Sex-biased genes expressed in the cricket brain evolve rapidly

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Abstract

Background

Sex-biased gene expression, particularly male-biased expression in the gonad, has often been linked to rapid protein sequence evolution (dN/dS) in animals. This evolutionary trend may arise from one or both of sexual selection pressures during mating or low pleiotropy. In insects, research on sex-biased transcription and dN/dS remains largely focused on a few holometabolous species, with variable findings on male and female gonadal effects. The brain is central to the mating process, and provides neurological foundation for mating behaviors, such as courtship, intrasex competition and mate choice. However, there is a striking paucity of research on sex-biased expression of genes in the brain and the rate of protein sequence evolution in such genes.

Results

Here, we studied sex-biased gene expression in a hemimetabolous insect, the cricket *Gryllus bimaculatus*. We generated novel RNA-seq data for two sexual tissue types, the gonad and somatic reproductive system, and for two core components of the nervous system, the brain and ventral nerve cord. From a genome-wide analysis of genes expressed in these tissues, we report the accelerated evolution of testis-biased genes and seminal fluid proteins (SFPs) genes, as compared to ovary-biased and unbiased genes in this cricket model, which includes an elevated frequency of positive selection events. With respect to the brain, while sex-biased brain genes were much less common than for the gonads, they exhibited exceptionally rapid evolution, an effect that was stronger for the female than for the male brain. Certain sex-biased brain genes were predicted to be involved in mating or sex-related functions, which we suggest may cause exposure to sexual selection. Moreover, the sex-biased brain genes exhibited remarkably low cross-tissue expression breadth, or pleiotropy. We speculate that this feature may permit relaxed purifying selection, and allow the freedom for adaptive protein functional changes in these brain-expressed genes.

Conclusions

Our results demonstrate that sex-biased expression in the male gonad, and sex-biased gene expression in the brain, especially the female brain, are associated with rapid protein sequence evolution in a cricket model system. We discuss the results with respect to our findings on pleiotropy and positive selection, and consider the potential role of the dynamic mating biology of this cricket model in shaping these patterns.

49 Keywords: *Gryllus*, sex-biased expression, dN/dS, pleiotropy, gonad, brain, mating biology

Background

Sexual dimorphism in animals is thought to be driven by differential gene expression, as most genes are common to both sexes [1-3]. Sex-biased gene expression, and particularly male-biased gene expression, has been widely linked to rapid protein sequence evolution in studied animals (reviewed by [1-3]. In the insects, studies have largely focused on the holometabolous insect *Drosophila*, and have repeatedly shown the rapid evolution (high nonsynonymous to synonymous substitution rates, dN/dS) of male-biased genes, particularly those from the male sex cells or gonads, as compared to their female counterparts and/or to sexually unbiased genes [1, 4-11] (but see also [12]). This pattern was also recently observed in red flour beetles (*T. castaneum*) [13]. The rapid divergence of male-biased genes has been proposed to be due to adaptive changes in amino acids arising from sexual selection pressures including male-male and sperm competition [4, 14-16], but could also reflect low pleiotropy that may relax purifying selection [7, 10, 17-19]. Nonetheless, despite a persistent pattern of accelerated evolution of male-biased genes, an opposite pattern of rapid evolution of female-biased, including ovary-biased, genes has been found in some holometabolous insects, namely mosquitoes (*Aedes*, *Anopheles*) [20, 21]. This difference from flies may reflect variation in their mating biology, whereby female-female competition for suitable males or male-mate choice may be more common in mosquitoes than in flies [20]. At present however, given the narrow scope of insects studied to date, further investigation of sex-biased expression and protein evolution is warranted, particularly in models outside the Holometabola.

A major understudied structure outside the reproductive system that is highly relevant in terms of sex-biased gene expression and protein evolution is the brain. The brain comprises a major tissue type providing the neurological basis for the mating behaviors of courtship, intrasex competition, mate-choice, and post-mating male-female responses [22-25]. Sex-biased expression *per se* in the brain has been examined in some insects and vertebrates [3, 5, 22, 23, 25-32]. Further, in *Drosophila*, analyses of a small number of neural genes have been found to be directly connected to mating functions and behaviors [33-35]. However, there is a striking paucity of data on the relationship between sex-biased expression in the brain and protein sequence evolution [22]. Moreover, the minimal research available from birds, humans and flies have suggested different types of male and female effects on rates of protein evolution, depending on the system [23, 29, 32] (see also some brain-related and composite-tissue analyses

81 [36, 37]), and the causes of those patterns remain poorly understood. It is therefore evident that
82 additional study is needed of sex-biased brain expression and its relationship to molecular
83 evolution.

84 An insect model system that offers significant opportunities to address these problems is
85 the cricket system *Gryllus* (Order Orthoptera). *Gryllus* is a hemimetabolous insect, and thus
86 phylogenetically in an outgroup order (Orthoptera) to the Holometabola [38]. The two-spotted
87 cricket *G. bimaculatus* in particular has emerged as a significant insect model in biology,
88 including for genetics, neuroscience and germ line establishment and development [39]. In fact,
89 many of the developmental mechanisms of *G. bimaculatus* appear more typical of arthropods
90 than the widely studied, and relatively derived, model *Drosophila melanogaster* [40, 41].
91 Moreover, many aspects of its mating biology are currently well understood. *G. bimaculatus*
92 exhibits intense male-male and sperm competition, including aggressive male-male fighting and
93 mate guarding [42, 43], increased rates of male transfer of spermatophores to females in the
94 presence of other males [44], and the complete mixing of sperm from multiple males in the
95 storage organ of the female reproductive tract, the spermathecae [45, 46]. In addition, females
96 have shown preferences for novel and young mating partners [47], and for males with larger
97 body size and higher quality auditory signals [47, 48]. Females also exhibit a post-mating
98 behaviour of removing spermatophores of non-favored males from their reproductive tract [46],
99 suggesting a propensity for female-mate choice in this organism. Moreover, in terms of the brain,
100 experiments in *G. bimaculatus* have shown that the brain is directly involved in male mating
101 behaviors such as courtship, copulation, spermatophore protrusion, mating intervals and male-
102 female auditory mating signalling [49-51]. The study of *Gryllus* therefore provides a valuable
103 avenue to advance our knowledge of sex-biased expression in reproductive and brain tissues,
104 including relationships to dN/dS and pleiotropy, in a taxon having well-studied mating biology.

105 Here, we rigorously assess sex-biased gene expression for two tissue types from the
106 reproductive system (gonad and somatic reproductive system) and from the nervous system
107 (brain and ventral nerve cord) and their relationships to protein sequence evolution in *G.*
108 *bimaculatus*. We report that male-biased gene expression in the gonad is linked to rapid protein
109 sequence evolution, including seminal fluid proteins (SFPs) [52], as compared to unbiased and
110 female-biased genes. However, we observed no significant effect of sex-biased expression in the
111 somatic reproductive system (non-germ line tissues) on dN/dS, despite the roles of these sexual

112 tissues in male-female interaction, mating and fertilization, and their potential exposure to sexual
113 selection pressures [4, 53-56]. With respect to the brain, we demonstrate that sex-biased genes
114 are uncommon as compared to the gonad, and that these genes typically evolve very rapidly,
115 especially the female-biased brain genes. Further, sex-biased brain genes are conspicuously
116 linked to predicted sex-related functions. The sex-biased brain genes exhibit especially low
117 cross-tissue expression, or pleiotropy, which may in itself accelerate evolution due to relaxed
118 purifying constraint. We propose that this low pleiotropy may also comprise a mechanism
119 potentially allowing greater freedom for these brain-expressed proteins to evolve adaptive
120 functional changes [13, 17-19, 57, 58]. We consider the putative roles of the male and female
121 mating biology of *G. bimaculatus* in shaping the present finings.
122

123 # Results and Discussion

124 The CDS of our main target species *G. bimaculatus* were obtained from its recently
125 available genome [59]. The annotated genome had a total of 17,714 predicted transcripts per
126 gene (longest CDS per gene; [59]). For this gene set, we extracted the CDS with a start codon, no
127 ambiguous nucleotides, and at least 150bp in length. The final list for the present study included
128 a total of 15,539 CDS (mean length=417.0 codons/CDS ±3.5 (standard error)) for *G.*
129 *bimaculatus*. For analysis of sex-biased gene expression in *G. bimaculatus* we isolated and
130 obtained RNA-seq data for four paired male and female tissue types from adult virgins
131 (Additional file 1: Table S1). The tissues included the gonad (testis for males, ovaries for
132 females), somatic reproductive system, brain and ventral nerve cord (Fig. 1A-F, Additional file
133 1: Table S1, for read counts). The somatic (non-germ line related) reproductive system herein for
134 males include the pooled vasa deferentia, seminal vesicles and ejaculatory duct, and for females
135 include the spermatheca, common and lateral oviducts, and bursa (Fig. 1A,B; note that a ninth,
136 unpaired, tissue type, the male accessory glands was also isolated and was used in pleiotropy
137 analysis and its own supplementary dN/dS analysis (Fig. 1G), as detailed in Methods). For each
138 tissue, reads were mapped to the entire *G. bimaculatus* CDS list to determine FPKM. We found
139 FPKM was strongly correlated between biological replicates, with Spearman's $R \geq 0.92$ ($P<0.05$;
140 Additional file 1: Fig. S1; one exception being the male somatic reproductive system, $R=0.71$,
141 $P<0.05$), indicating high reproducibility of results between biological replicates. Sex-biased gene

142 expression was then determined separately for each of the four paired tissue types using a cut-off
143 of two-fold higher expression in one sex versus the other and P<0.05 (see Methods).
144 As shown in Fig. 2, we found that that sex-biased gene expression was most common in
145 the gonadal tissues, where 4,822 (31.1%) of all *G. bimaculatus* genes under study were sex-
146 biased in expression: 2,698 (17.4%) and 2,124 (13.7%) genes had ovary-biased and testis-biased
147 expression respectively, and a total of 10,717 (69.0%) were unbiased in expression. By
148 comparison, sex-biased gene expression was markedly less common in the somatic reproductive
149 system, where only 5.6% of genes were sex-biased, with 353 (2.3%) and 520 (3.3%) genes
150 showing female- and male-bias respectively. As compared to the gonad, markedly fewer genes
151 exhibited female-biased and male-biased expression in the neural tissues, where 4.5% of 15,539
152 *G. bimaculatus* genes had sex-biased expression in the ventral nerve cord: 279 (1.8%) and 425
153 (2.7%) were female- and male-biased respectively (Fig. 2). For the brain, only 1.0% of genes
154 were sex-biased in expression, with 51 (0.33%) and 106 (0.68%) being female- and male-biased
155 respectively. Together, using the present criteria, it is evident that sex-biased gene expression is
156 most common in the gonad, which is consistent with high phenotypic and transcriptional
157 dimorphism of these sex organs in animals [8, 13, 14, 20, 26, 60-64]. In contrast, sex-biased gene
158 expression is markedly less common in the somatic reproductive system and ventral nerve cord,
159 and least common in the brain of *G. bimaculatus*.
160
161 **Molecular evolution of sex-biased genes**
162 We aimed to assess whether and how protein sequence divergence, measured as
163 synonymous to nonsynonymous substitutions, or dN/dS, varied with sex-biased gene expression.
164 Unlike *Drosophila, Gryllus* is currently an emerging model genus with few genomic resources
165 outside the aforementioned recent *G. bimaculatus* genome [59]. Thus, to measure dN/dS, we
166 generated and assembled novel RNA-seq data for its sister species *G. assimilis* to obtain a CDS
167 list for that organism (Additional file 1: Table S2). Two-species assessments of dN/dS have been
168 repeatedly shown to be an effective means to study divergence of sex-biased genes (*cf.* [8, 19,
169 20, 23, 65, 66] including for organisms with few available genomes, as is the case with *Gryllu*s.
170 Details of the *G. assimilis* assembly, including BUSCO scores (Seppey, Manni et al. 2019), and
171 ORF predictions [67] are provided in Additional file 1: Text File S1. Following reciprocal
172 BLASTX [68] between *G. bimaculatus* and *G. assimilis* CDS and retention of genes with

173 unsaturated dN and dS values (<1.5) after alignment, we identified 7,220 high confidence *G.*
174 *bimaculatus*-*G. assimilis* orthologs that were used for all dN/dS analyses. Across all 7,220
175 orthologs under study, we found that the alignments with gaps removed were on average 68.0%
176 (standard error=0.3) of the original *G. bimaculatus* CDS length (that pre-alignment had an
177 average length of 577.0 codons, standard error=5.9 for the subset of its genes with between-
178 species orthologs), and that the median dN/dS was 0.1152. The median dN was 0.0042 and
179 median dS was 0.0396, values that were substantially <1, consistent with a close phylogenetic
180 relatedness between these two sister *Gryllus* species from the same genus.
181 To precisely reveal the relationship between sex-biased gene expression for each
182 individual tissue type and dN/dS, we identified genes that were sex-biased in expression in only
183 one of four of the female-male paired tissues (gonad, somatic reproductive system, brain or
184 ventral nerve cord) and unbiased in all three remaining tissues in *G. bimaculatus*. These genes
185 are hereafter denoted as tissue-specific sex biased, or TSSB genes (N_{TSSB} values provided in
186 Additional file 1: Table S3). We also identified those genes with universally unbiased expression
187 in all four tissues types as a control (N=3,449; Additional file 1: Table S3). The vast majority of
188 the 7,220 genes (with orthologs in both species) fell into one of these two categories (94.5% of
189 7,220 genes had TSSB or universally unbiased status).
190
191 ***dN/dS of sex-biased genes in the four tissue types and pleiotropy***
192 The dN/dS values of sex-biased$_{TTSB}$ genes for the four core *Gryllus* tissue types under
193 study, and for universally unbiased genes, are shown in Fig. 3A. In turn, for completeness, the
194 dN/dS values of all sex-biased genes per tissue, regardless of status in other tissues (sex-
195 biased$_{ALL}$), are shown in Fig. 3B. The results show that in this cricket model, testis-biased $_{TTSB}$
196 genes evolved faster than ovary-biased $_{TTSB}$ and universally unbiased genes (MWU-tests
197 P<0.05). Further, sex-biased brain genes, while uncommon (Fig. 2), evolved exceptionally
198 rapidly, particularly with faster evolution of the female-biased$_{ALL}$ brain genes than of
199 unbiased$_{ALL}$ genes (MWU-test P<0.05, Fig. 3B, for details see below section "*Rapid evolution of*
200 *sex-biased brain genes*"). In turn, no differences in dN/dS of male-biased, female-biased, or
201 unbiased genes from the somatic reproductive system or ventral nerve cords were observed
202 (using TSSB and universally unbiased genes (Fig. 2A) or ALL genes per tissue type (Fig. 3B,
203 MWU-tests P>0.05). In this regard, it is evident that sex-biased expression in this cricket system

204 is associated with rapid evolution of testis-biased genes and of sex-biased brain genes,
205 particularly those with biased expression in the female brain.
206 We next assessed the expression breadth across tissues (using nine tissues, the eight
207 paired tissues and the male accessory glands, see Methods), as a proxy for pleiotropy, or
208 multifunctionality of a gene, which is thought to strengthen purifying selection and in turn
209 restrict adaptive evolutionary potential [8, 17-20, 57, 58, 69]. Genes were categorized into genes
210 expressed at >5FPKM in 1-2, 3-4,5-6,7-9 tissues. As shown in Fig. 4A, when studying all 7,220
211 genes with high confidence orthologs, we found that the rate of evolution of *Gryllus* genes was
212 strongly inversely correlated with pleiotropy. The slowest rate of evolution was found in genes
213 transcribed in all tissues under study (median dN/dS=0.161), and the fastest rate in genes
214 expressed in one to two tissues (median=0.221, Ranked ANOVA and Dunn's paired contrasts
215 P<0.05). Further, as indicated in Fig. 4B, with respect to sex-biased gene expression, we found
216 that testis-biased genes had markedly lower expression breadth than ovary-biased genes and than
217 universally unbiased genes (MWU-tests P<0.05), while female-biased brain genes had the
218 smallest median expression breadth of all studied categories, with statistically significantly lower
219 values than universally unbiased genes (MWU-test P<0.05). Thus, this suggests a plausible
220 connection between rapid protein evolution of sex-biased genes in the brain and their narrow
221 pleiotropy, either due to relaxed constraint in itself, and/or due to an associated freedom to
222 evolve functional changes under low purifying constraint [8, 17-20, 57, 58, 69]. In sum, the rapid
223 evolution of testis-biased genes (as compared to ovary-biased and unbiased genes) and of sex-
224 biased brain genes may be at least partly be a result of their pleiotropy, and in turn, potential
225 adaptive changes (see evidence in agreement with a pleiotropy-adaptive evolution relationship in
226 the below section "***Evidence of A History of Positive Selection in Sex-Biased Gonadal and***
227 ***Brain Genes"***). In the following sections, we describe in greater detail the sex-biased genes in
228 the brain and in the reproductive system in Fig. 3 and Fig. 4, and consider the putative roles of
229 pleiotropy and positive selection in affecting their molecular evolution.
230
231 **Rapid evolution of sex-biased genes from the brain**
232 With respect to the brain, female-biased$_{TSSB}$ genes had markedly higher median dN/dS
233 values (median=0.295) than male-biased$_{TSSB}$ genes (0.203, Fig. 3A), although that contrast was
234 not statistically significant (MWU-test P=0.739). This may reflect the low statistical power of

235 this comparison due to the rarity of genes with brain specific sex-biases in expression (sex-
236 biased$_{TSSB}$, Additional file 1: Table S3). However, when studying all genes with sex-biased$_{ALL}$
237 expression in the brain, regardless of their expression status in other tissues (Fig. 3B), we found
238 that the 20 female-biased$_{ALL}$ brain genes had substantially higher median dN/dS values (median=
239 0.245) than the 45 male-biased$_{ALL}$ (0.169) and the 7,155 unbiased$_{ALL}$ brain-expressed genes
240 (0.115), wherein its contrast to the unbiased set was statistically significant (MWU-test
241 P=0.047). The dN/dS of every sex-biased brain$_{ALL}$ gene is shown in Table 1. 11 of the 20
242 female-biased$_{ALL}$ brain genes (Fig. 3B) and 19 of 45 male-biased$_{ALL}$ brain genes had dN/dS
243 values more than twice as high (>0.236) as the median observed for universally unbiased genes
244 (median=0.118, Fig. 3A). This suggests that these genes share a strong propensity to evolve
245 rapidly, with the effect being greatest in the female brain (Fig. 3A, Table 1). While the study of
246 protein evolution of sex-biased brain genes (brains *sensu stricto*, rather than simply heads, or
247 pooled brain-eye tissues as considered by previous studies [36, 37]) remains rare, rapid evolution
248 of female-biased brain genes has been reported in some bird embryos [23], and in some
249 autosomal genes in flies [32]. However, an opposite pattern of rapid evolution of male-biased
250 brain genes for several stages of development was reported in humans [29]. The avian result was
251 interpreted as possibly reflecting selective pressures arising from brain-regulated mating
252 behaviors [23]. We suggest that this may also be a main factor contributing to the trend of rapid
253 evolution of sex-biased brain genes here for crickets.

254 With respect to the ventral nerve cord, while more sex-biased$_{TSSB}$ genes were observed
255 than for the brain (greater than seven-fold higher, Additional file 1: Table S3), not even mild
256 differences in dN/dS were observed as a group between male-biased$_{TSSB}$ and female-biased$_{TSSB}$
257 genes (Medians= 0.160 and 0.162 respectively, MWU-test P=0.628, Fig. 3). In sum, the results
258 in Fig. 3 indicate that the main effect of sex-biased expression in the nervous system is the rapid
259 divergence of male- and female-biased$_{ALL}$ brain genes, with particularly rapid evolution in the
260 female-biased group.

261 As shown in Table 1, we examined individual GO functions for each female-biased$_{ALL}$
262 and male-biased$_{ALL}$ brain gene (Fig. 3B). including the subset that had TSSB status (Fig. 3A,
263 Additional file 1: Additional file 1: Table S3; any brain genes that had the same sex-biased
264 expression status in the gonad are also shown as gonad sex bias "GSB"). For this, we used
265 single-direction BLASTX [68] of the *G. bimaculatus* genome to the well-studied insect model *D.*

266 *melanogaster* [70] to identify its putative orthologs to be used for functional analysis in the tool
267 DAVID [71] (note that we chose single direction BLASTX for functional analysis, rather than
268 the reciprocal BLASTX approach that was used for *G. bimaculatus*-*G. assimilis* contrasts for
269 dN/dS, as we considered the latter overly conservative for predictive functional analysis, as it
270 might impede detection of putative paralogs to a gene in crickets; see Methods). We found that
271 the predicted functions of female-biased brain genes included involvement in neurotransmission
272 (*AP-1-2beta*), sensory organ development (*crinkled*), apoptosis (*D. melanogaster CG2681*), and
273 DNA binding (*CG11403*) (Table 1). Remarkably, certain brain expressed genes were involved in
274 sexual processes, including multicellular reproduction (*CG10407*), inter-male aggressive
275 behavior (*tramtrack*) and included an ejaculatory bulb protein (*EbpIII*) (Table 1). Each of these
276 genes had exceptionally elevated dN/dS values of 0.460, 0.384 and 0.244 respectively (Table 1),
277 as compared to the median for universally unbiased genes (median=0.118, Fig. 3A). The fastest
278 evolving female-biased brain gene (dN/dS=0.970) was a putative ortholog of *kekkon-3*, a
279 member of a *kekkon* gene family known to be involved in neuron function and differentiation of
280 the central nervous system in flies [72], and conserved in files and mosquitoes [73]. The rapid
281 evolution of numerous female-biased brain genes in Table 1, combined with their low pleiotropy
282 (Fig. 4), and a propensity for sex-related functions (Table 1) suggest the possibility that they may
283 share a common history of relaxed selection and/or adaptive evolution due to sexual selection
284 pressures in the cricket clade.

285 Nonetheless, not every female-biased$_{TSSB}$ *G. bimaculatus* brain gene evolved rapidly
286 (Table 1). For instance, one highly constrained gene (GBI_02686-RA, (dN/dS=0 (dN=0
287 dS=0.041)) was a likely ortholog match to *D. melanogaster crinkled*, which is involved in
288 hearing (vibration sensing) in both flies and vertebrates [74, 75]. We speculate that a history of
289 strong constraint on dN/dS of this female-biased$_{TSSB}$ brain gene could reflect an essential role of
290 negative phonotaxis (potentially relevant to avoiding predators [76]), perhaps an effect enhanced
291 in females. However, the sex-biased expression of this putative *crinkled* gene may also suggest it
292 has a sexual role. A fundamental factor underlying male-female attraction in *G. bimaculatus* is
293 song, which is used by males to attract females (positive phonotaxis), and is thought to be
294 regulated by the auditory neural pathways involving the brain [50, 77]. Thus, it is tempting to
295 speculate that the strong purifying selection on this female biased $_{TSSB}$ gene could reflect an
296 important role in receiving male auditory signals for courtship and mating. Further studies in

297 crickets should assess sex-biased gene expression in the brain of males and females from mixed
298 mating populations (virgin males and females were studied herein, see Methods) to identify
299 brain-related auditory genes potentially involved in mating. Additional valuable directions could
300 include study of sex-biased expression in the male and female auditory organs located on the
301 tibia of the forelegs in crickets [76, 77], in the antennae, which are involved in male-female
302 attraction and male-male aggression and contain neurons involved in sex-related pheromonal
303 signalling [74, 78, 79], and in the terminal abdominal ganglion, which has been linked to mating
304 behaviors [50]. Such studies will help further identify and evaluate the evolutionary rates of
305 brain and neural genes linked to mating and sex-related auditory and pheromonal signalling in
306 this taxon.

307 With regard to the male-biased$_{ALL}$ brain genes, a range of predicted functions were
308 observed. For instance, multiple genes were involved in phagocytosis (six of 45 genes), and early
309 embryo development (three genes). In addition, some genes had predicted sexual roles. In
310 particular, a putative *G. bimaculatus* ortholog (GBI_17358-RA) to a *D. melanogaster* ejaculatory
311 bulb protein *EbpIII* had a dN/dS value of 0.449, which was nearly four-fold higher than the
312 median for universally unbiased genes (0.118, Table 1). This same *EbpIII* related gene
313 (GBI_17358-RA) was also found to be testis-biased in expression (Table 1), which is consistent
314 with a putative significant role in testicular function in *G. bimaculatus*. As described above, a
315 different *G. bimaculatus* gene (GBI_17348-RA) that was also an ortholog match to *D.*
316 *melanogaster EbpIII* was sex-biased in the female-brain (dN/dS=0.243, Table 1), suggesting the
317 possibility that there are two distinct paralogs to this gene, which may have different roles in
318 male and female brains in crickets (note that as the *G. bimaculatus* to *D. melanogaster* BLASTX
319 used for functional analysis was one-directional, and thus more than one cricket gene could
320 match a single *D. melanogaster* gene, see Methods). These two genes matching *EbpIII*, one
321 biased in the male-brain and the other in the female brain, are candidates to be involved in male-
322 female attraction, mating or sexual behaviors. In *D. melanogaster,* while the exact functions of
323 *EbpIII*u remains largely unknown, its key predictive classifications include olfactory function,
324 post-mating behavior, and mating plugs (flybase.org, [70]); further suggesting a possible
325 function in male-female brain mediated sexual behaviors in *G bimaculatus*. We also discovered
326 that the male-biased$_{ALL}$ brain genes included a putative ortholog of *Angiotensin converting*
327 *enzyme*, a gene involved in *D. melanogaster* spermatid nucleus differentiation and sperm

328 individualization. This gene had a dN/dS value of 0.236, which is double the median of
329 universally unbiased genes (Table 1). In this regard, multiple male-biased brain genes exhibit
330 rapid divergence and thus are candidates to have potential sex-related roles in this taxon.
331 While the rapid evolution of sex-biased brain genes in Table 1 could partly result from
332 relaxed purifying constraint and neutral protein sequence changes, as suggested by their low
333 pleiotropy (Fig. 4), the low pleiotropy could in principle also act to accelerate protein changes by
334 more readily allowing adaptive functional changes [8, 17-20, 57, 58, 69]. We suggest here that
335 several features of the mating biology of *G. bimaculatus* might cause episodic adaptive evolution
336 and underlie the high dN/dS values observed herein (see also below section "***Evidence of A***
337 ***History of Positive Selection in Sex-Biased Gonadal and Brain Genes***"). For instance, *G.*
338 *bimaculatus* exhibits aggressive male-male fighting and mate guarding [42, 43] and males
339 transfer larger spermatophores to females when in the company of rival males [44]. Such
340 behaviors are likely mediated by the male brain. This could, in principle, lead to sexual selection
341 pressures on the male-biased brain genes shown in Table 1, which might give rise to adaptive
342 changes in dN/dS. It is also feasible that inter-locus sexual conflict could contribute to rapid
343 evolution of both sets of male- and female-biased brain genes [80-82]. In other words, it is
344 possible that aggressive male-male behaviors in *G. bimaculatus* [42, 43], directed by male-biased
345 brain genes, may negatively affect female fitness. This might be predicted to lead to an adaptive
346 response in female-biased brain genes (e.g., genes regulating the behavior of removal of
347 spermatophores of certain males by females after mating [48]), causing an evolutionary "arms
348 race" that could in principle accelerate evolution of proteins of both types of genes [1, 81]. Taken
349 together, we suggest that there are several plausible mechanisms related to mating biology of this
350 taxon that may underlie the observed patterns for sex-biased brain genes (Table 1), mediated by
351 low pleiotropy and an enhanced potential for adaptive evolution.
352
353 **Rates of Evolution of Sex-biased Genes from the Reproductive System**
354
355 ***Rapid evolution of testis-biased genes***
356 For the gonads, as shown in Fig. 3A, we found marked differences in dN/dS among sex-
357 biased$_{TSSB}$ genes. First, dN/dS decreased progressively from testis-biased$_{TSSB}$ (median=0.128), to
358 universally unbiased genes (median=0.118) to ovary-biased genes (median=0.097, each paired

359 MWU-test P<0.05; see also Fig. 3B). Thus, the rate differences were most marked between
360 testis-biased$_{TSSB}$ and ovary-biased$_{TSSB}$ genes, with intermediate values for those with universally
361 unbiased expression.

362 While rapid evolution of whole male- or of testis-related genes versus their female
363 counterparts may have become a largely accepted paradigm for studied animals [1, 2, 83], in
364 insects this notion has been almost entirely based on repeated studies in *Drosophila* [1, 2, 6-8,
365 11, 14, 15, 19, 61]. Thus, it is worthwhile to consider the present gonadal result from *Gryllus* in
366 the context of the comparable data available from its fellow insects. For example, the pattern in
367 *Gryllus* (Fig. 3) is consistent with observations of rapid evolution of testis-biased and slow
368 evolution of ovary-biased genes in *Drosophila* [7, 11, 14, 61] (see also results in a related fly,
369 [37]). In addition, this pattern also matches our recent results from beetles (*Tribolium*
370 *castaneum*) that showed rapid evolution of testis-biased genes [13], a taxon which like
371 *Drosophila* species' is polyandrous, and has evidence of pre- and post-mating female choice
372 mechanisms [84]. These collective results in crickets, fruit flies and beetles, however are
373 opposite to the rapid evolution of ovary-biased (or ovary-specific) genes that we previously
374 reported in the mosquitoes *Aedes* and *Anopheles* [20, 21], and thus the former pattern is not
375 universal to insects.

376 Given that *Gryllus* (Orthoptera) is a distant outgroup to the two Diptera groups
377 (*Drosophila* and *Aedes/Anopheles*) and the Coleoptera (*Tribolium*) [38] it may be suggested,
378 based on the collective anecdotal evidence, that there could be a shared ancestral effect of testis-
379 biased expression in *Drosophila-Tribolium-Gryllus* [1, 13, 14, 60]) and a derived effect of rapid
380 evolution of ovary-biased genes in *Aedes/Anopheles* [20, 21]. Under this hypothesis, the pattern
381 observed for studied *Aedes* and *Anopheles* species would be a derived feature, and could reflect
382 variation in mating biology among these insects. As an example, although like *Drosophila,*
383 *Aedes aegypti* (the species studied in *Aedes* [20] is polyandrous and thus prone to sperm
384 competition, the polyandry is thought to be relatively weak [85]. Further, this mosquito can
385 exhibit intensive male swarming during courtship that may involve female-female mosquito
386 competition and/or male-mate choice [20, 86]. In addition, nonporous mating plugs are formed in
387 the female mosquito reproductive tract after mating, which prevent sperm competition [86] and
388 thus differ both from the mating plugs formed in *Drosophila*, which allows sperm transfer from
389 competitor males [87, 88], and from observations of complete sperm mixing from multiple males

390 in *Gryllus* [46]. Any of these mating-related features could in principle underlie the relatively
391 faster evolution of ovary-biased than testis-biased genes in mosquitoes [20], and not in the other
392 studied insects. Studies in even more insect models, particularly in monogamous versus
393 polyandrous species [60], and in additional insects with various degrees of male-male or female-
394 female competition and with and without impermeable mating plugs [20], would help elucidate
395 whether and how and why the effects of sex-biased transcription on protein evolution vary
396 among insects.

397 Functional predictions of testis-biased$_{TSSB}$ and ovary-biased$_{TSSB}$ genes are shown in Table
398 2 (using *D. melanogaster* orthologs and GO clustering). Testis-biased$_{TSSB}$ genes were predicted
399 to be preferentially involved in cilium functions, potentially reflecting roles in sperm motility
400 [89]. Ovary-biased$_{TSSB}$ genes were particularly involved in fundamental processes such as
401 transcription and protein synthesis functions. Thus, the former may be linked to specialized
402 functions of the male gonad, and sperm functionality, while the latter may include genes
403 involved in broader functions in addition to their roles in the female gonad. In terms of GO
404 functions of the universally unbiased genes, these genes were preferentially involved in core
405 cellular and nuclear functions including protein structure (coiled coil), nucleotide binding and
406 splicing (Table S4), differing from more specialized functions of testis-biased genes.

407 It is worth mentioning that in Fig. 3A, while testis-biased$_{TSSB}$ genes had higher dN/dS
408 values than ovary-biased$_{TSSB}$ genes and than the universally unbiased genes, they did not exhibit
409 any statistically significant differences with respect to the less common male-biased genes from
410 the three other tissues, including from the brain (MWU-test $P>0.05$). Significantly, however,
411 given the much greater abundance of testis-biased$_{TSSB}$ genes than male-biased$_{TSSB}$ genes from
412 other tissues (8- to 65- fold more common, Fig. 2, Additional file 1: Table S3, Fig. 3A), it may
413 be inferred that testis-biased gene expression plays a substantial role in shaping the portion of the
414 genome that is fast evolving in *G. bimaculatus* (as compared to sex-biased genes from other
415 tissues).

416

417 ***Sex-biased gonadal expression in G. assimilis***

418 While our main target for expression analyses was *G. bimaculatus*, and *G. assimilis* was
419 used primarily as a reference point to measure rates of protein divergence, we considered the
420 degree of conservation of gene expression between the two species for the 7,220 genes with

421 orthologs for the gonads (which had the largest N values of all tissues, Additional file 1: Table
422 S3). The results are shown in Additional file 1: Fig. S2 and are described in Additional file 1:
423 Text File S1. We observed that the finding of elevated dN/dS of testis-biased versus ovary-
424 biased genes was robust to whether the sex-biased status (testis-biased, ovary-biased) was
425 observed in one species or was conserved both of these species. Thus, testis-biased expression in
426 one species (i.e., *G. bimaculatus*, Additional file 1: Fig. S2) is sufficient to predict elevated
427 pairwise dN/dS.
428
429 ***Lack of different rates of evolution in sex-biased genes from the somatic reproductive system***
430 In contrast to the gonad, for the sex-biased$_{TSSB}$ genes from the somatic reproductive
431 system (N values in Additional file 1: Table S3), no statistically significant differences were
432 observed in dN/dS of male-biased$_{TSSB}$ and female-biased $_{TSSB}$ genes, nor between those groups
433 and the universally unbiased genes (MWU-tests P>0.05, Fig. 3A). This result may be considered
434 surprising, given the roles of these sexual tissues in reproductive success and fitness, particularly
435 for the female tissues (common oviduct, spermathecae, and vagina).
436 Few comparable insect data of sex-biases in somatic reproductive system tissues are
437 available. Some specific genes involved in the female reproductive tract in *Drosophila* have been
438 linked to rapid and/or adaptive evolution, which may be due to their dynamic roles in receiving
439 and maintaining sperm after mating [54, 55]. However, a separate assessment of genes broadly
440 defined as female reproductive tract proteins in *D. melanogaster* (based on expression data from
441 mixed or mated flies) showed those genes exhibited slow protein evolution (dN/dS), below the
442 genome-wide average [4]. Thus, our results from *Gryllus* (unmated) showing no differences in
443 dN/dS between female-biased $_{TTSB}$ somatic reproductive system genes and the universally
444 unbiased genes or the genome as a whole (Fig. 3), differs from those findings in *Drosophila*
445 (note: see below section "*Positive Selection Tests in the Sex-biased Gonadal and Brain Genes*"
446 which suggests a small number of female somatic reproductive system genes evolve adaptively)
447 [4]. Significantly, it is evident that the lack of effect of sex-biased transcription in the somatic
448 reproductive system stands in contrast to the substantial effects observed for the gonad (Fig. 3).
449
450 ***Rapid divergence of genes from the male accessory glands and seminal fluid proteins***

451 As a supplementary analysis with respect to the reproductive structures, given that sex-
452 biased genes from the male-accessory glands, including seminal fluid protein (SFPs), have been
453 strongly linked to rapid evolution in species of *Drosophila*, we assessed the evolutionary rates of
454 these genes in *Gryllus.* The results are described in detail in Additional file 1: Text File 1, Table
455 S5 and Table S6. In brief, we show that *G. bimaculatus* genes with expression specific to the
456 male accessory glands (0 FPKM in all eight other tissues studied here) had few orthologs
457 detected (well below the genome-wide average of ortholog detection) in its sister species *G.*
458 *assimilis*, and in *D. melanogaster*, suggesting a history of rapid evolution potentially so extensive
459 that it prevents protein similarity detection by these methods, and/or a history of lineage-specific
460 gene losses or gains of genes involved in this sexual tissue [4, 90].
461 Further, for SFPs, we used the recently available list of 134 SFPs for the species *D.*
462 *melanogaster* (shown in Additional file 1: Table S6, [52]) as a reference, and found that only 20
463 SFP genes had identifiable putative orthologs in *G. bimaculatus* (14.9%). Only seven of those
464 were included among the subset of 7,220 genes with between-species orthologs in *Gryllus*. The
465 dN/dS values of these seven genes are shown in Table 3; all were above the genome-wide
466 median dN/dS value (0.115, Fig 2A). Positive selection was indicated for the odorant binding
467 SFP protein *Obp56g*, with dN/dS>1 (Table 3). Together, we conclude that the putative SFPs in
468 the crickets studied here have evolved very rapidly, a feature shared with SFPs of *D.*
469 *melanogaster* [4, 52], and that could be due to their potential subjection to sex-related selection
470 pressures. For instance, in flies SFPs may enhance sperm competitive ability in the female
471 reproductive tract or egg release from the ovary [91, 92], and males may alter relative production
472 of different SFPs when exposed to male rivals [91]. If similar types of mechanisms of sexual
473 selection exist in crickets, then they could contribute to fast evolution of SFP genes. Another
474 potentially significant behavioural factor in *G. bimaculatus*, is the tendency of females to
475 preferentially retain deposited spermatophores of certain (larger) males [46, 48], which
476 comprises a mechanism of female-choice in this species [48]. This behaviour might lead to
477 sexual selection pressures on SFPs contained in those spermatophores, and accelerate their
478 evolution.
479
480 **Evidence of A History of Positive Selection in Sex-Biased Gonadal and Brain Genes**

481 Finally, we considered the incidences of positive selection among those genes with
482 between-species *Gryllus* orthologs. The use of dN/dS>1 is a conservative means to assess
483 adaptive evolution, as positive selection must be frequent enough across all codon sites to be
484 detected. We found that 1.63 % of all the 7,220 *G. bimaculatus*-*G. assimilis* gene orthologs
485 (N=118 genes) showed dN/dS>1 (retaining only genes where both dN and dS >0).
486 We then considered whether dN/dS values of the sex-biased gonad$_{TTSB}$ genes, which had
487 the highest N values of all tissues analysed (Additional file 1: Table S3), were consistent with the
488 aforementioned hypothesis that reduced gene pleiotropy, or expression breadth (and thus
489 purifying selection), may enhance a gene's functional evolvability [8, 13, 17, 19, 57, 58, 69]. We
490 found that the percent of genes with positive selection (dN/dS>1) increased from ovary-
491 biased$_{TTSB}$ genes (1.02%, 19 of 1,858) to universally unbiased genes (1.91%, 66 of 3,449) and
492 testis-biased$_{TTSB}$ genes (2.09%, 22 of 1,055; Chi^2 P with Yates' correction was <0.05 for each
493 paired contrast to ovary-biased$_{TSSB}$ genes, Table 4). In turn, we assessed gene pleiotropy for each
494 group. Expression breadth of genes decreased from all ovary-biased$_{TTSB}$ (average expression
495 breadth of 7.97±0.04 (standard error)), to universally unbiased (6.95±0.05) and to testis-
496 biased$_{TTSB}$ genes (5.90±0.18 tissues; (MWU-tests P<0.001 for each of three paired contrasts).
497 Strikingly, the differences were even more magnified in the subset of genes with dN/dS>1 shown
498 in Table 4, with markedly higher average expression breadth (2.5 fold) for ovary-biased$_{TSSB}$
499 (6.74±0.74) than for testis-biased$_{TSSB}$ (2.73±0.72) genes (Chi^2 P<0.05, Table 4). Crucially, these
500 patterns observed using whole-gene dN/dS values in this cricket system provide empirical
501 support for the theoretical proposition that that the fewer tissues a gene is expressed in, the more
502 its adaptive evolutionary potential may be enhanced, likely by reducing putative constraint
503 imposed by multiple cross-tissue functions [8, 17, 57, 58, 69]. Our data thus specifically show
504 that this hypothesis can apply to sex-biased genes [17].
505 We further assessed whether there was evidence of positive selection for sex-biased brain
506 genes, which were much less common that those from the gonad (Additional file 1: Table S3,
507 Fig. 2). The only gene with whole-gene dN/dS >1 (=3.675, GBI_19557-RB, Table 1) was of
508 unknown function and specifically expressed in the male brain (expression breadth=1 tissue).
509 Thus, this result is also concordant with adaptive evolution facilitated by low pleiotropy. The
510 female-biased brain gene with the highest dN/dS of 0.9735 matched *D. melanogaster kekkon3*.
511 This value near one could suggest a history of neutral evolution, but may also reflect positive

512 selection at multiple codon sites in that gene; we cannot distinguish between these two
513 possibilities using gene-wide dN/dS.
514 As a follow-up analysis to gene-wide dN/dS, we examined positive selection among
515 species at specific codon sites using branch-site analysis (with *G. bimaculatus* as the target
516 branch)[93], based on three-way alignments of *G. bimaculatus*, *G. assimilis* and an available
517 cricket outgroup species *L. kohalensis* [59, 94]. The results are described in Additional file 1:
518 Text File S1 and Table S7. It should be emphasized the assessment is highly conservative given
519 it only includes genes with high confidence three-way reciprocal orthologs between species (see
520 Methods). Nonetheless, we found that substantial portion of the male$_{TSSB}$- and female$_{TSSB}$-biased
521 gonadal genes showed positive selection (≥9.6%), and that only minor variation was observed
522 between groups, perhaps due to the conserved nature of the analysis (Additional file 1: Table
523 S7). Three sex-biased brain genes that were studied in Table 1 (among ten of the 65 in Table 1
524 that had three-species orthologs available for analysis, Additional file 1: Table S7) showed
525 positive selection using branch-site analysis (GBI_05906-RA, GBI_09477-RB, GBI_05452-RB,
526 Additional file 1: Table S7). This result is consistent with the hypothesis of a history of adaptive
527 evolution, which may be due to sex-related evolutionary pressures, in the brain (Fig. 3AB).
528 It is worth noting that for the branch-site analysis, we found that a small subset of genes
529 that were female-biased in the somatic reproductive system (six of 33 genes (18.2%) with three-
530 species orthologs), which includes the reproductive tract and/or spermathecae, tended to evolve
531 adaptively using branch-site analysis (Additional file 1: Table S7). In this context, the result
532 suggests that a small number of female-biased reproductive system genes may evolve adaptively,
533 potentially in response to sexual selection pressures [55, 95], in this cricket taxon. Further studies
534 using more powerful branch-site positive selection tests [93] as more species genomic data
535 emerges, and/or population genetics analysis of frequencies of codon mutations [96], may further
536 reveal the scale of positive selection at specific codon sites in the sex-biased genes from various
537 tissues of this cricket.
538

539 # Conclusions

540 Here, we have conducted a comprehensive assessment of sex-biased gene expression in
541 reproductive and nervous system tissues, and revealed their relationships to rates of protein
542 evolution, in a cricket model system. We have shown rapid evolution of testis-biased genes and

543 sex-biased brain genes, particularly female-biased brain genes, in *G. bimaculatus* (Fig. 3, Table
544 1), and suggested how these rates of protein evolution (dN/dS) may be shaped by pleiotropy
545 (Fig. 4). Further, our data suggest a direct link between relaxed purifying constraint and the
546 frequency of adaptive evolution (Table 4). We further suggested that cricket mating biology
547 might underlie putative roles of sexual selection in accelerating evolution of these genes.
548 Future studies should assess sex-biased gene expression in the brain and gonad of *G.*
549 *bimaculatus* adult males and females in a courtship environment with male-male rivals, and/or
550 with multiple females exposed to few males (female-female competition) to identify how genes
551 specifically associated distinct mating conditions may have evolved in this taxon. In addition,
552 attaining additional *Gryllus* genomes and/or population data for *G. bimaculatus* to allow the
553 application of MacDonald-Kreitman tests may allow even more powerful positive selection tests
554 [93, 96] for genes linked to the tissues studied herein, particularly for those with small sample
555 sizes such as the brain. Further, studies of sex-biased gene expression different brain regions may
556 also provide insights into male and female differences in protein evolution [97]. A particularly
557 meaningful avenue for future investigations will include the study of sex-biased gene expression
558 in the reproductive and nervous system tissues among insects that have known differences in
559 their mating biology (variation in testis-size, sperm mixing, degree of female-female
560 competition, mate-choice, *cf.* [60]), including among additional species of *Gryllus*, to further
561 decipher how evolutionary rates may be shaped by these various mechanisms of sexual selection
562 across a phylogeny.
563

564 Materials and Methods

565 **Biological samples and RNA-seq**

566 For our RNA-seq assessment of *G. bimaculatus* we isolated the male and female gonad
567 (testis for males, ovaries for females), somatic reproductive system, brain and ventral nerve cord
568 (Fig. 1, Additional file 1: Table S1; Fig. 1A,B schematic is based on [98] and simplified from
569 Fox 2001; http://lanwebs.lander.edu/faculty/rsfox/invertebrates/acheta.html)). A ninth, unpaired
570 tissue type, the male accessory gland, was also extracted as a supplementary tissue for study (see
571 section "*Rapid divergence of seminal fluid proteins and genes from the male accessory glands*").
572 Because this relatively large glandular sexual tissue (as compared to somatic reproductive system

573 tissues defined herein, Fig. 1) is directly involved in reproduction, its gene expression has been
574 linked to protein changes (Additional file 1: Table S5) [4, 16, 53], and it provides an additional
575 sexual tissue type for our analysis of cross-tissue expression, or pleiotropy (see section "*dN/dS of*
576 *sex-biased genes in the four tissue types and pleiotropy*"). Further, we considered that its
577 inclusion in the male somatic reproductive system sample might overwhelm the transcript
578 population of that tissue type upon by RNA-seq, making it incomparable to the female somatic
579 reproductive system.

580 The rearing of specimens for tissue sampling was as follows: post hatching, wild type *G.*
581 *bimaculatus* nymphs from a previously laboratory colony inbred for at least 14 years [99] were
582 grown at 29°C until adulthood in well-ventilated plastic cages on a 12 hour day/ 12 hour night
583 cycle [99]. Plastic cages were provided with egg cartons for shelter, and the animals were fed
584 with ground cat food (Purina item model number 178046) and water. Prior to the final nymphal
585 molt, animals were sexed based on the presence (female) or absence (male) of an ovipositor and
586 separated into male and female cages to avoid any mating and thus obtain virgin samples.
587 Dissections were then performed on the unmated adults within a week after their final molt, by
588 briefly anesthetizing the animals on ice for 5-10 minutes prior to dissection. Different tissue
589 types (gonad, somatic reproductive system, brain, ventral nerve cord, male accessory
590 reproductive glands) were dissected per animal using sterile equipment wiped with ethanol and
591 RNaseZap (Ambion, catalog number AM9780), in ice-cold 1x Phosphate Buffer Saline (PBS),
592 and the tissue cleaned of any unwanted contaminating material. Each tissue was then transferred
593 immediately into individual 1.5ml Eppendorf tubes containing 500µl of pre-frozen Trizol
594 (Thermo Fisher, catalog number 15596018) on dry ice, and stored at -80°C until further use.
595 RNA extractions and library processing for RNA-seq were then performed as described
596 previously [13]. The same procedure was conducted for specimens of *G. assimilis*, which was
597 used to obtain for RNA-seq for an assembly to be used for dN/dS analysis (Additional file 1:
598 Table S2; which also included a carcass tissue type, see below section "Assembly of *G. assimilis*
599 RNA-seq data and protein sequence divergence analysis"). The *G. assimilis* eggs were obtained
600 from the Hedwig lab (University of Cambridge, U.K) and reared to adulthood, using the same
601 animal husbandry protocols as published previously for *G. bimaculatus* [40, 100, 101].

602 The RNA-seq procedures for single-end reads was conducted for each tissue type as
603 described previously [13]. The complete RNA-seq data are available at the Short Read Archive

604 (SRA) under the project identifier PRJNA564136 (Tables S1, S2). The RNA-seq reads (76bp in
605 length) for each sample were trimmed of adapters and poor quality bases using the program
606 BBduk available from the Joint Genome Institute (https://jgi.doe.gov/data-and-tools/bbtools/)
607 using default parameters.
608
609 **CDS of *G. bimaculatus* and sex-biased gene expression**
610 The expression level per *G. bimaculatus* gene was determined by mapping reads from
611 each RNA-seq dataset per tissue to the full CDS list using Geneious Read Mapper [102], a
612 program we previously found to be as effective as other read mappers (c.f. [13]), to obtain
613 FPKM per gene. To further confirm that FPKM was robust to mapping programs, we compared
614 FPKM values obtained when using mapping from Geneious and other common mappers
615 including BBmap (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbmap-guide/)
616 and Bowtie2 [103], which yielded Spearman R correlations in FPKM across all genes of R>0.94
617 (e.g., for male 1 brain RNA-seq). Read counts per CDS were converted to FPKM.
618 Expression level between sexes was compared separately for each tissue-type. We
619 compared expression between males and females for the gonad, somatic reproductive system,
620 brain, and ventral nerve cord across replicates using the program Deseq2 to obtain P-values
621 [104]. The degree of sex-biased expression was obtained using the ratio of average FPKM of the
622 replicates for female and male tissues. Any genes having a two-fold or greater ratio in average
623 expression in one sex and a statistically significant P-value (P<0.05) as well as a FPKM of at
624 least 1 in one tissue type was defined as sex-biased [13, 19, 20]. All other genes were defined as
625 unbiased.
626
627 **Assembly of *G. assimilis* RNA-seq data and protein sequence divergence analysis**
628 To study dN/dS, we generated and assembled RNA-seq CDS for the *G. bimaculatus*
629 sister species *G. assimilis* (Additional file 1: Table S2). Accordingly, we assembled the RNA-seq
630 datasets for *G. assimilis* shown in Additional file 1: Table S2 (490,414,291 trimmed reads in
631 total). For this, the *G. assimilis* reads were *de novo* assembled into contigs using Trinity [105] set
632 to default parameters using Galaxy (https://usegalaxy.org/). We then identified CDS using the
633 Plant tribes pipeline tools [106]. To assess the completeness of the assembled transcriptome, we
634 used BUSCO 3.0.1 [107] to reveal the percentage of the single-copy CDS that was observed in

635 the standardized Arthropod conserved gene set, and as employed in gVolante ([108]
636 https://gvolante.riken.jp/analysis.html). To refine the CDS for *G. assimilis* we then assessed each
637 CDS in ORF predictor, using its downloadable Perl script [67], to identify the highest quality
638 reading frame per sequence. In ORF predictor, we used the option to include the best-hit (lowest
639 e-value) BLASTX alignment (conducted in BLAST+ v2.7.1, https://blast.ncbi.nlm.nih.gov)
640 [109] of *G. assimilis* versus the reference *G. bimaculatus* protein database (i.e., its translated
641 15,539 CDS) to define reading frames, and retained all *G. assimilis* CDS at least 150bp long and
642 a start codon.
643 It is worth noting that while paired-end reads have often been used for RNA-seq
644 assembly, transcriptome assemblies from single-end reads have been successfully employed to
645 obtain CDS (not requiring isoforms) as studied herein [110, 111]. Further to this point, single-
646 end reads have even been applied for *de novo* assemblies in non-traditional model systems [110,
647 111]. Here, we have the additional advantage of a closely related reference genome to *G.*
648 *assimilis*, namely *G. bimaculatus* [59], to confirm/identify orthologs.
649
650 ***Ortholog identification and dN/dS***
651 Gene ortholog matches between *G. bimaculatus* and *G. assimilis* were identified using
652 reciprocal BLASTX of the full CDS list between the two species in the program BLAST+ v2.7.1
653 (https://blast.ncbi.nlm.nih.gov) [109]. Genes having an identical best match sequence (lowest e-
654 value) in both forward and reverse contrasts and $e<10^{-6}$ were defined as putative orthologs. The
655 identified orthologous gene sequences in *G. bimaculatus* and *G. assimilis* were aligned by
656 codons using MUSCLE [112] set to default parameters in the program Mega-CC v7 [113] and
657 gaps removed. Removal of divergent regions from alignments, despite partial loss of sequence
658 regions, improves quantification of protein divergence; thus, highly divergent segments were
659 removed using the program GBlocks v. 0.91b set at default parameters [114, 115].
660 Using the aligned *G. bimaculatus* and *G. assimilis* CDS, we employed yn00 of PAML
661 using the Yang and Nielson 2000 substitution model, which comprises a maximum likelihood
662 method that accounts for codon usage biases [93, 116], to measure dN, dS, and dN/dS [93] (note
663 that dN/dS measures using Yang and Neilson 2000 [116] were strongly correlated to those using
664 other models; e.g., values from the Pamilo and Bianchi 1993 method [117] had Spearman's
665 $R=0.95$ $P<2X10^{-7}$). Values of dN/dS >1, =1, and <1 suggest a prevalent history of positive

666 selection, neutral evolution and purifying selection respectively [93]. However, even when <1
667 for gene-wide measures of dN/dS, elevated values suggest greater roles of positive selection
668 and/or relaxed purifying constraint. Genes that were best matches by reciprocal BLASTX, and
669 for which both values of dN and dS values were <1.5 (and thus were unsaturated [118, 119]),
670 were defined as high confidence orthologs (N=7,220) between *G. bimaculatus* and *G. assimilis*
671 for dN/dS analysis. Thus, the paired alignments and dN, dS, and dN/dS measures herein are
672 conservative.
673
674 **Positive selection tests**
675 In our core assessments of gene-wide dN/dS using paired contrasts of *G. bimaculatus* and
676 *G. assimilis* from the same genus, any values >1 were interpreted as an indicator of a potential
677 history of positive selection [93]. For conservative analysis, we included only those genes with
678 both dN and dS>0.
679 In addition to this assessment, we examined positive selection at specific codon sites for
680 the *G. bimaculatus* branch using branch-site analysis in codeml of PAML [93]. As an outgroup
681 species was required for this assessment, we used the recently available assembled and annotated
682 *Laupala kohalensis* genome [59]. Three-way orthologs between *G. bimaculatus*, *G. assimilis*,
683 and *L. kohalensis* were identified using reciprocal BLASTX ($e<10^{-6}$) among each of the three
684 paired species contrasts (our criterion was that for each *G. bimaculatus*-*G. assimilis* paired
685 ortholog, the same matching *L. kohalensis* CDS must be found using reciprocal BLASTX to *G.*
686 *bimaculatus* CDS and to *G. assimilis* CDS). Genes were aligned by codons using all three-
687 species CDS and filtered using GBlocks [114, 115] and gaps removed as described in "*Ortholog*
688 *identification and dN/dS*" (note: alignments using this relatively distant outgroup were conducted
689 independently of the paired *Gryllus* alignments). The phylogeny was (*G. bimaculatus*, *G.*
690 *assimilis*), *L. kohalensis*)) and was unrooted for the PAML free-ratio analysis (Model=1,
691 NSsites=0 in codeml) that was used to determine dN and dS per branch. Only those genes with
692 dN and dS below three [23] in the *L. kohalensis* branch were defined as high confidence
693 orthologs and used for branch-site analysis (unlike the two-species contrasts within *Gryllus*
694 which were more closely related and had a cut-off of 1.5). For genes meeting these criteria,
695 positive selection was assessed on the *G. bimaculatus* branch using Chi-square values for 2XΔln
696 Likelihood between models with and without positive selection was determined as described in

697 the PAML manual [93]. We note that our stringent approach to defining three-way orthologs
698 favors study of the more conservative portion of the genome for branch-site analysis. Further,
699 some studies have suggested that branch-site analysis can lack sensitivity to detect functional
700 changes [120, 121], and/or may generate false positives [121, 122], the latter likely being
701 sensitive to the stringency of alignment. We thus aimed to control this factor by our conservative
702 approach to this assessment (excluding genes with any signs of dN or dS saturation).
703

704 **Pleiotropy analysis**

705 Expression breadth across tissues can serve as a proxy to study pleiotropy, or
706 multifunctionality of a gene, which is thought to strengthen purifying selection, and in turn
707 restrict adaptive evolutionary potential [8, 17-20, 57, 58, 69]. In other words, in theory genes
708 with low pleiotropy are hypothesized to experience relatively relaxed purifying selection, and
709 thus may be freer to evolve adaptive (mutational) changes. To assess the relationship between
710 pleiotropy and molecular evolution in *G. bimaculatus*, we determined the breadth of expression
711 of each studied gene across all nine tissues that had available RNA-seq reads (at a level of
712 ≥5FKPM per tissue, Additional file 1: Table S1) including the male and female gonad, somatic
713 reproductive system, brain and ventral nerve cord and the male accessory glands. Expression
714 breadth was evaluated with respect to dN/dS, and particularly adaptive evolution (dN/dS>1), of
715 the sex-biased genes.
716

717 **Sex-biased expression between *G. bimaculatus* and *G. assimilis***

718 As a supplementary analysis to our core assessment of sex-biased expression in our main
719 target taxon *G. bimaculatus*, we also examined sex-biased transcription of genes in *G. assimilis*.
720 For this, we focused on the gonads, which had the highest number of sex-biased genes among
721 tissues in *G. bimaculatus* (see Results). We assessed the correlation in expression for orthologs
722 between the two species using Spearman's ranked correlations. In turn, we determined those
723 genes with conserved and variable sex-biased expression status in the gonads between species,
724 and their relationships to dN/dS.
725

726 **Gene ontology**

727 Gene ontology (GO) was characterized using the tool DAVID [71]. For this, we
728 identified orthologs to *G. bimaculatus* in the insect model *D. melanogaster*, which has the most
729 well-studied insect genome to date (CDS v6.24 available from www.flybase.org [70]), using
730 BLASTX (https://blast.ncbi.nlm.nih.gov) [109] and the best match (lowest e-value with cut off
731 of $e<10^{-3}$ of *D. melanogaster*). Single direction BLASTX with *G. bimaculatus* CDS as the query
732 to the *D. melanogaster* protein database was used for these assessments (unlike for the more
733 rigorous reciprocal BLASTX analysis used to identify orthologs between the two *Gryllus* species
734 for dN/dS analysis), as reciprocal BLASTX would be overly conservative between these insects
735 from different orders for the purpose of functional characterization and analysis. *D.*
736 *melanogaster* gene identifiers were input into DAVID [71] to obtain gene putative GO functions
737 and/or classifications.
738 It is important to note that for functional analysis, more than one *G. bimaculatus* gene
739 could match a single *D. melanogaster* gene (as non-reciprocal BLASTX was used). In such
740 cases, this would suggest two or more paralogs of the same *D. melanogaster* gene existed in the
741 cricket species.
742
743 **Seminal fluid proteins**
744 As a supplemental reproductive assessment in *G. bimaculatus*, we examined seminal
745 fluid proteins (SFPs). The SFPs are included in the fluids transferred with the sperm to the
746 female reproductive tract, and are thought to play crucial roles in sperm vitality, sperm storage in
747 female organs and in fertilization [52]. A recent proteome analysis of sexual structures in *D.*
748 *melanogaster* confirmed functions for 125 previously identified SFPs in that insect, and revealed
749 nine newly identified SFPs [52], that may be used as a reference to study SFPs in crickets. Thus,
750 using this very recent list of 134 SFPs in *D. melanogaster* we identified putative orthologs of
751 SFPs in *G. bimaculatus* and between *G. bimaculatus* and *G. assimilis* and assessed the dN/dS
752 values of these genes. All between-order comparisons between *Gryllus bimaculatus* to *D.*
753 *melanogaster* genes herein, including for these SPFs, are single direction BLASTX.
754

755 # List of abbreviations

756 TSSB, tissue-specific sex bias

757 FPKM, frequency per kilobase million

758

759 # Declarations

760

761 ***Ethics approval and consent to participate***

762 Not applicable.

763

764 ***Consent for publication***

765 Not applicable.

766

767 ***Availability of data and material***

768 All RNA-seq data under study are described in Additional file 1: Table S1 and Table S2 and are
769 available at the Short Read Archive (SRA) under the project identifier PRJNA564136.

770

771 ***Competing interests***

772 The authors declare they have no competing interests.

773

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776

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781

782 ***Authors' contributions***

783 CAW, AK and CGE designed the study. CAW analyzed data and wrote the manuscript with
784 contributions by AK and CGE. AK reared G. *bimaculatus* and *G. assimilis* and sampled tissues
785 for RNA-seq. All authors read and approved the final manuscript.

786

Additional Files

Additional File 1: The file contains the supplementary Tables, Figures and Text which are denoted and Tables S1 to S7, Figures S1 to S2, and Text File S1.

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Table 1. The dN/dS values and GO functions of all female-biased brain genes and male-biased brain genes among the 7,220 genes with *G. bimaculatus*-*G assimilis* orthologs (Fig 2B). GO terms are from DAVID software [71] using those *G. bimaculatus* genes with putative *D. melanogaster* (Dmel) orthologs. TSSB indicates genes that have tissue-specific sex-biased expression in the brain and are unbiased in all other tissues (shown by "*"; Fig. 3A). Gonad sex bias (GSB) indicates the gene has the same female- or male-biased expression in the gonad as in brain and is unbiased in other tissues ("**"). Genes are listed by highest to lowest dN/dS values.

***G. bimaculatus* ID**	**dN/dS**	**TSSB**	**GSB**	**Matching Dmel ID**	**Dmel gene name[a]**	**GO terms**
Female-biased in brain (N=20)						
GBI_10990-RA	0.9739	*		FBgn0028370	*Kekkon-3 (kek3)*	Plasma membrane
GBI_06557-RA	0.8282			FBgn0035082	CG2811	AIG2-like, Butirosin biosynthesis, Acyltransferase
GBI_06507-RA	0.564			FBgn0035951	CG5068	Plasma membrane, Alpha/beta hydrolase fold-1
GBI_00147-RA	0.527			No match		
GBI_11079-RA	0.5226			FBgn0031265	CG2794	Metabolic process
GBI_14015-RA	0.4598			FBgn0038395	CG10407	Multicellular reproduction
GBI_14708-RA	0.3835	*		FBgn0003870	*tramtrack (ttk)*	Inter-male aggressive behavior, nervous system
GBI_01688-RA	0.3273	*		FBgn0011604	*Imitation SWI (Iswi)*	Chromatin organization
GBI_16251-RA	0.2633	*		FBgn0052432	CG32432	Integral component of membrane
GBI_04158-RA	0.2452			FBgn0027582	CG6230	Plasma membrane
GBI_17348-RA	0.2439			FBgn0011695	*Ejaculatory bulb protein III (EbpIII)*	Insect pheromone-binding protein, post-mating behavior
GBI_05906-RA	0.2258			FBgn0033215	CG1942	Mesoderm development
GBI_13745-RB	0.1525	*		FBgn0010380	*Adaptor protein (AP-1-2beta)*	Neurotransmitter secretion, synaptic vesicle coating
GBI_09497-RB	0.1433		**	No match		
GBI_00160-RA	0.0692			FBgn0026876	CG11403	DNA binding
GBI_07457-RC	0.0558			FBgn0037659	*Lysine (K)- demethylase 2 (Kdm2)*	Transcription, DNA-templated
GBI_04405-RA	0.0451		**	FBgn0024997	CG2681	Apoptotic process, multicellular development
GBI_06070-RA	0.0357			FBgn0035724	CG10064	WD40 repeat, dehydrogenase-like superfamily
GBI_02686-RA	0		**	FBgn0000317	*crinkled (ck)*	Sensory organ development
GBI_09453-RB	0	*		FBgn0031550	*Intraflagellar transport 57 (IFT57)*	Apoptotic process
Male-biased in brain (N=45)						
GBI_19557-RB	3.675	*		FBgn0030947	CG6696	Proteolysis, metalloendopeptidase activity
GBI_01683-RA	0.7988	*		FBgn0039590	CG10011	Intracellular protein transport
GBI_10265-RB	0.6262			FBgn0035132	*methuselah-like 10 (mthl10)*	G-protein coupled receptor activity, alternative splicing
GBI_09477-RB	0.6208			FBgn0004364	*18-wheeler (18w)*	Transmembrane signaling receptor activity
GBI_01684-RA	0.5977	*		FBgn0031473	CG3104	Ankyrin repeat, ER to Golgi vesicle transport
GBI_17358-RA	0.4488		**	FBgn0011695	*Ejaculatory bulb protein III (EbpIII)*	Insect pheromone-binding protein, post-mating behavior

GBI_03471-RA	0.4445			FBgn0019972	*Death rel. ICE-like caspase (Drice)*	Apoptotic process, proteolysis, adult lifespan
GBI_07016-RA	0.4422	*		FBgn0053196	*dumpy (dpy)*	Transcription factor activity, epithelial/tracheal develop
GBI_08544-RB	0.3989	*		No match		
GBI_09470-RA	0.3951		**	FBgn0039478	*Neprilysin 5 (Nep5)*	Membranes, posttranslational modification
GBI_01935-RB	0.3929	*		FBgn0012051	*Calpain-A (CalpA)*	Neuronal cell body, BMP spinal cord patterning
GBI_17696-RA	0.3765			No match		
GBI_05452-RB	0.3402			FBgn0036877	CG9452	Acid phosphatase activity, extracellular exosome
GBI_07279-RA	0.3265			FBgn0025874	*Meiotic central spindle (Meics)*	Transcription/cell division and chromosome partitioning
GBI_11920-RB	0.31	*		FBgn0000083	*Annexin B9 (AnxB9)*	Cell polarity, wing disc dorsal/ventral pattern formation
GBI_04818-RB	0.2852			FBgn0051217	*modular serine protease (modSP)*	Autocatalytic cleavage
GBI_14462-RA	0.2756	*		No match		
GBI_04545-RA	0.2414			FBgn0012051	*Calpain-A (CalpA)*	Phagocytosis, adult lifespan, larval locomotory behavior
GBI_12729-RA	0.2362			FBgn0012037	*Angiotensin converting enzyme (Ance)*	Spermatid nucleus differentiation, sperm individualization
GBI_11067-RA	0.2248			FBgn0033250	CG14762	Axonogenesis, neuron projection morphogenesis
GBI_15926-RA	0.2248		**	FBgn0030778	CG4678	Peptidase M14, proteolysis, peptide metabolic process
GBI_04544-RA	0.2013			FBgn0012051	*Calpain-A (CalpA) CalpA*	Phagocytosis, adult lifespan, larval locomotory behavior
GBI_17460-RA	0.1685			FBgn0038047	CG5245	Zinc finger, nucleic acid binding
GBI_01710-RA	0.1497			FBgn0004638	*downstream of receptor kinase (drk)*	Embryonic development/syncytial blastoderm
GBI_03557-RA	0.1337			FBgn0037802	*Sirtuin 6 (Sirt6)*	Adult lifespan, chromatin silencing,
GBI_07735-RA	0.13			FBgn0041713	*yellow-c*	Melanin biosynthetic process, cuticle pigmentation
GBI_00231-RA	0.1299	*		FBgn0259736	CG42390	Cell division/chromosome partitioning
GBI_08685-RA	0.126	*		FBgn0036454	CG17839	Immunoglobulin subtype 2, Immunoglobulin
GBI_10295-RA	0.0921			No match		
GBI_15959-RA	0.0902			FBgn0013348	*Troponin C at 41C (TpnC41C)*	EF-hand domain, calcium-binding site
GBI_01504-RC	0.089			FBgn0037665	Sulfotransferase 2 (St2)	Sulfotransferase activity
GBI_14634-RB	0.0721	*		FBgn0032979	*Chromatin-linked adaptor (Clamp)*	Zinc finger, nucleic acid binding
GBI_09694-RB	0.0652		**	FBgn0032768	CG17564	Domain of unknown function
GBI_07712-RA	0.0492	*		FBgn0263025	CG43320	JmjC domain, cell division and chromosome partitioning
GBI_08082-RA	0.0489			FBgn0030304	*Cytochrome P450 (Cyp4g15)*	Neural
GBI_11047-RB	0.0435		**	FBgn0264907	CG44098	Transmembrane transport
GBI_07069-RB	0.043			FBgn0002524	CG4162	Imaginal disc development, Wnt signaling pathway
GBI_14322-RA	0.0227			FBgn0243514	*Eater*	Phagocytosis, scavenger receptor activity
GBI_00965-RA	0		**	FBgn0034909	CG4797	Hexose transmembrane transport
GBI_02270-RA	0		**	FBgn0260439	*Protein phosphatase 2A (Pp2A-29B)*	Protein complex assembly, phagocytosis
GBI_03078-RA	0	*		FBgn0002789	*Muscle protein 20 (Mp20)*	Actin binding
GBI_06961-RA	0	*		FBgn0031800	CG9497	Integral component of membrane
GBI_07963-RA	0	*		FBgn0036316	CG10960	Sugar/inositol transporter, hexose transmembrane transport
GBI_14909-RA	0	*		FBgn0038385	*F-box and leucine repeat 7 (Fbxl7)*	Proximal/distal pattern formation, imaginal disc
GBI_15287-RA	0			FBgn0034267	CG4984	PMP-22/EMP/MP20/Claudin, membrane

[a], some gene names are abbreviated.

Table 2. Top GO functional groups for testis-biased$_{TSSB}$ and ovary-biased$_{TSSB}$ genes identified in *G. bimaculatus* (those with orthologs in *G. assimilis*) in Fig 2A. Genes were sex-biased only in the gonads and not in the somatic reproductive system, brain or ventral nerve cords (tissue-specific sex biased, TSSB). The top six clusters with the greatest enrichment (abundance) scores are shown per category. *P*-values are derived from a modified Fisher's test, where lower values indicate greater enrichment. Data is from DAVID software [71] using those *G. bimaculatus* genes with predicted *D. melanogaster* orthologs.

Ovary-biased genes (N=1,858)		**Testis-biased genes (N=1,055)**	
GO Function	**P-value**	**GO Function**	**P-value**
Cluster 1: Enrichment Score 10.31		**Cluster 1 Enrichment Score: 5.38**	
nucleotide-binding	1.00E-15	ubiquitin-protein transferase activity	1.20E-07
ATP-binding	2.00E-14	**Cluster 2 Enrichment Score: 3.66**	
Cluster 2: Enrichment Score 7.19		cilium assembly	3.70E-06
WD40/YVTN repeat-like-containing domain	7.70E-09	cilium morphogenesis	6.90E-05
Cluster 3: Enrichment Score 5.41		**Cluster 3 Enrichment Score: 3.28**	
transcription, DNA-templated	5.70E-03	Nucleotide-binding	2.50E-04
Cluster 4: Enrichment Score 5.03		ATP binding	6.40E-04
zinc-finger	1.30E-05	**Cluster 4 Enrichment Score: 3.19**	
Cluster 5: Enrichment Score 4.28		nonmotile primary cilium assembly	2.20E-05
ligase	5.00E-09	intraciliary retrograde transport	3.10E-03
Aminoacyl-tRNA synthetase	1.20E-06	**Cluster 5 Enrichment Score: 2.56**	
protein biosynthesis	2.50E-02	mitochondrial inner membrane	3.00E-03
Cluster 6: Enrichment Score 3.30		**Cluster 6 Enrichment Score: 2.39**	
transcription initiation	1.80E-05	cell projection	1.00E-02
RNA polymerase II transcription cofactor	1.10E-03	flagellum	2.60E-02

Table 3. The *D. melanogaster* seminal fluid proteins (SFPs) [52] that were found to have putative orthologs in *G. bimaculatus* (GB) among the subset of 7,220 genes with intra-*Gryllus* orthologs used for dN/dS analysis. Expression levels (FPKM) for each gene are shown for the three male sexual tissues under study.

SFP gene in *D. melanogaster*	**Gene name or ID**	**Gene match in *G. bimaculatus****	**dN/dS**	**Male sexual tissue expression** (FPKM)		
				Accessory glands	Testis	Male somatic reproductive system
FBgn0034474	*Obp56g*	GBI_14450-RA	2.4819	41.495	0	0.32
FBgn0028986	*Spn38F*	GBI_05353-RD	0.3435	0.565	4.13	43.58
FBgn0028987	*Spn28F*	GBI_00301-RB	0.2866	36.84	270.87	94.46
FBgn0030362	*regucalcin*	GBI_08029-RA	0.2496	37.63	15.08	23.19
FBgn0030932	*Ggt-1*	GBI_03406-RA	0.2302	9.845	8.60	21.73
FBgn0038198	*Npc2b*	GBI_06029-RA	0.2197	7.5	0.50	793.96
FBgn0283509	*Phm*	GBI_06121-RA	0.1496	71.82	32.28	86.185

Table 4. The proportion of genes with sex-biased$_{TSSB}$ gonadal expression in *G. bimaculatus* that had dN/dS>1 and their expression breadth (exp. breadth) across tissues (average number of nine tissues with expression >5 FPKM). Only genes with dN/dS>1 and both dN and dS >0 are included.

Gene category	**N dN/dS >1**	**N Genes**	**Percent of genes**	**Chi^2 P[a]**	**Ave. Exp. breadth**	**SE**	**MWU-test P (exp. breadth)[a]**
Ovary-biased$_{TTSB}$	19	1,858	1.02	a	6.74	0.74	a
Testis-biased$_{TTSB}$	22	1,055	2.09	b	2.73	0.72	b
Universally unbiased	66	3,449	1.91	b	5.62	0.76	a

[a] Different letters in columns with P values indicate a statistically significant difference between categories with P<0.05. SE=standard error.

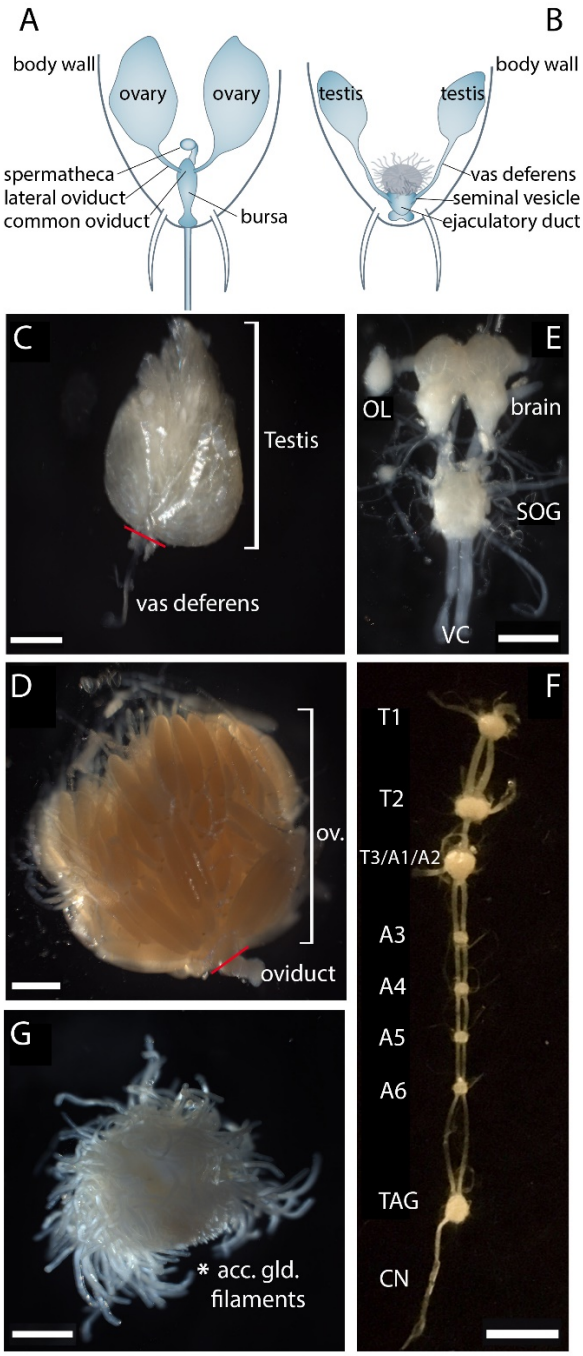

Fig. 1. *Gryllus bimaculatus* reproductive and nervous system tissues studied herein. A schematic of the female (A) and male (B) reproductive systems. In A and B, the gonads and the somatic tissues included in the somatic reproductive system under study are indicated. C-G provide micrographs of various tissue types studied herein. C) the testis (one testis shown here; both testes from a given male were used for sampling), including a part of its attached vas deferens (boundary indicated by red line; the vasa deferentia were not included in testis samples). D) the ovary (ov; one ovary shown here; both ovaries from a given female were used for sampling) and an

immediately attached segment of oviduct (boundary indicated by red line; the oviducts were not included in ovary samples). E) the brain, including an optic lobe (OL) (one OL shown here; both OLs from a given individual were included in brain samples). For context, the attached suboesophageal ganglion (SOG) and upper portion of the ventral nerve cord (VC) are also shown; these structures were not included in brain samples. F) the ventral nerve cord including the three thoracic ganglia (T1: prothoracic, T2: mesothoracic, T3/A1/A2: metathoracic ganglion complex), and five abdominal ganglia (A3-A6 and the terminal abdominal ganglion TAG) [123, 124]. The cercal nerve (CN) of one side is also shown. For the ventral nerve cord samples, all tissues in F and the SOG were pooled. G) The male accessory gland consisting of numerous accessory gland filaments (asterisk). Scale bars: 500μm in C and E, 1000μm in D and G, 2500μm in F.

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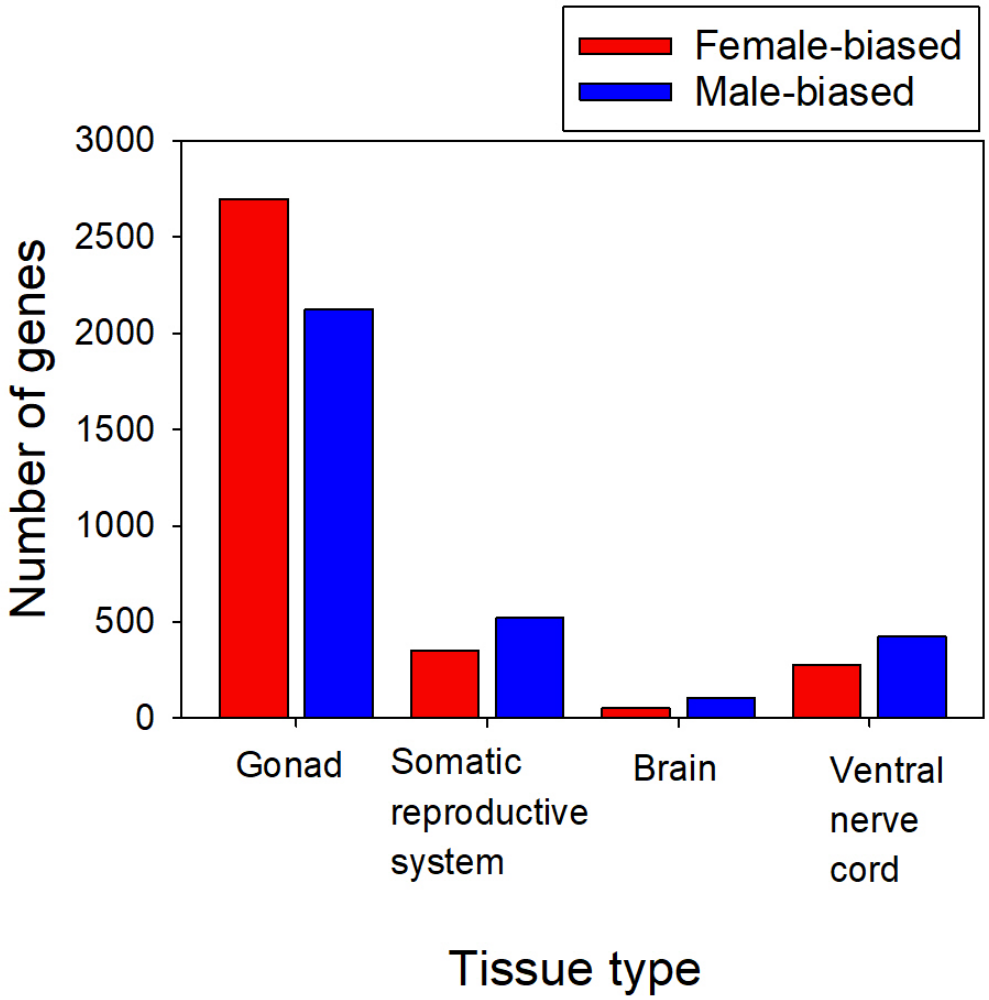
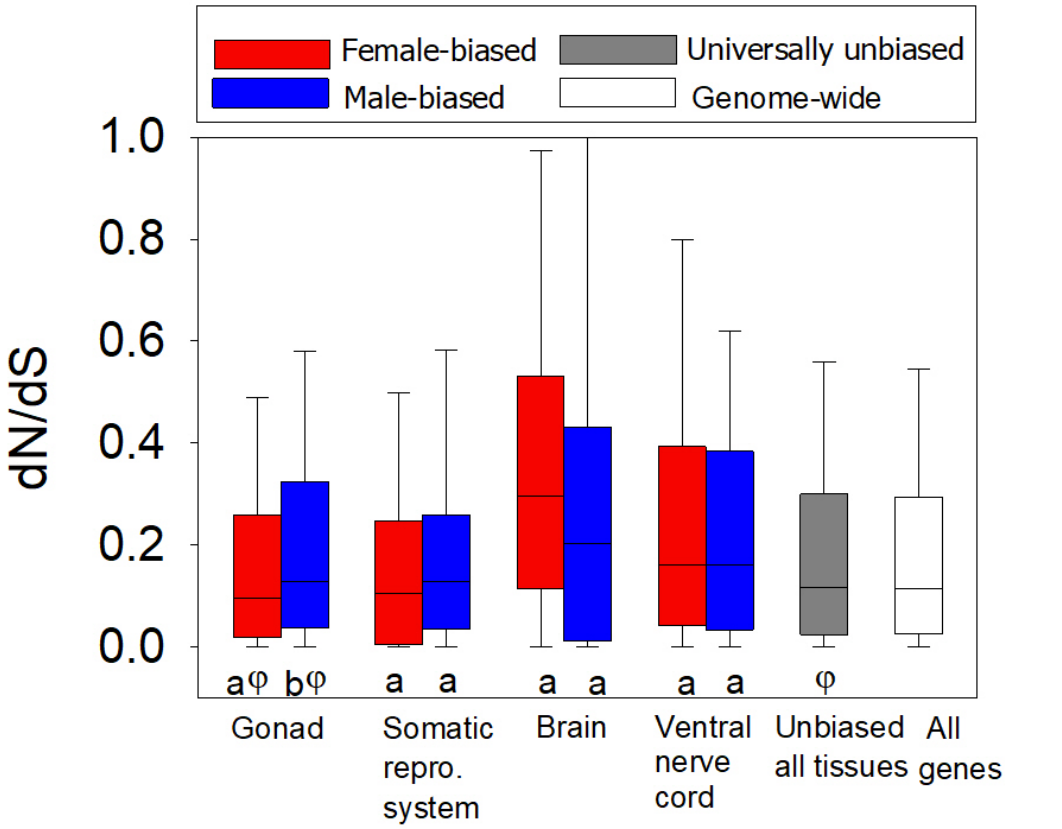
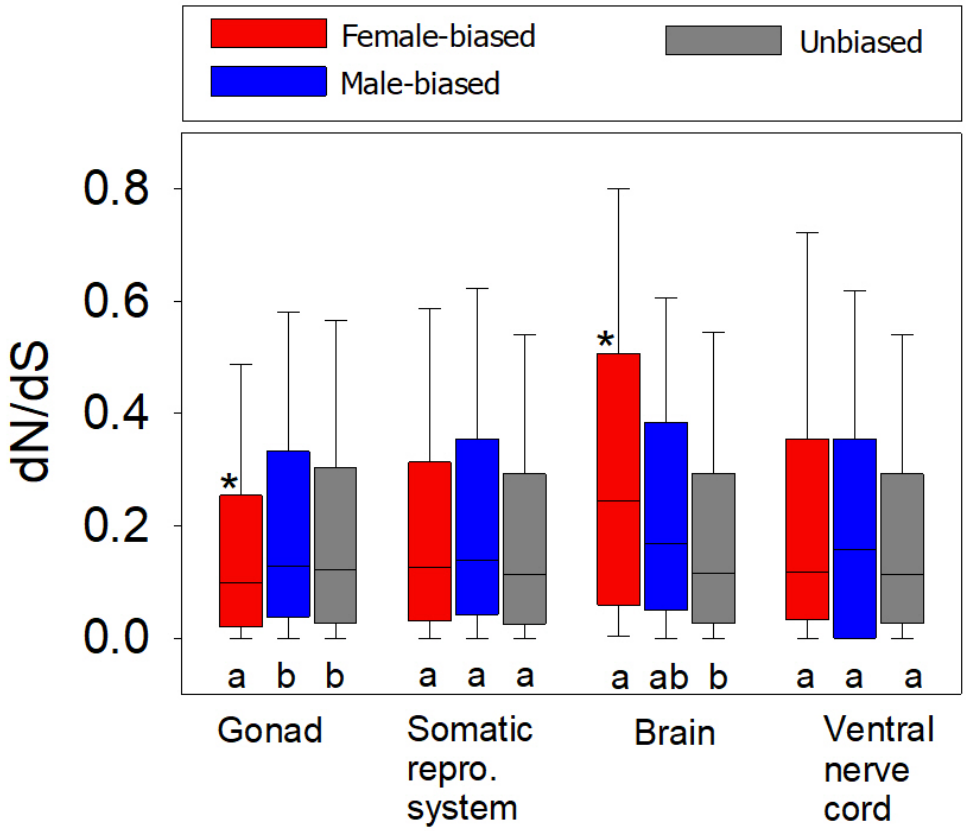


Fig. 2. The number of male-biased and female-biased genes identified in the gonad, somatic reproductive system, brain, and ventral nerve cord across all 15,539 *G. bimaculatus* genes under study (sex-biased indicates a two-fold difference in expression and P<0.05). All remaining genes not shown per tissue type had unbiased status as follows: gonad (N=10,717), somatic reproductive system (N=14,666), brain (N=15,382) and ventral nerve cord (N=14,835).



A. Genes sex-biased only in one tissue type



B. dN/dS and sex-biased status

Fig. 3. The dN/dS values of genes with female- or male-biased expression in *G. bimaculatus*. A) Genes with

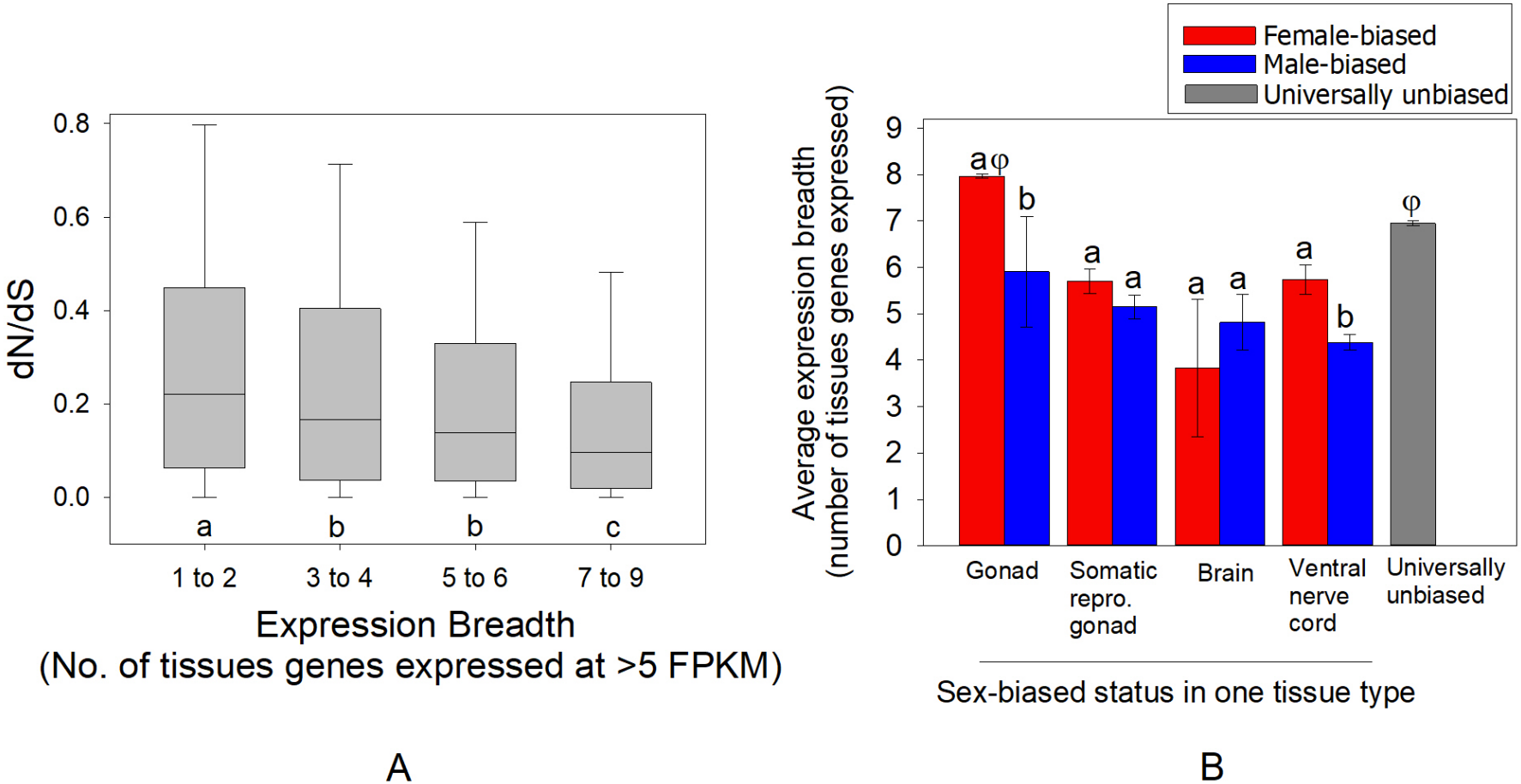


Fig. 4. A) Box and whisker plots of the dN/dS values of genes with respect to their expression breadth, or pleiotropy, for *G. bimaculatus* (N=7,220 genes); B) The expression breadth (average number of tissues with expression of a gene) of genes with female- or male-biased expression in one tissue type only (TSSB, as shown in Fig. 3A). In A, different letters below bars indicate a statistically significant difference using ranked ANOVA with Dunn's paired contrast (P<0.05). In B, different letters in each pair of bars indicate a difference using MWU-tests. φ for ovary-biased and universally unbiased genes indicates a statistically significant difference from each other and from all other bars. Error bars in B indicate standard errors. repro. = reproductive.