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

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

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

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

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

Results and Discussion

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

unsaturated dN and dS values (<1.5) after alignment, we identified 7,220 high confidence *G. bimaculatus*-*G. assimilis* orthologs that were used for all dN/dS analyses. Across all 7,220 orthologs under study, we found that the alignments with gaps removed were on average 68.0% (standard error=0.3) of the original *G. bimaculatus* CDS length (that pre-alignment had an average length of 577.0 codons, standard error=5.9 for the subset of its genes with between-species orthologs), and that the median dN/dS was 0.1152. The median dN was 0.0042 and median dS was 0.0396, values that were substantially <1, consistent with a close phylogenetic relatedness between these two sister *Gryllus* species from the same genus.

To precisely reveal the relationship between sex-biased gene expression for each individual tissue type and dN/dS, we identified genes that were sex-biased in expression in only one of four of the female-male paired tissues (gonad, somatic reproductive system, brain or ventral nerve cord) and unbiased in all three remaining tissues in *G. bimaculatus*. These genes are hereafter denoted as tissue-specific sex biased, or TSSB genes (N_{TSSB} values provided in Additional file 1: Table S3). We also identified those genes with universally unbiased expression in all four tissues types as a control (N=3,449; Additional file 1: Table S3). The vast majority of the 7,220 genes (with orthologs in both species) fell into one of these two categories (94.5% of 7,220 genes had TSSB or universally unbiased status).

dN/dS of sex-biased genes in the four tissue types and pleiotropy

The dN/dS values of sex-biased$_{TTSB}$ genes for the four core *Gryllus* tissue types under study, and for universally unbiased genes, are shown in Fig. 3A. In turn, for completeness, the dN/dS values of all sex-biased genes per tissue, regardless of status in other tissues (sex-biased$_{ALL}$), are shown in Fig. 3B. The results show that in this cricket model, testis-biased $_{TTSB}$ genes evolved faster than ovary-biased $_{TTSB}$ and universally unbiased genes (MWU-tests P<0.05). Further, sex-biased brain genes, while uncommon (Fig. 2), evolved exceptionally rapidly, particularly with faster evolution of the female-biased$_{ALL}$ brain genes than of unbiased$_{ALL}$ genes (MWU-test P<0.05, Fig. 3B, for details see below section "*Rapid evolution of sex-biased brain genes*"). In turn, no differences in dN/dS of male-biased, female-biased, or unbiased genes from the somatic reproductive system or ventral nerve cords were observed (using TSSB and universally unbiased genes (Fig. 2A) or ALL genes per tissue type (Fig. 3B, MWU-tests P>0.05). In this regard, it is evident that sex-biased expression in this cricket system

is associated with rapid evolution of testis-biased genes and of sex-biased brain genes, particularly those with biased expression in the female brain.

We next assessed the expression breadth across tissues (using nine tissues, the eight paired tissues and the male accessory glands, see Methods), as a proxy for pleiotropy, or multifunctionality of a gene, which is thought to strengthen purifying selection and in turn restrict adaptive evolutionary potential [8, 17-20, 57, 58, 69]. Genes were categorized into genes expressed at >5FPKM in 1-2, 3-4,5-6,7-9 tissues. As shown in Fig. 4A, when studying all 7,220 genes with high confidence orthologs, we found that the rate of evolution of *Gryllus* genes was strongly inversely correlated with pleiotropy. The slowest rate of evolution was found in genes transcribed in all tissues under study (median dN/dS=0.161), and the fastest rate in genes expressed in one to two tissues (median=0.221, Ranked ANOVA and Dunn's paired contrasts $P<0.05$). Further, as indicated in Fig. 4B, with respect to sex-biased gene expression, we found that testis-biased genes had markedly lower expression breadth than ovary-biased genes and than universally unbiased genes (MWU-tests $P<0.05$), while female-biased brain genes had the smallest median expression breadth of all studied categories, with statistically significantly lower values than universally unbiased genes (MWU-test $P<0.05$). Thus, this suggests a plausible connection between rapid protein evolution of sex-biased genes in the brain and their narrow pleiotropy, either due to relaxed constraint in itself, and/or due to an associated freedom to evolve functional changes under low purifying constraint [8, 17-20, 57, 58, 69]. In sum, the rapid evolution of testis-biased genes (as compared to ovary-biased and unbiased genes) and of sex-biased brain genes may be at least partly be a result of their pleiotropy, and in turn, potential adaptive changes (see evidence in agreement with a pleiotropy-adaptive evolution relationship in the below section "***Evidence of A History of Positive Selection in Sex-Biased Gonadal and Brain Genes"***). In the following sections, we describe in greater detail the sex-biased genes in the brain and in the reproductive system in Fig. 3 and Fig. 4, and consider the putative roles of pleiotropy and positive selection in affecting their molecular evolution.

Rapid evolution of sex-biased genes from the brain

With respect to the brain, female-biased$_{TSSB}$ genes had markedly higher median dN/dS values (median=0.295) than male-biased$_{TSSB}$ genes (0.203, Fig. 3A), although that contrast was not statistically significant (MWU-test P=0.739). This may reflect the low statistical power of

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

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

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

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

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

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

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

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

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

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

Sex-biased gonadal expression in G. assimilis

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

orthologs for the gonads (which had the largest N values of all tissues, Additional file 1: Table S3). The results are shown in Additional file 1: Fig. S2 and are described in Additional file 1: Text File S1. We observed that the finding of elevated dN/dS of testis-biased versus ovary-biased genes was robust to whether the sex-biased status (testis-biased, ovary-biased) was observed in one species or was conserved both of these species. Thus, testis-biased expression in one species (i.e., *G. bimaculatus*, Additional file 1: Fig. S2) is sufficient to predict elevated pairwise dN/dS.

Lack of different rates of evolution in sex-biased genes from the somatic reproductive system

In contrast to the gonad, for the sex-biased$_{TSSB}$ genes from the somatic reproductive system (N values in Additional file 1: Table S3), no statistically significant differences were observed in dN/dS of male-biased$_{TSSB}$ and female-biased $_{TSSB}$ genes, nor between those groups and the universally unbiased genes (MWU-tests $P>0.05$, Fig. 3A). This result may be considered surprising, given the roles of these sexual tissues in reproductive success and fitness, particularly for the female tissues (common oviduct, spermathecae, and vagina).

Few comparable insect data of sex-biases in somatic reproductive system tissues are available. Some specific genes involved in the female reproductive tract in *Drosophila* have been linked to rapid and/or adaptive evolution, which may be due to their dynamic roles in receiving and maintaining sperm after mating [54, 55]. However, a separate assessment of genes broadly defined as female reproductive tract proteins in *D. melanogaster* (based on expression data from mixed or mated flies) showed those genes exhibited slow protein evolution (dN/dS), below the genome-wide average [4]. Thus, our results from *Gryllus* (unmated) showing no differences in dN/dS between female-biased $_{TTSB}$ somatic reproductive system genes and the universally unbiased genes or the genome as a whole (Fig. 3), differs from those findings in *Drosophila* (note: see below section "*Positive Selection Tests in the Sex-biased Gonadal and Brain Genes*" which suggests a small number of female somatic reproductive system genes evolve adaptively) [4]. Significantly, it is evident that the lack of effect of sex-biased transcription in the somatic reproductive system stands in contrast to the substantial effects observed for the gonad (Fig. 3).

Rapid divergence of genes from the male accessory glands and seminal fluid proteins

As a supplementary analysis with respect to the reproductive structures, given that sex-biased genes from the male-accessory glands, including seminal fluid protein (SFPs), have been strongly linked to rapid evolution in species of *Drosophila*, we assessed the evolutionary rates of these genes in *Gryllus.* The results are described in detail in Additional file 1: Text File 1, Table S5 and Table S6. In brief, we show that *G. bimaculatus* genes with expression specific to the male accessory glands (0 FPKM in all eight other tissues studied here) had few orthologs detected (well below the genome-wide average of ortholog detection) in its sister species *G. assimilis*, and in *D. melanogaster*, suggesting a history of rapid evolution potentially so extensive that it prevents protein similarity detection by these methods, and/or a history of lineage-specific gene losses or gains of genes involved in this sexual tissue [4, 90].

Further, for SFPs, we used the recently available list of 134 SFPs for the species *D. melanogaster* (shown in Additional file 1: Table S6, [52]) as a reference, and found that only 20 SFP genes had identifiable putative orthologs in *G. bimaculatus* (14.9%). Only seven of those were included among the subset of 7,220 genes with between-species orthologs in *Gryllus*. The dN/dS values of these seven genes are shown in Table 3; all were above the genome-wide median dN/dS value (0.115, Fig 2A). Positive selection was indicated for the odorant binding SFP protein *Obp56g*, with dN/dS>1 (Table 3). Together, we conclude that the putative SFPs in the crickets studied here have evolved very rapidly, a feature shared with SFPs of *D. melanogaster* [4, 52], and that could be due to their potential subjection to sex-related selection pressures. For instance, in flies SFPs may enhance sperm competitive ability in the female reproductive tract or egg release from the ovary [91, 92], and males may alter relative production of different SFPs when exposed to male rivals [91]. If similar types of mechanisms of sexual selection exist in crickets, then they could contribute to fast evolution of SFP genes. Another potentially significant behavioural factor in *G. bimaculatus*, is the tendency of females to preferentially retain deposited spermatophores of certain (larger) males [46, 48], which comprises a mechanism of female-choice in this species [48]. This behaviour might lead to sexual selection pressures on SFPs contained in those spermatophores, and accelerate their evolution.

Evidence of A History of Positive Selection in Sex-Biased Gonadal and Brain Genes

Finally, we considered the incidences of positive selection among those genes with between-species *Gryllus* orthologs. The use of dN/dS>1 is a conservative means to assess adaptive evolution, as positive selection must be frequent enough across all codon sites to be detected. We found that 1.63 % of all the 7,220 *G. bimaculatus*-*G. assimilis* gene orthologs (N=118 genes) showed dN/dS>1 (retaining only genes where both dN and dS >0).

We then considered whether dN/dS values of the sex-biased gonad$_{TTSB}$ genes, which had the highest N values of all tissues analysed (Additional file 1: Table S3), were consistent with the aforementioned hypothesis that reduced gene pleiotropy, or expression breadth (and thus purifying selection), may enhance a gene's functional evolvability [8, 13, 17, 19, 57, 58, 69]. We found that the percent of genes with positive selection (dN/dS>1) increased from ovary-biased$_{TTSB}$ genes (1.02%, 19 of 1,858) to universally unbiased genes (1.91%, 66 of 3,449) and testis-biased$_{TTSB}$ genes (2.09%, 22 of 1,055; Chi^2 P with Yates' correction was <0.05 for each paired contrast to ovary-biased$_{TSSB}$ genes, Table 4). In turn, we assessed gene pleiotropy for each group. Expression breadth of genes decreased from all ovary-biased$_{TTSB}$ (average expression breadth of 7.97±0.04 (standard error)), to universally unbiased (6.95±0.05) and to testis-biased$_{TTSB}$ genes (5.90±0.18 tissues; (MWU-tests P<0.001 for each of three paired contrasts). Strikingly, the differences were even more magnified in the subset of genes with dN/dS>1 shown in Table 4, with markedly higher average expression breadth (2.5 fold) for ovary-biased$_{TSSB}$ (6.74±0.74) than for testis-biased$_{TSSB}$ (2.73±0.72) genes (Chi^2 P<0.05, Table 4). Crucially, these patterns observed using whole-gene dN/dS values in this cricket system provide empirical support for the theoretical proposition that that the fewer tissues a gene is expressed in, the more its adaptive evolutionary potential may be enhanced, likely by reducing putative constraint imposed by multiple cross-tissue functions [8, 17, 57, 58, 69]. Our data thus specifically show that this hypothesis can apply to sex-biased genes [17].

We further assessed whether there was evidence of positive selection for sex-biased brain genes, which were much less common that those from the gonad (Additional file 1: Table S3, Fig. 2). The only gene with whole-gene dN/dS >1 (=3.675, GBI_19557-RB, Table 1) was of unknown function and specifically expressed in the male brain (expression breadth=1 tissue). Thus, this result is also concordant with adaptive evolution facilitated by low pleiotropy. The female-biased brain gene with the highest dN/dS of 0.9735 matched *D. melanogaster kekkon3*. This value near one could suggest a history of neutral evolution, but may also reflect positive

selection at multiple codon sites in that gene; we cannot distinguish between these two possibilities using gene-wide dN/dS.

As a follow-up analysis to gene-wide dN/dS, we examined positive selection among species at specific codon sites using branch-site analysis (with *G. bimaculatus* as the target branch)[93], based on three-way alignments of *G. bimaculatus*, *G. assimilis* and an available cricket outgroup species *L. kohalensis* [59, 94]. The results are described in Additional file 1: Text File S1 and Table S7. It should be emphasized the assessment is highly conservative given it only includes genes with high confidence three-way reciprocal orthologs between species (see Methods). Nonetheless, we found that substantial portion of the male$_{TSSB}$- and female$_{TSSB}$-biased gonadal genes showed positive selection (≥9.6%), and that only minor variation was observed between groups, perhaps due to the conserved nature of the analysis (Additional file 1: Table S7). Three sex-biased brain genes that were studied in Table 1 (among ten of the 65 in Table 1 that had three-species orthologs available for analysis, Additional file 1: Table S7) showed positive selection using branch-site analysis (GBI_05906-RA, GBI_09477-RB, GBI_05452-RB, Additional file 1: Table S7). This result is consistent with the hypothesis of a history of adaptive evolution, which may be due to sex-related evolutionary pressures, in the brain (Fig. 3AB).

It is worth noting that for the branch-site analysis, we found that a small subset of genes that were female-biased in the somatic reproductive system (six of 33 genes (18.2%) with three-species orthologs), which includes the reproductive tract and/or spermathecae, tended to evolve adaptively using branch-site analysis (Additional file 1: Table S7). In this context, the result suggests that a small number of female-biased reproductive system genes may evolve adaptively, potentially in response to sexual selection pressures [55, 95], in this cricket taxon. Further studies using more powerful branch-site positive selection tests [93] as more species genomic data emerges, and/or population genetics analysis of frequencies of codon mutations [96], may further reveal the scale of positive selection at specific codon sites in the sex-biased genes from various tissues of this cricket.

Conclusions

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

sex-biased brain genes, particularly female-biased brain genes, in *G. bimaculatus* (Fig. 3, Table 1), and suggested how these rates of protein evolution (dN/dS) may be shaped by pleiotropy (Fig. 4). Further, our data suggest a direct link between relaxed purifying constraint and the frequency of adaptive evolution (Table 4). We further suggested that cricket mating biology might underlie putative roles of sexual selection in accelerating evolution of these genes.

Future studies should assess sex-biased gene expression in the brain and gonad of *G. bimaculatus* adult males and females in a courtship environment with male-male rivals, and/or with multiple females exposed to few males (female-female competition) to identify how genes specifically associated distinct mating conditions may have evolved in this taxon. In addition, attaining additional *Gryllus* genomes and/or population data for *G. bimaculatus* to allow the application of MacDonald-Kreitman tests may allow even more powerful positive selection tests [93, 96] for genes linked to the tissues studied herein, particularly for those with small sample sizes such as the brain. Further, studies of sex-biased gene expression different brain regions may also provide insights into male and female differences in protein evolution [97]. A particularly meaningful avenue for future investigations will include the study of sex-biased gene expression in the reproductive and nervous system tissues among insects that have known differences in their mating biology (variation in testis-size, sperm mixing, degree of female-female competition, mate-choice, *cf.* [60]), including among additional species of *Gryllus*, to further decipher how evolutionary rates may be shaped by these various mechanisms of sexual selection across a phylogeny.

Materials and Methods

Biological samples and RNA-seq

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

(SRA) under the project identifier PRJNA564136 (Tables S1, S2). The RNA-seq reads (76bp in length) for each sample were trimmed of adapters and poor quality bases using the program BBduk available from the Joint Genome Institute (https://jgi.doe.gov/data-and-tools/bbtools/) using default parameters.

CDS of *G. bimaculatus* and sex-biased gene expression

The expression level per *G. bimaculatus* gene was determined by mapping reads from each RNA-seq dataset per tissue to the full CDS list using Geneious Read Mapper [102], a program we previously found to be as effective as other read mappers (c.f. [13]), to obtain FPKM per gene. To further confirm that FPKM was robust to mapping programs, we compared FPKM values obtained when using mapping from Geneious and other common mappers including BBmap (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbmap-guide/) and Bowtie2 [103], which yielded Spearman R correlations in FPKM across all genes of $R>0.94$ (e.g., for male 1 brain RNA-seq). Read counts per CDS were converted to FPKM.

Expression level between sexes was compared separately for each tissue-type. We compared expression between males and females for the gonad, somatic reproductive system, brain, and ventral nerve cord across replicates using the program Deseq2 to obtain P-values [104]. The degree of sex-biased expression was obtained using the ratio of average FPKM of the replicates for female and male tissues. Any genes having a two-fold or greater ratio in average expression in one sex and a statistically significant P-value ($P<0.05$) as well as a FPKM of at least 1 in one tissue type was defined as sex-biased [13, 19, 20]. All other genes were defined as unbiased.

Assembly of *G. assimilis* RNA-seq data and protein sequence divergence analysis

To study dN/dS, we generated and assembled RNA-seq CDS for the *G. bimaculatus* sister species *G. assimilis* (Additional file 1: Table S2). Accordingly, we assembled the RNA-seq datasets for *G. assimilis* shown in Additional file 1: Table S2 (490,414,291 trimmed reads in total). For this, the *G. assimilis* reads were *de novo* assembled into contigs using Trinity [105] set to default parameters using Galaxy (https://usegalaxy.org/). We then identified CDS using the Plant tribes pipeline tools [106]. To assess the completeness of the assembled transcriptome, we used BUSCO 3.0.1 [107] to reveal the percentage of the single-copy CDS that was observed in

the standardized Arthropod conserved gene set, and as employed in gVolante ([108] https://gvolante.riken.jp/analysis.html). To refine the CDS for *G. assimilis* we then assessed each CDS in ORF predictor, using its downloadable Perl script [67], to identify the highest quality reading frame per sequence. In ORF predictor, we used the option to include the best-hit (lowest e-value) BLASTX alignment (conducted in BLAST+ v2.7.1, https://blast.ncbi.nlm.nih.gov) [109] of *G. assimilis* versus the reference *G. bimaculatus* protein database (i.e., its translated 15,539 CDS) to define reading frames, and retained all *G. assimilis* CDS at least 150bp long and a start codon.

It is worth noting that while paired-end reads have often been used for RNA-seq assembly, transcriptome assemblies from single-end reads have been successfully employed to obtain CDS (not requiring isoforms) as studied herein [110, 111]. Further to this point, single-end reads have even been applied for *de novo* assemblies in non-traditional model systems [110, 111]. Here, we have the additional advantage of a closely related reference genome to *G. assimilis*, namely *G. bimaculatus* [59], to confirm/identify orthologs.

Ortholog identification and dN/dS

Gene ortholog matches between *G. bimaculatus* and *G. assimilis* were identified using reciprocal BLASTX of the full CDS list between the two species in the program BLAST+ v2.7.1 (https://blast.ncbi.nlm.nih.gov) [109]. Genes having an identical best match sequence (lowest e-value) in both forward and reverse contrasts and $e<10^{-6}$ were defined as putative orthologs. The identified orthologous gene sequences in *G. bimaculatus* and *G. assimilis* were aligned by codons using MUSCLE [112] set to default parameters in the program Mega-CC v7 [113] and gaps removed. Removal of divergent regions from alignments, despite partial loss of sequence regions, improves quantification of protein divergence; thus, highly divergent segments were removed using the program GBlocks v. 0.91b set at default parameters [114, 115].

Using the aligned *G. bimaculatus* and *G. assimilis* CDS, we employed yn00 of PAML using the Yang and Nielson 2000 substitution model, which comprises a maximum likelihood method that accounts for codon usage biases [93, 116], to measure dN, dS, and dN/dS [93] (note that dN/dS measures using Yang and Neilson 2000 [116] were strongly correlated to those using other models; e.g., values from the Pamilo and Bianchi 1993 method [117] had Spearman's $R=0.95$ $P<2X10^{-7}$). Values of dN/dS >1, =1, and <1 suggest a prevalent history of positive

selection, neutral evolution and purifying selection respectively [93]. However, even when <1 for gene-wide measures of dN/dS, elevated values suggest greater roles of positive selection and/or relaxed purifying constraint. Genes that were best matches by reciprocal BLASTX, and for which both values of dN and dS values were <1.5 (and thus were unsaturated [118, 119]), were defined as high confidence orthologs (N=7,220) between *G. bimaculatus* and *G. assimilis* for dN/dS analysis. Thus, the paired alignments and dN, dS, and dN/dS measures herein are conservative.

Positive selection tests

In our core assessments of gene-wide dN/dS using paired contrasts of *G. bimaculatus* and *G. assimilis* from the same genus, any values >1 were interpreted as an indicator of a potential history of positive selection [93]. For conservative analysis, we included only those genes with both dN and dS>0.

In addition to this assessment, we examined positive selection at specific codon sites for the *G. bimaculatus* branch using branch-site analysis in codeml of PAML [93]. As an outgroup species was required for this assessment, we used the recently available assembled and annotated *Laupala kohalensis* genome [59]. Three-way orthologs between *G. bimaculatus*, *G. assimilis*, and *L. kohalensis* were identified using reciprocal BLASTX ($e<10^{-6}$) among each of the three paired species contrasts (our criterion was that for each *G. bimaculatus*-*G. assimilis* paired ortholog, the same matching *L. kohalensis* CDS must be found using reciprocal BLASTX to *G. bimaculatus* CDS and to *G. assimilis* CDS). Genes were aligned by codons using all three-species CDS and filtered using GBlocks [114, 115] and gaps removed as described in "*Ortholog identification and dN/dS*" (note: alignments using this relatively distant outgroup were conducted independently of the paired *Gryllus* alignments). The phylogeny was (*G. bimaculatus*, *G. assimilis*), *L. kohalensis*)) and was unrooted for the PAML free-ratio analysis (Model=1, NSsites=0 in codeml) that was used to determine dN and dS per branch. Only those genes with dN and dS below three [23] in the *L. kohalensis* branch were defined as high confidence orthologs and used for branch-site analysis (unlike the two-species contrasts within *Gryllus* which were more closely related and had a cut-off of 1.5). For genes meeting these criteria, positive selection was assessed on the *G. bimaculatus* branch using Chi-square values for 2XΔln Likelihood between models with and without positive selection was determined as described in

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

Pleiotropy analysis

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

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

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

Gene ontology

Gene ontology (GO) was characterized using the tool DAVID [71]. For this, we identified orthologs to *G. bimaculatus* in the insect model *D. melanogaster*, which has the most well-studied insect genome to date (CDS v6.24 available from www.flybase.org [70]), using BLASTX (https://blast.ncbi.nlm.nih.gov) [109] and the best match (lowest e-value with cut off of $e<10^{-3}$ of *D. melanogaster*). Single direction BLASTX with *G. bimaculatus* CDS as the query to the *D. melanogaster* protein database was used for these assessments (unlike for the more rigorous reciprocal BLASTX analysis used to identify orthologs between the two *Gryllus* species for dN/dS analysis), as reciprocal BLASTX would be overly conservative between these insects from different orders for the purpose of functional characterization and analysis. *D. melanogaster* gene identifiers were input into DAVID [71] to obtain gene putative GO functions and/or classifications.

It is important to note that for functional analysis, more than one *G. bimaculatus* gene could match a single *D. melanogaster* gene (as non-reciprocal BLASTX was used). In such cases, this would suggest two or more paralogs of the same *D. melanogaster* gene existed in the cricket species.

Seminal fluid proteins

As a supplemental reproductive assessment in *G. bimaculatus*, we examined seminal fluid proteins (SFPs). The SFPs are included in the fluids transferred with the sperm to the female reproductive tract, and are thought to play crucial roles in sperm vitality, sperm storage in female organs and in fertilization [52]. A recent proteome analysis of sexual structures in *D. melanogaster* confirmed functions for 125 previously identified SFPs in that insect, and revealed nine newly identified SFPs [52], that may be used as a reference to study SFPs in crickets. Thus, using this very recent list of 134 SFPs in *D. melanogaster* we identified putative orthologs of SFPs in *G. bimaculatus* and between *G. bimaculatus* and *G. assimilis* and assessed the dN/dS values of these genes. All between-order comparisons between *Gryllus bimaculatus* to *D. melanogaster* genes herein, including for these SPFs, are single direction BLASTX.

List of abbreviations

TSSB, tissue-specific sex bias

FPKM, frequency per kilobase million

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

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

Competing interests

The authors declare they have no competing interests.

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Authors' contributions

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

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.

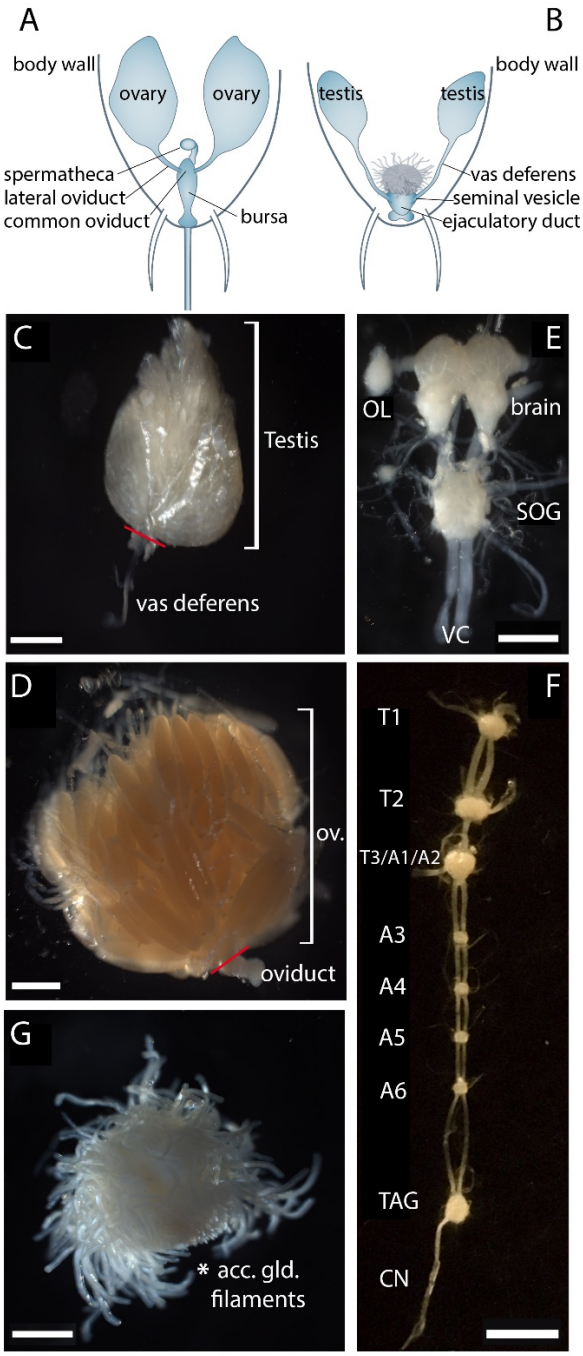

Fig. 1. *Gryllus bimaculatus* reproductive and nervous system tissues studied herein. A schematic of the female (A) and male (B) reproductive systems. In A and B, the gonads and the somatic tissues included in the somatic reproductive system under study are indicated. C-G provide micrographs of various 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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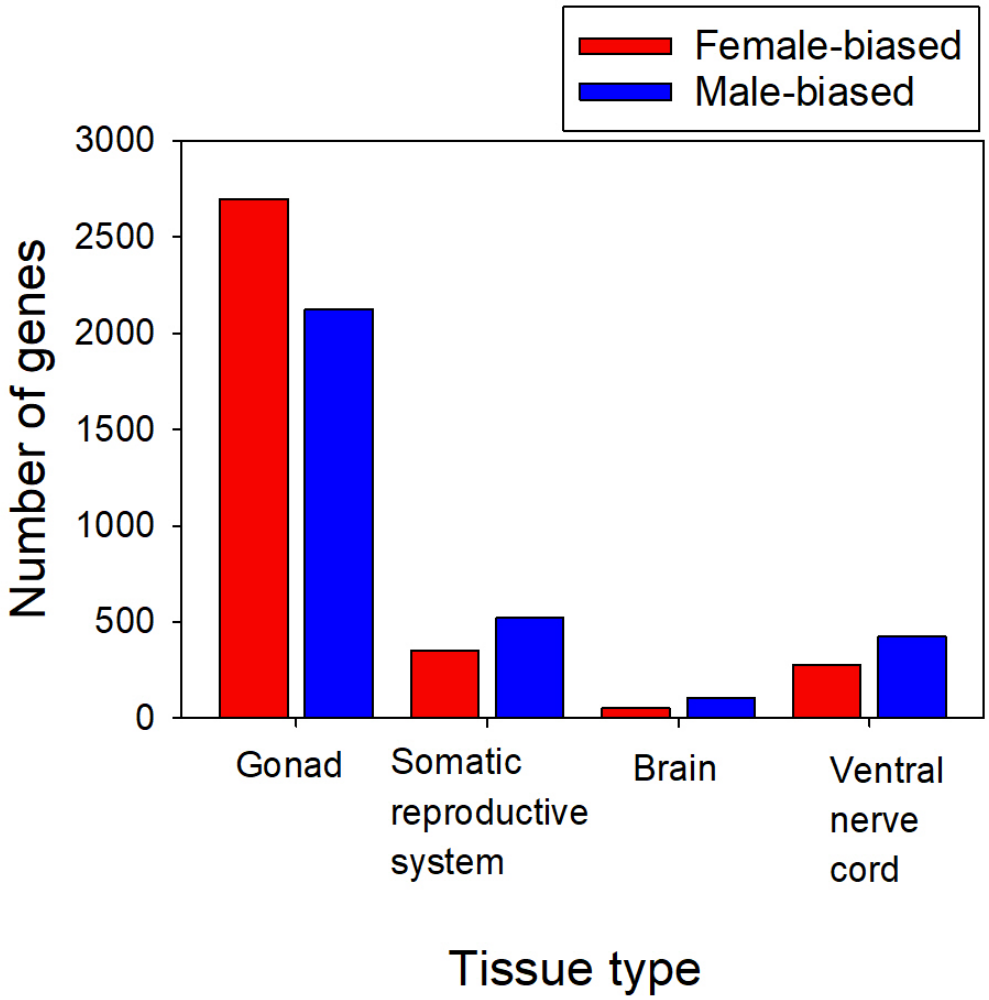
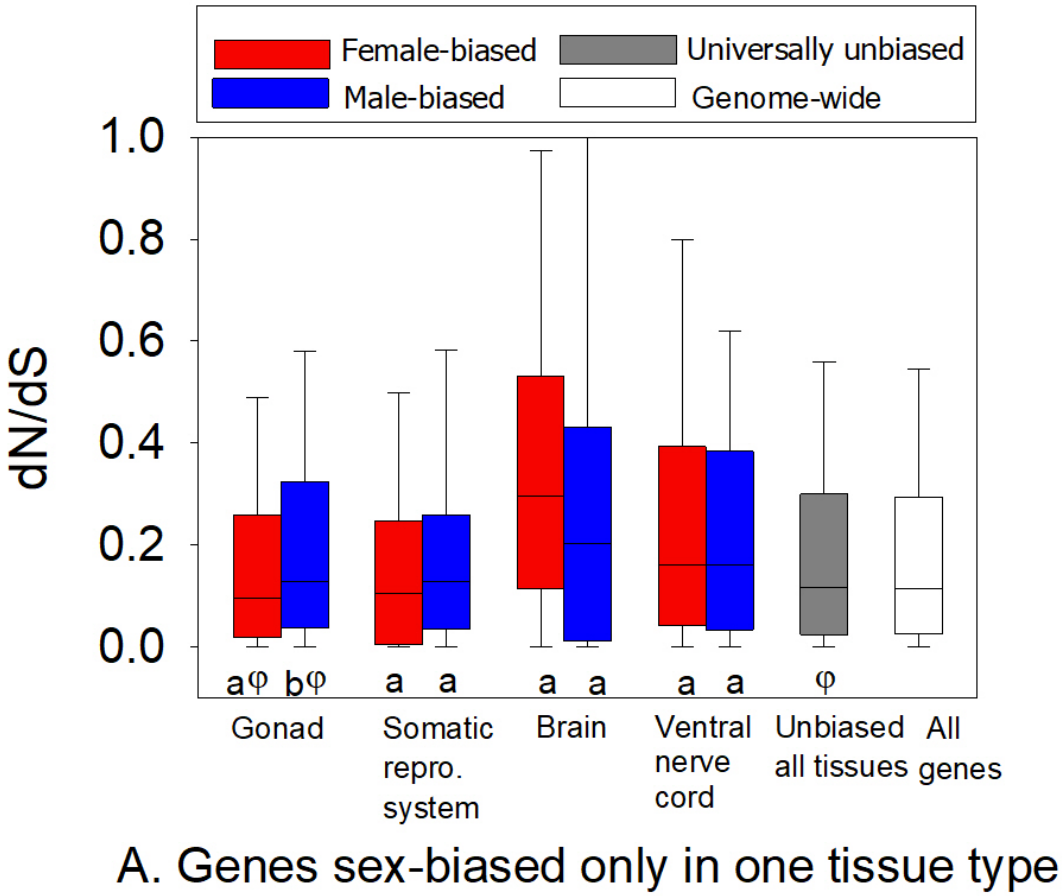
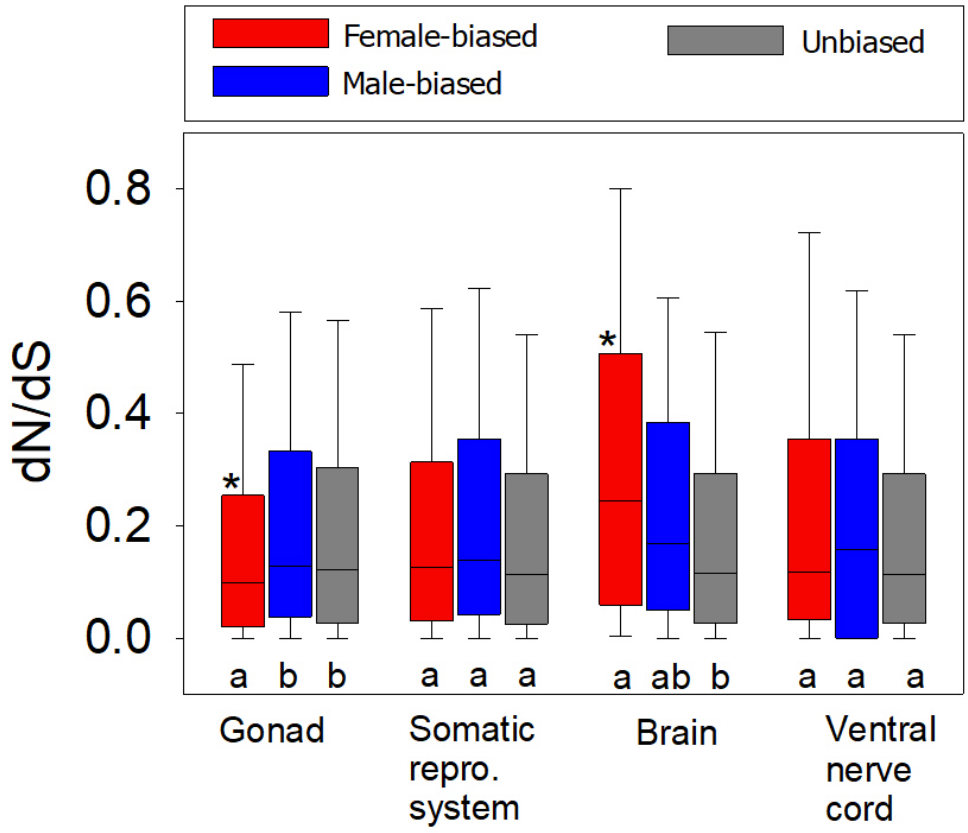


Fig. 2. The number of male-biased and female-biased genes identified in the gonad, somatic reproductive system, brain, and ventral nerve cord across all 15,539 *G. bimaculatus* genes under study (sex-biased indicates a two-fold difference in expression and $P<0.05$). All remaining genes not shown per 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

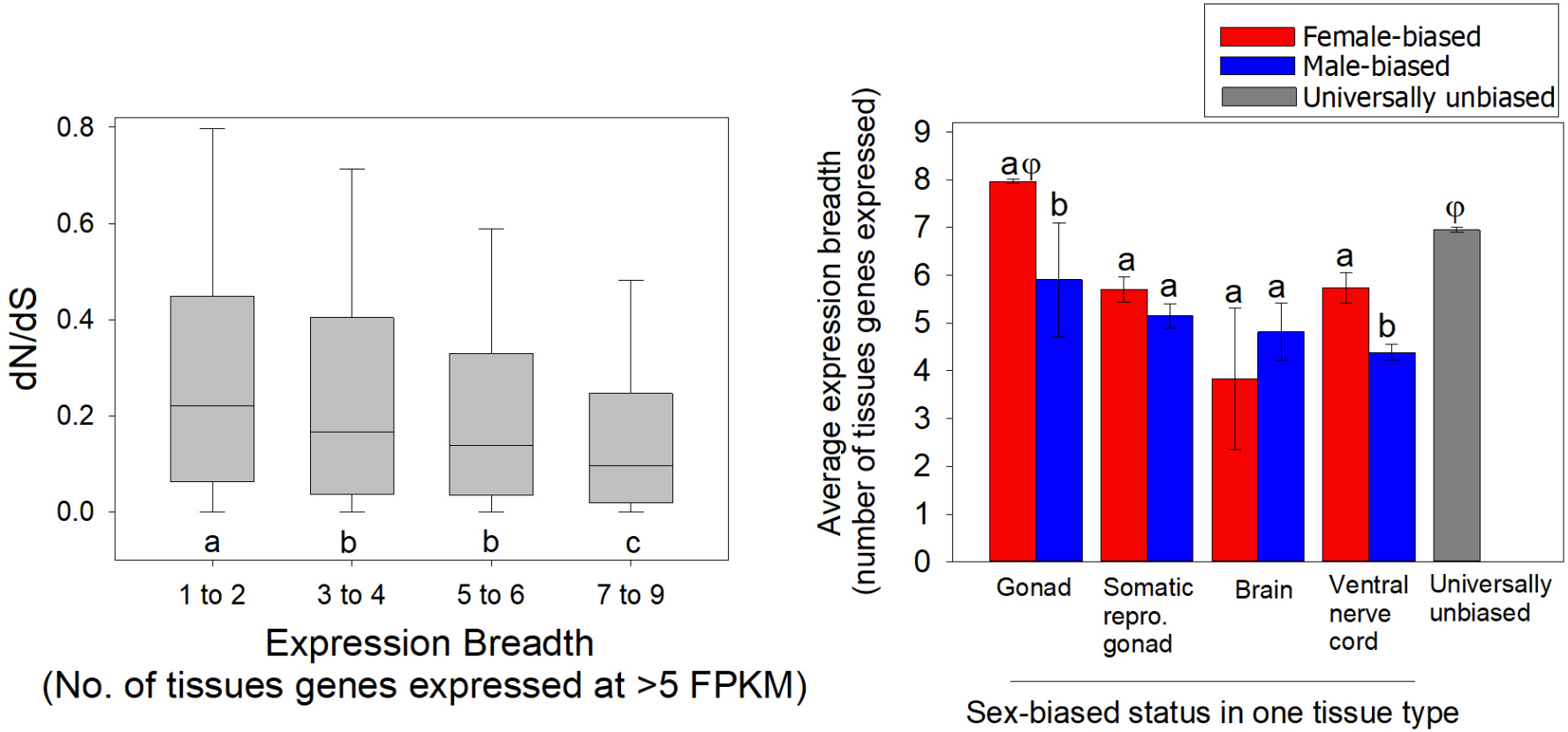


Fig. 4. A) Box and whisker plots of the dN/dS values of genes with respect to their expression breadth, or pleiotropy, for *G. bimaculatus* (N=7,220 genes); B) The expression breadth (average number of tissues with expression of a gene) of genes with female- or male-biased expression in one tissue type 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.