stop codon was added by using the RNA-seq data, which occurred for N=1,249 CDS, we replaced the original Augustus-based CDS (among the 12,432) with the latter RNA-seq-mapped version CDS. Original CDS that were identified in *T. freemani* (and not found in the 12,432 list) only after using the RNA-seq mapping approach (N=196) were also included in the species final CDS list.