Sex-biased genes expressed in the cricket brain evolve rapidly

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Abstract

Sex-biased gene expression, particularly male-biased expression in the gonad, has often been linked to rapid protein sequence evolution (nonsynonymous to synonymous substitutions, dN/dS) in animals. This evolutionary trend may arise from adaptive evolution and/or from relaxed purifying selection due to 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 provides the neurological foundation for male and female mating behaviors. However, little is known about sex-biased expression of brain genes and their rates of protein sequence evolution. Here, we studied sex-biased gene expression in a hemimetabolous insect, the cricket *Gryllus bimaculatus*. We generated novel male and female 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 gene, as compared to ovary-biased and unbiased genes, which was associated with 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. A tendency for narrow cross-tissue expression breadth, suggesting low pleiotropy, was observed for sex-biased brain genes. We speculate that this feature may permit relaxed purifying selection, which in turn, may allow enhanced freedom for adaptive protein functional changes in these sex-biased genes. Our results demonstrate that sex-biased expression in the male gonad, and sex-biased 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 plausible roles of the mating biology of this cricket in shaping the observed patterns.

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

1 INTRODUCTION

Sexual dimorphism in animals is thought to be driven by differential gene expression, as most genes are common to both sexes (Ellegren & Parsch, 2007, Grath & Parsch, 2016, Ingleby et al., 2014). Sex-biased gene expression, and particularly male-biased gene expression, has been widely linked to rapid protein sequence evolution in studied animals (reviewed by (Ingleby et al., 2014, Grath & Parsch, 2016, Ellegren & Parsch, 2007)). 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 (Haerty et al., 2007, Ellegren & Parsch, 2007, Jagadeeshan & Singh, 2005, Jiang & Machado, 2009, Zhang et al., 2007, Meisel, 2011, Grath & Parsch, 2012, Whittle & Extavour, 2019b, Perry et al., 2015) (but see also (Dorus et al., 2006)). This pattern was also recently observed for the gonads of red flour beetles (*T. castaneum*) (Whittle et al., 2020). 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 (Zhang et al., 2004, Haerty et al., 2007, Proschel et al., 2006, Swanson et al., 2001), but could also reflect low pleiotropy that may relax purifying selection (Mank & Ellegren, 2009, Dean & Mank, 2016, Assis et al., 2012, Zhang et al., 2007, Whittle & Extavour, 2019b). Nonetheless, the pattern of accelerated evolution of male-biased genes is not universal, as an opposite pattern of rapid evolution of female-biased, including ovary-biased, genes has been found in some holometabolous insects, namely mosquitoes (*Aedes*, *Anopheles*) (Whittle & Extavour, 2017, Papa et al., 2017). 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, and/or reflect variation in male- and female-related purifying selection among insects (Whittle & Extavour, 2017). At present however, given the narrow scope of insects studied to date, further investigation of sex-biased expression in the reproductive system and protein evolution is warranted, particularly in models outside the Holometabola.

While studies of sex-biased expression and its link to protein sequence evolution have largely focused on the reproductive system, a major, and markedly understudied structure, in terms of molecular evolution, is the brain. The brain is a major tissue type providing the neurological basis for the mating behaviors of courtship, intrasex competition, mate-choice, and post-mating male-female responses (Wright & Mank, 2013, Mank et al., 2007, Dalton et al., 2010, Naurin et al., 2011). Male and female differences in gene expression *per se* in the brain have been examined in some insects and vertebrates (Mank et al., 2007, Small et al., 2009, Santos et al., 2008, Naurin et al., 2011, Catalan et al., 2012, Shi et al., 2016, Tomchaney et al., 2014, Ingleby et al., 2014, Wright & Mank, 2013, Yang et al., 2016, Jagadeeshan & Singh, 2005, Khodursky et al., 2020, Huylmans & Parsch, 2015). Further, in *Drosophila*, analyses of a small number of neural genes are directly

connected to mating functions and behaviors (Drapeau et al., 2003, Kadener et al., 2006, Dauwalder, 2008). However, there is a striking paucity of data on whether and how sex-biased expression in the brain is associated with protein sequence evolution (Wright & Mank, 2013). Moreover, the minimal research available from birds, humans and flies have suggested that male and female expression may have different effects on the rates of protein evolution, depending on the system (Mank et al., 2007, Shi et al., 2016, Khodursky et al., 2020) (see also some brain-related (Biswas et al., 2016) and composite-tissue analyses (Catalan et al., 2018, Congrains et al., 2018)), and the causes of those patterns remain poorly understood. It is therefore evident that additional study of sex-biased expression in the brain, is needed, particularly with respect to 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 in an outgroup order to the Holometabola (Misof et al., 2014). 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 (Kulkarni & Extavour, 2019). 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* (Mito & Noji, 2008, Donoughe & Extavour, 2016). 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 (Vedenina & Shestakov, 2018, Gee, 2019), increased rates of male transfer of spermatophores to females in the presence of other males (Lyons & Barnard, 2006), and the complete mixing of sperm from multiple males in the storage organ of the female reproductive tract, the spermathecae (Morrow & Gage, 2001, Simmons, 1986). In addition, females have shown preferences for novel and young mating partners (Zhemchuzhnikov et al., 2017), and for males with larger body size and higher quality auditory signals (Zhemchuzhnikov et al., 2017, Bateman et al., 2001). Females also exhibit a post-mating behaviour of removing spermatophores of non-favored males from their reproductive tract (Simmons, 1986), 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 (Matsumoto & Sakai, 2000, Sakai et al., 2017, Haberkern & Hedwig, 2016). 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) in *G. bimaculatus,* and evaluate their relationships to protein sequence evolution. We report that male-biased gene

expression in the gonad is linked to rapid protein sequence evolution (dN/dS), as compared to unbiased and female-biased genes. However, we observed no consistent 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 (Haerty et al., 2007, Clark & Swanson, 2005, Swanson & Vacquier, 2002, Swanson et al., 2004, Panhuis & Swanson, 2006). 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, a proxy for pleiotropy (Mank & Ellegren, 2009), which may in itself accelerate protein sequence 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, an evolutionary dynamic that has been suggested in some studies (Mank & Ellegren, 2009, Otto, 2004, Larracuente et al., 2008, Mank et al., 2008, Meisel, 2011). We consider the putative roles of the male and female mating biology of *G. bimaculatus* in shaping the present finings.

2 RESULTS AND DISCUSSION

2.1 Identification of sex-biased genes

The CDS of our main target species *G. bimaculatus* were obtained from its recently available genome (Ylla et al., 2020). The annotated genome had 17,714 predicted transcripts (after selecting the longest CDS per gene; (Ylla et al., 2020)). For this gene set, we extracted the CDS with a start codon, no ambiguous nucleotides, and at least 150bp in length, yielding 15,539 CDS for study (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 (biological replicates (Congrains et al., 2018) and read counts in Table S1). The tissues included the gonad (testis for males, ovaries for females), somatic reproductive system, brain and ventral nerve cord (Fig. 1A-F). The somatic (non-germ line related) reproductive system herein for males included the pooled vasa deferentia, seminal vesicles and ejaculatory duct, and for females included 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 sample, reads were mapped to the entire *G. bimaculatus* CDS list to determine FPKM. We found FPKM was strongly correlated between the biological replicates, with Spearman's $R \geq 0.92$ ($P<0.05$; Fig. S1; one exception being the male somatic reproductive system, $R=0.71$, $P<0.05$), indicating high reproducibility of expression profiles. Sex-biased gene expression was then determined separately for each of the four paired tissue types using a cut-off of

two-fold higher expression in one sex versus the other and a P value of <0.05 (see Methods). All genes per tissue that were not sex-biased in expression were defined as unbiased, such that all studied genes were assigned to one of three categories (male-biased, female-biased, unbiased; see Methods for details).

As shown in Fig. 2, sex-biased gene expression was most common in the gonadal tissues, where 4,822 (31.0%) of all *G. bimaculatus* genes under study were sex-biased in expression: 2,698 (17.4%) and 2,124 (13.7%) genes had ovary-biased and testis-biased expression respectively, and a total of 10,717 (69.0%) were unbiased in expression. By comparison, sex-biased gene expression was markedly less common in the somatic reproductive system, where only 5.6% of genes were sex-biased, with 353 (2.3%) and 520 (3.3%) genes showing female- and male-bias respectively. As compared to the gonad, markedly fewer genes exhibited female-biased and male-biased expression in the nervous system tissues, where 4.5% of 15,539 *G. bimaculatus* genes had sex-biased expression in the ventral nerve cord: 279 (1.8%) and 425 (2.7%) were female- and male-biased respectively (Fig. 2). For the brain, only 1.0% of genes were sex-biased in expression, with 51 (0.33%) and 106 (0.68%) being female- and male-biased respectively, an uncommonness that notably has also been suggested for brains of *D melanogaster* (Huylmans & Parsch, 2015). Together, using the present criteria, it is evident that sex-biased gene expression is most common in the gonad, which is consistent with high phenotypic and transcriptional dimorphism of these sex organs in animals (Whittle & Extavour, 2017, Harrison et al., 2015, Meisel, 2011, Zhang et al., 2004, Whittle & Extavour, 2019a, Whittle et al., 2020, Parisi et al., 2004, Arbeitman et al., 2004, Oliver et al., 2010, Small et al., 2009). In contrast, sex-biased gene expression is markedly less common in the somatic reproductive system and ventral nerve cord, and least common in the brain of *G. bimaculatus*.

2.2 Molecular evolution of sex-biased genes

2.2.1 Rates of evolution

We aimed to assess whether and how evolutionary pressures on protein sequence divergence, measured as dN/dS, varied with sex-biased gene expression. Unlike *Drosophila, Gryllus* is currently an emerging model genus with limited genomic resources outside the recent *G. bimaculatus* genome (Ylla et al., 2020). Thus, to measure dN/dS, we generated and assembled novel RNA-seq data for its sister species *G. assimilis* to obtain a CDS list for that organism (Table S2). Two-species assessments of dN/dS have been repeatedly shown to be an effective means to study divergence of sex-biased genes (*cf.* (Meisel, 2011, Jaquiery et al., 2018, Baines et al., 2008, Mank et al., 2007, Assis et al., 2012, Whittle & Extavour, 2017) including for organisms with few available genomes, as is the case with *Gryllu*s. Details of the *G. assimilis* assembly, including BUSCO scores (Seppey, Manni et al. 2019), and ORF predictions (Min et al., 2005) are provided in Text File S1. Following reciprocal BLASTX (Altschul et al., 1990) 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, 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 unsaturated substitution rates and a close phylogenetic relatedness between these two sister *Gryllus* species. Notably, the 90th percentile of dN values was 0.042 and 95th percentile was 0.094, also each well below 1, which facilitates precise ortholog detection (by a protein similarity search, reciprocal BLASTX (Altschul et al., 1997)), and indicates the studied gene set includes rapidly evolving genes in the genome(s) (those with 22-fold higher dN than the median).

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 the four 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 Table S3). We emphasize that the TSSB status of a gene indicates that there is a tissue-specific sex difference in expression (female-biased or male-biased status) that is not observed in other tissues (unbiased status), and does not imply that this gene is not expressed in any other tissue. Further, we identified those genes with universally unbiased expression in all four tissues types as a control (N=3,449; 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, while the remainder had mixed statuses among tissues).

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

The dN/dS values of sex-biased$_{TSSB}$ genes for each of the four paired *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 for each 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$_{TSSB}$ genes evolved faster than ovary-biased$_{TSSB}$ and universally unbiased genes (MWU-tests P<0.001 and 0.05 respectively, Fig. 3A). Further, sex-biased brain genes, while uncommon (Fig. 2, Table S3), evolved exceptionally rapidly, particularly with faster evolution of the female-biased$_{ALL}$ brain genes than of unbiased$_{ALL}$ genes (MWU-test P=0.047, Fig. 3B, for further details see below section "*Rapid evolution of sex-biased brain genes*"). In turn, no statistically significant differences in dN/dS were observed among male-biased, female-biased, or unbiased genes from the somatic reproductive system or ventral nerve cords (using the TSSB genes and universally unbiased genes in Fig. 3A, or using ALL genes per tissue type in Fig. 3B (MWU-tests P>0.05)). In this regard, it is evident that the primary patterns in

this cricket system include the rapid evolution of testis-biased genes and of sex-biased brain genes, particularly female-biased brain genes.

We next assessed the expression breadth across tissues (using nine tissues, the four paired female and male 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 (Whittle & Extavour, 2017, Meisel, 2011, Mank et al., 2008, Dean & Mank, 2016, Mank & Ellegren, 2009, Otto, 2004, Larracuente et al., 2008, Assis et al., 2012). Genes were categorized into bins based on expression at >5 FPKM in 1-2, 3-4, 5-6, and 7-9 tissues (note: we choose a direct determination of expression breadth (Haerty et al., 2007, Meisel, 2011, Duret & Mouchiroud, 2000), see also (Yanai et al., 2005)). 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 expression breadth. The lowest dN/dS values were found in genes transcribed in 7-9 tissues under study (median dN/dS=0.096), and the highest in genes expressed in 1-2 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$_{TSSB}$ genes had markedly lower expression breadth than ovary-biased$_{TSSB}$ genes and than universally unbiased genes (MWU-tests $P<0.001$). Female-biased$_{TSSB}$ brain genes, had the smallest median expression breadth of all studied categories, which despite their low N value (Table S3), was statistically significantly lower than that of the universally unbiased genes (MWU-test $P=0.021$, Fig. 4B). Thus, this suggests a plausible connection between rapid protein sequence evolution and pleiotropy for sex-biased genes from the brain and gonad, either due to relaxed constraint in itself, and/or due to an associated freedom to evolve functional changes under low purifying constraint (Whittle & Extavour, 2017, Meisel, 2011, Mank et al., 2008, Dean & Mank, 2016, Mank & Ellegren, 2009, Otto, 2004, Larracuente et al., 2008, Assis et al., 2012)(see below section "*Evidence of A History of Positive Selection in Sex-Biased Gonadal and Brain Genes*"). Given these results, in the following sections, we focus in detail on the dN/dS patterns for sex-biased genes in the brain and the reproductive system in Fig. 3 and Fig. 4, and consider further the putative roles of pleiotropy and positive selection in affecting their molecular evolution.

2.3 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 sex-biased$_{TSSB}$ brain status (Table S3). 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 unbiased$_{ALL}$ brain-expressed genes (0.115), wherein its contrast to the unbiased set was, as aforementioned, statistically significant (MWU-test P=0.047). Thus, the statistical tests suggest significant patterns in the brain (Fig. 3AB). Nonetheless, given the growing recognition that P-values alone may not always provide a full perspective to discern important biological patterns (Amrhein et al., 2019), particularly for samples with small sizes (such as for sex-biased brain genes studied here, Fig. 2), we aimed to further assess these results by examining the sex-biased brain$_{ALL}$ genes on a gene-by-gene basis, including their rates of evolution and their putative functions, as shown in Table 1. Using this approach, we show that 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; this value is shown in 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 some previous studies (Catalan et al., 2018, Congrains et al., 2018)) remains rare, rapid evolution of female-biased brain genes has been reported in some bird embryos (Mank et al., 2007), and in some autosomal genes in flies (Khodursky et al., 2020). However, an opposite pattern of rapid evolution of male-biased brain genes for several stages of development was reported in humans (Shi et al., 2016). The avian result was interpreted as possibly reflecting selective pressures arising from brain-regulated mating behaviors (Mank et al., 2007). 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.

As shown in Table 1, we examined putative GO functions for the sex-biased brain genes (Fig. 3B), including ALL (sex-biased) genes and the subset of genes that had TSSB status (Fig. 3A, 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 (Altschul et al., 1990) of the *G. bimaculatus* entire CDS list to the CDS of well-studied insect model *D. melanogaster* (Gramates et al., 2017) to identify its putative orthologs, which were assessed in the GO tool DAVID (Huang da et al., 2009) (note that single direction BLASTX was used for functional analysis, rather than the reciprocal BLASTX approach that was used for *G. bimaculatus* and *G. assimilis* contrasts for dN/dS, as we considered the latter approach overly conservative for predictive functional analysis, and that it might impede detection of putative paralogs to a gene in *G. bimaculatus*; see details in Methods). We found that the predicted functions of female-biased brain genes included involvement in neurotransmission (*AP-1-2β*), apoptosis (*D. melanogaster* ID number CG2681), and DNA binding (CG11403) (Table 1). Remarkably, certain brain expressed genes were predicted to be involved in sexual processes or organs, including multicellular reproduction (CG10407), inter-male aggressive behavior (*tramtrack*) (Yamamoto et al., 1998) and the ejaculatory bulb (*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 (Musacchio & Perrimon, 1996), and conserved in flies and mosquitoes (MacLaren et al., 2004). Collectively, the genes that are upregulated in the cricket female brain may play significant roles in female behaviors, such as mating functions, possibly contributing to their rapid divergence.

Despite a tendency for accelerated evolution, not every female-biased *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 (Boekhoff-Falk & Eberl, 2014, Todi et al., 2005). We speculate that a history of strong constraint reflected in dN/dS of this female-biased brain gene could indicate an essential role of negative phonotaxis (potentially relevant to avoiding predators (Schneider et al., 2017)), 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 (Sakai et al., 2017, Lankheet et al., 2017). Thus, it is tempting to speculate that the strong purifying selection on this female-biased 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 (Schneider et al., 2017, Lankheet et al., 2017), in the antennae, which are involved in male-female attraction and male-male aggression and contain neurons involved in sex-related pheromonal signalling (Boekhoff-Falk & Eberl, 2014, Yoritsune & Aonuma, 2012, Murakami & Itoh, 2003), and in the terminal abdominal ganglion, which has been linked to mating behaviors (Sakai et al., 2017). 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 brain genes, a range of predicted functions were observed. For instance, multiple genes were associated with phagocytosis (six of 45 genes), and early-stage development (three genes). In addition, some genes had predicted sexual roles. In particular, a putative *G. bimaculatus* ortholog (GBI_17358-RA) of 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 putatively significant roles in both brain and testicular functions 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 the *G. bimaculatus* to *D. melanogaster* BLASTX used for functional analysis was one-directional, and thus allowed more than one cricket gene to 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* remain under assessment, its key predictive classifications include olfactory function, post-mating behavior, and mating plugs (flybase.org, (Gramates et al., 2017)); further suggesting a possible function in male-female brain mediated sexual behaviors in *G bimaculatus*. We also discovered that the male-biased brain genes included a putative ortholog of *Angiotensin converting enzyme*, a gene whose functions include involvement in *D. melanogaster* spermatid nucleus differentiation and sperm individualization (Hurst et al., 2003). This gene had a dN/dS value of 0.236, which is double the median of universally unbiased genes (Table 1). In this regard, multiple male-biased brain genes exhibit rapid protein-level divergence and thus are candidates to have potential sex-related roles in this taxon.

While the rapid protein sequence evolution of sex-biased brain genes in Table 1 could largely result from relaxed purifying constraint and neutral protein sequence changes, as suggested by their low pleiotropy (Fig. 4), the low pleiotropy could in principle also act to accelerate protein changes by more readily allowing adaptive functional changes (Whittle & Extavour, 2017, Meisel, 2011, Mank et al., 2008, Dean & Mank, 2016, Mank & Ellegren, 2009, Otto, 2004, Larracuente et al., 2008, Assis et al., 2012). We suggest here that several features of the mating biology of *G. bimaculatus* might cause episodic adaptive evolution and underlie the high dN/dS values observed herein (see also below section "*Evidence of A History of Positive Selection in Sex-Biased Gonadal and Brain Genes*"). For instance, *G. bimaculatus* exhibits aggressive male-male fighting and mate guarding (Vedenina & Shestakov, 2018, Gee, 2019) and males transfer larger spermatophores to females when in the company of rival males (Lyons & Barnard, 2006). Such behaviors are likely mediated by the male brain. This could, in principle, lead to sexual selection pressures on the male-biased brain genes, which might give rise to adaptive changes in dN/dS. It is also feasible that inter-locus sexual conflict could contribute to rapid evolution of both sets of male- and female-biased brain genes (Pennell et al., 2016, Mank et al., 2013, Koene et al., 2013). In other words, it is possible that aggressive male-male behaviors in *G. bimaculatus* (Vedenina & Shestakov, 2018, Gee, 2019), directed by male-biased brain genes, may negatively affect female fitness. This might be predicted to lead to an adaptive response in female-biased brain genes (e.g., genes

regulating the behavior of removal of spermatophores of certain males by females after mating (Bateman et al., 2001)), causing an evolutionary "arms race" that could in principle accelerate evolution of proteins of both types of genes (Mank et al., 2013, Ellegren & Parsch, 2007). Taken together, we suggest that there are several plausible mechanisms related to mating biology of this taxon that may underlie the observed patterns for sex-biased brain genes (Table 1), mediated by low pleiotropy and, in turn, an enhanced potential for adaptive evolution.

It should be recognized that while sex-biased brain genes, by definition, exhibit differences in gene expression between the female and male brain, these sex-biases may reflect differences in cellular expression and/or allometric scaling differences in male and female brains. As an example, the female-biased brain gene *crinkled* (Table 1) may be more highly expressed in all female than male brain cells, the female brain may typically contain more cells that express this gene (Montgomery & Mank, 2016), and/or the gene may be more highly expressed in cells from a particular sub-region(s) of the brain (Tuller et al., 2008), whereby the size or cell composition of the subsections may vary between females and males (Montgomery & Mank, 2016). Further studies of gene expression, and the allometry, of subsections of the brain in males and females would be needed to distinguish among these possibilities, and to better understand the factors underlying differences in male and female brain expression.

2.4 Rates of Evolution of Sex-biased Genes from the Reproductive System

2.4.1 Rapid evolution of testis-biased genes

With respect to sex-biased expression in the gonads and dN/dS, which has been more commonly studied as compared to the brain in insects, we observed marked differences in rates of protein sequence evolution among sex-biased$_{TSSB}$ genes. First, dN/dS decreased progressively from testis-biased$_{TSSB}$ (median=0.128), to universally unbiased genes (median=0.118) to ovary-biased genes (median=0.097, each paired MWU-test $P<0.05$; see also Fig. 3B). Thus, the rate differences were most marked between testis-biased$_{TSSB}$ and ovary-biased$_{TSSB}$ genes, with intermediate values for those with universally unbiased expression. The rapid evolution of testis-biased genes in this cricket concurs with patterns observed for *Drosophila* (Ellegren & Parsch, 2007, Zhang et al., 2007, Meisel, 2011, Whittle & Extavour, 2019a, Grath & Parsch, 2016, Zhang et al., 2004, Proschel et al., 2006, Perry et al., 2015, Assis et al., 2012, Jiang & Machado, 2009) (see results in a related fly (Congrains et al., 2018)), and recent findings in beetles (*Tribolium castaneum*) (Whittle et al., 2020). However, the results are opposite to the rapid evolution of ovary-biased (or ovary-specific) genes previously reported in the mosquitoes *Aedes* and *Anopheles* (Whittle & Extavour, 2017, Papa et al., 2017). In this regard, it is worth considering possible reasons for variation in the effects of sex-biased gonadal expression among these insect taxa.

Given that *Gryllus* (Orthoptera) is a distant outgroup to the two Diptera groups (*Drosophila* and *Aedes/Anopheles*) and the Coleoptera (*Tribolium*) (Misof et al., 2014) it may be suggested, based on the collective anecdotal evidence, that there could be a shared ancestral effect of testis-biased expression in *Drosophila-Tribolium-Gryllus* (Ellegren & Parsch, 2007, Harrison et al., 2015, Zhang et al., 2004, Whittle et al., 2020)) and a derived effect of rapid evolution of ovary-biased (or ovary-specific) genes in *Aedes/Anopheles* (Whittle & Extavour, 2017, Papa et al., 2017). Under this hypothesis, the pattern observed for studied *Aedes* and *Anopheles* species would be a derived feature, and could reflect variation in mating biology among these insects. For example, although both *Drosophila* and *Aedes aegypti* (the species studied in *Aedes* (Whittle & Extavour, 2017)) are polyandrous and thus prone to sperm competition, the polyandry is thought to be relatively weak in the mosquitoes (Helinski et al., 2012). Further, this mosquito can exhibit intensive male swarming during courtship that may involve female-female mosquito competition and/or male-mate choice (Oliva et al., 2014, Whittle & Extavour, 2017). In addition, nonporous mating plugs are formed in the female mosquito reproductive tract after mating, which prevent sperm competition (Oliva et al., 2014) and thus differ both from the mating plugs formed in *Drosophila*, which allows sperm transfer from competitor males (Avila et al., 2015, Manier et al., 2010), and from observations of complete sperm mixing from multiple males in *Gryllus* (Simmons, 1986). Any of these mating-related features could in principle give rise to sexual selection and the relatively faster evolution of ovary-biased than testis-biased genes in mosquitoes (Whittle & Extavour, 2017), and not in the other studied insects. In addition, relaxed purifying selection, possibly due to low pleiotropy, may be more common for ovary-biased genes in the mosquitoes (Whittle & Extavour, 2017), as inferred for testis-biased (or male-biased) genes in some organisms, including flies (Ghiselli et al., 2018, Allen et al., 2018) , and suggested for the crickets studied here (Fig. 4B). Studies in even more insect models, particularly in monogamous versus polyandrous species (Harrison et al., 2015), and in additional insects with various degrees of male-male or female-female competition and with and without impermeable mating plugs (Whittle & Extavour, 2017), 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 in *G. bimaculatus* 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 (Trotschel et al., 2019). Ovary-biased$_{TSSB}$ genes were particularly involved in fundamental processes such as transcription 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 male-biased genes from the three other tissues, including from the brain (MWU-tests 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, Table S3), 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*.

2.4.2 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, Table S3). The results are shown in Fig. S2 and are described in 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* or *G. assimilis*, Fig. S2) is sufficient to predict elevated pairwise dN/dS.

2.5 Sex-biased genes from the somatic reproductive system

In contrast to the gonad, the lack of differences in dN/dS of male-biased$_{TSSB}$ and female-biased$_{TSSB}$ genes, and between those groups and the universally unbiased genes, for the somatic reproductive system (MWU-tests P>0.05, Fig. 3A; and when using ALL genes, Fig. 3B) is surprising, given the roles of these sexual tissues in reproductive success and fitness, including for the female tissues (oviducts, spermathecae, and bursa). 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 (Swanson et al., 2004, Swanson & Vacquier, 2002) (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). 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 (Haerty et al., 2007). Our results from unmated

Gryllus suggest no differences in dN/dS between female-biased$_{TSSB}$ somatic reproductive system genes and the universally unbiased genes or the genome as a whole (Fig. 3).

It is also notable that markedly fewer genes were sex-biased in expression in the somatic reproductive system as compared to the gonads (Fig. 2). One possible reason is that there may be an inherent variation in expression among individuals for the male somatic reproductive system (which had the least strongly correlated FPKM among replicates of all nine tissue types, Fig S1H), such that a consistent male to female difference in expression may be less apt to be observed for those tissues. Another possibility is that the gonads in adults are continuously supporting the dynamic process of gametogenesis (Williamson & Lehmann, 1996, Pauli & Mahowald, 1990) causing high female and male expression differentiation (Fig. 2), while the somatic reproductive system, particularly in unmated tissues as studied here, may be less dynamic, and thus exhibit less potential for female to male differential transcription.

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

For thoroughness in the study of reproductive structures, given that genes from the male-accessory glands, including seminal fluid protein (SFPs), have been linked to rapid evolution in species of *Drosophila* (Sepil et al., 2019, Haerty et al., 2007), and in some identified cricket SFPs based on partial gene sets attained from assembled reproductive transcriptome sequences for species such as *G. firmus*, *G. pennsylvanicus* and *Allonemobius fasciatus* (Andres et al., 2013, Braswell et al., 2006, Andres et al., 2006), we assessed expression and evolution of such genes in *G. bimaculatus*. The findings for the male-accessory glands are described in detail in Text File S1 and Table S5 and showed that *G. bimaculatus* genes that had expression solely in the male accessory glands rarely had a high confidence ortholog in its sister species *G. assimilis*. Thus, this suggests 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 particular sexual tissue (Tautz & Domazet-Loso, 2011, Haerty et al., 2007).

For the study of SFPs, we used the recently available gene list of 134 SFPs from the species *D. melanogaster* as the reference, as a species has the most intensively-studied insect genome and expression/proteomics data for the identification of these genes (Sepil et al., 2019). The results are described in Text File S1 and Table S6. We found that only 20 *D. melanogaster* 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 the two species of *Gryllus* (note the stringent criteria used for the intra-*Gryllus* ortholog matches, see Methods). 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). Positive selection was indicated for the gene matching an odorant binding SFP protein *Obp56g*, with dN/dS>1 (Table 3). Together, we conclude that the putative SFPs in

the crickets studied herein have evolved very rapidly, a feature shared with SFPs of *D. melanogaster* (Sepil et al., 2019, Haerty et al., 2007), 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 (Fedorka et al., 2011, Heifetz et al., 2000), and males may alter relative production of different SFPs when exposed to male rivals (Fedorka et al., 2011). 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 (Simmons, 1986, Bateman et al., 2001), which comprises a mechanism of female-choice in this species (Bateman et al., 2001), potentially accelerating SFP evolution.

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

Finally, we considered the incidences of positive selection among the 7,220 genes with between-species *Gryllus* orthologs. Gene-wide dN/dS>1 was taken as evidence of positive selection (Clark et al., 2006, Hill et al., 2019, Hunt et al., 2011, Ghiselli et al., 2018, Yang, 2007, Nielsen et al., 2005, Torgerson et al., 2002, Swanson et al., 2001, Buschiazzo et al., 2012)). The use of dN/dS>1 across a gene is a stringent and conservative means to identify positive selection (Buschiazzo et al., 2012, Swanson et al., 2001), as nonsynonymous codon changes should be sufficiently common to cause the ratio to exceed 1. We found that 1.63% of all the 7,220 *G. bimaculatus*-*G. assimilis* gene orthologs (N=118 genes) showed dN/dS>1.

We then considered whether dN/dS values of the sex-biased$_{TSSB}$ genes from the gonad (Table 4), which had the highest N values of all tissues analysed (Table S3), were consistent with the aforementioned hypothesis that reduced gene pleiotropy, or expression breadth (and thus purifying selection), may lead to an enhanced opportunity for functional evolution of genes (Mank & Ellegren, 2009, Whittle et al., 2020, Mank et al., 2008, Otto, 2004, Assis et al., 2012, Larracuente et al., 2008, Meisel, 2011). We found that the percent of genes with positive selection increased from ovary-biased$_{TSSB}$ genes (1.02%, 19 of 1,858) to universally unbiased genes (1.91%, 66 of 3,449) and testis-biased$_{TSSB}$ 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, expression breadth of these genes decreased from all ovary-biased$_{TSSB}$ (average expression breadth of 7.97±0.04 (standard error)), to universally unbiased (6.95±0.05) and to testis-biased$_{TSSB}$ genes (5.90±0.18 tissues; (MWU-tests P<0.001 for each of three paired contrasts (Fig. 4B). 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 (MWU-test P<0.05, Table 4). These patterns observed using whole-gene dN/dS values in this cricket system provide empirical data consistent with the theoretical proposition that that the fewer tissues a gene is expressed in, the more its adaptive evolutionary potential may

be enhanced, likely by relaxing purifying selection that may be imposed by multiple cross-tissue functions (Mank & Ellegren, 2009, Otto, 2004, Larracuente et al., 2008, Mank et al., 2008, Meisel, 2011). Our data thus specifically suggest that this hypothesis can apply to sex-biased genes (Mank & Ellegren, 2009). We note nonetheless that given the close relatedness between these two *Gryllus* species studied here, this may potentially elevate observed dN/dS for some genes (Mugal et al., 2014) , and thus further studies of dN.dS using additional *Gryllus* species as data becomes available will help test the rigor of these patterns across the genus.

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 (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 was expressed primarily in the male brain (number tissues with >5 FPKM =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 supplemental 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)(Yang, 2007), based on three-way alignments of *G. bimaculatus*, *G. assimilis* and an available cricket outgroup species *Laupala kohalensis* (Ylla et al., 2020, Blankers et al., 2018). The results are described in Text File S1 and Table S7. It should be emphasized the assessment is inherently very conservative given it only includes the subset of genes with high confidence three-way reciprocal orthologs among the three species (that is, only 26,7% of the 7,220 genes with orthologs in the two *Gryllus* species had three-species orthologs, see Methods, and Text File S1). Nonetheless, we found that a non-negligible portion of the male- and female-biased$_{TSSB}$ 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 (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, Table S7) showed positive selection using branch-site analysis (GBI_05906-RA, GBI_09477-RB, GBI_05452-RB, Table S7). This result is consistent with the hypothesis of a history of adaptive evolution in the brain, possibly elevating dN/dS (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 (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, as suggested in flies (Swanson et

al., 2004, Prokupek et al., 2008), in this cricket taxon. Further studies using more powerful branch-site positive selection tests (Yang, 2007) as genomic data emerges in even more crickets, and/or population genetics analysis of frequencies of codon mutations (McDonald & Kreitman, 1991), will further reveal the scale of positive selection at specific codon sites in the sex-biased genes from various tissues. Such analyses will also allow further evaluation the link between positive selection (dN/dS>1) and gene pleiotropy that was suggested for gonads using the gene-wide dN/dS herein (Table 4), and permit additional evaluation this relationship for the brain, which had relatively few sex-biased genes to consider this specific relationship (of dN/dS>1 and pleiotropy) using gene-wide dN/dS (Fig. 2, Table 1).

3 CONCLUSIONS

Here, we have conducted a comprehensive assessment of sex-biased gene expression in reproductive and nervous system tissues, and revealed their relationships to pressures on protein sequence evolution, in a cricket model system. We have demonstrated the rapid evolution sex-biased brain genes, particularly female-biased brain genes, (Fig. 3, Table 1), and of male-biased genes from the gonad, in *G. bimaculatus*. Further, our data suggested a direct link between low pleiotropy and elevated dN/dS of sex-biased genes in the brain and the gonad (Fig. 2, Fig. 3) that may reflect relaxed purifying selection, which in turn, may permit elevated instances of positive selection (Table 4) (Mank & Ellegren, 2009, Otto, 2004, Larracuente et al., 2008, Mank et al., 2008, Meisel, 2011). We speculate that the features of this cricket's mating biology may give rise to sexual selection and thus contribute at least partly towards the accelerated evolution of the sex-biased brain genes, and male-biased gonadal genes, in this taxon.

Significant directions for future studies include the following approaches. First, research on sex-biased gene expression from different brain regions may further decipher its relationship to protein evolution (Tuller et al., 2008), and the possible roles of allometric scaling (Montgomery & Mank, 2016). Second, investigation of the involvement of sex-biased brain genes in gene pathways and networks, and their expression breadth across even more tissue types than those studied herein, may help elucidate why they often evolve rapidly. Third, similar studies as conducted herein in more divergent *Gryllus* species and in other genera such as *Drosophila* may help reveal whether the relationship between sex-biased expression and dN/dS varies over evolutionary time (Mugal et al., 2014), and/or is affected by the turnover in sex-biased expression status (Whittle & Extavour, 2019b, Zhang et al., 2007). Fourth, additional studies should consider potential differences in sex-biased expression of alternately spliced mRNAs, some of which may be involved in sexual differentiation (Nagoshi et al., 1988, Wexler et al., 2019). Fifth, the sequencing of additional *Gryllus* genomes and/or generation of population sequence data for *G. bimaculatus* may allow MacDonald-Kreitman tests and more powerful positive branch-site selection tests (McDonald & Kreitman, 1991, Yang, 2007) than available herein,

particularly for those with small sample sizes of sex-biased genes such as the brain. Sixth, assessment of sex-biased gene expression in the brains and gonads of *G. bimaculatus* adult males and females should be conducted in a courtship environment with male-male rivals, and/or with multiple females exposed to few males (female-female competition). Finally, the study of sex-biased expression in brains and gonads among insects that have known differences in their mating biology (including for example variation in testis-size, sperm mixing, degree of female-female competition, mate-choice, *cf.* (Harrison et al., 2015)), including among additional species of *Gryllus*, will help further decipher whether and how protein sequence evolutionary rates may be shaped by these various mechanisms of sexual selection across a phylogeny.

4 METHODS

4.1 Biological samples and RNA-seq

For our RNA-seq assessment of *G. bimaculatus* we isolated the male and female gonad, somatic reproductive system, brain and ventral nerve cord (shown in Fig. 1, Table S1; Fig. 1A,B schematic is based on (Kumashiro & Sakai, 2001) and simplified from Fox 2001; http://lanwebs.lander.edu/faculty/rsfox/invertebrates/acheta.html)). A ninth, unpaired reproductive tissue type, the male accessory gland, was also extracted, as its gene expression has been linked to protein sequence changes (Haerty et al., 2007, Clark & Swanson, 2005, Swanson et al., 2001), and it provides an additional sexual tissue type for the analysis of cross-tissue expression breadth. Further, we considered that its inclusion in the male somatic reproductive system sample might overwhelm the transcript population of that tissue type upon RNA-seq, and make it incomparable to the female reproductive system.

The rearing of specimens for tissue sampling was as follows: post hatching, wild type *G. bimaculatus* nymphs from a previously laboratory colony inbred for at least 14 years (Kainz et al., 2011a) were grown at 29°C until adulthood in well-ventilated plastic cages on a 12 hour day/ 12 hour night cycle (Kainz et al., 2011a). Plastic cages were provided with egg cartons for shelter, and the animals were fed with ground cat food (Purina item model number 178046) and water. Prior to the final nymphal molt, animals were sexed based on the presence (female) or absence (male) of an ovipositor and separated into male and female cages to avoid any mating and thus obtain virgin samples. Dissections were then performed on the unmated adults within a week after their final molt, by briefly anesthetizing the animals on ice for 5-10 minutes prior to dissection. Different tissue types (gonad, somatic reproductive system, brain, ventral nerve cord, male accessory reproductive glands) were dissected per animal using sterile equipment wiped with ethanol and RNaseZap (Ambion, catalog number AM9780), in ice-cold 1x Phosphate Buffer Saline (PBS), and the tissue cleaned of any unwanted contaminating material. Each tissue was then transferred immediately into individual 1.5ml Eppendorf tubes containing 500µl of pre-frozen Trizol (Thermo Fisher, catalog number 15596018) on dry ice, and stored at -

80°C until further use. RNA extractions, library processing and RNA-seq were then performed as described previously (Whittle et al., 2020). The same procedure was conducted for specimens of *G. assimilis*, which was used to obtain for RNA-seq for an assembly to be used for dN/dS analysis (Table S2; which also included a carcass tissue type). The *G. assimilis* eggs were obtained from the Hedwig lab (University of Cambridge, UK) and reared to adulthood, using the same animal husbandry protocols as published previously for *G. bimaculatus* (Kainz et al., 2011b, Mito & Noji, 2008, Kochi et al., 2016).

The complete RNA-seq data are available at the Short Read Archive (SRA) under the project identifier PRJNA564136 (under species name and Study ID SRP220521, 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.

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

The expression level for each of 15,539 *G. bimaculatus* genes was determined by mapping reads from each RNA-seq dataset per tissue to the full CDS list using Geneious Read Mapper (Kearse et al., 2012), a program previously found to be as effective as other common read mappers (*cf.* (Whittle et al., 2020)). We compared expression between males and females for the gonad, somatic reproductive system, brain, and ventral nerve cord using the program DESeq2, which uses the mapped reads across biological replicates and the negative binomial distribution to quantify the P-values of expression differences (Love et al., 2014). In addition, the degree of sex-biased expression per gene was determined using the ratio of average in FPKM across the replicates for female and male tissues. Any genes that had a two-fold or greater ratio in average expression in one sex (as compared to the other) and a statistically significant P-value from DESeq ($P<0.05$) as well as a FPKM of at least 1 in one tissue type was defined as sex-biased (*cf.* on a two-fold cutoff, (Assis et al., 2012, Whittle & Extavour, 2017, Whittle et al., 2020, Proschel et al., 2006, Whittle & Extavour, 2019b, Parker et al., 2019). Given the use of two biological replicates for the large-scale RNA-seq (Table S1) and our high threshold cutoff (two-fold), the identification of sex-biased genes herein is conservative. All genes not defined as sex-biased per tissue type were defined as unbiased (Zhang et al., 2010, Whittle et al., 2020, Whittle & Extavour, 2019b, Darolti et al., 2018, Parker et al., 2019, Whittle & Extavour, 2017), which herein includes all genes with less than two-fold sex-biased expression or with <1 FPKM (including undetectable expression, and apt not to play sex-related roles) in both females and males (Whittle & Extavour, 2019b, Whittle & Extavour, 2017, Whittle et al., 2020). Thus, all 15,539 genes belonged to one of these three categories per tissue (Fig. 2). We note that 95.4% of the 15,539 *G. bimaculatus* genes were expressed in at least one of the nine tissues in Additional file 1; Table S1, suggesting the vast majority of genes have putative roles in some or all of these studied tissues.

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

4.3.1 Assembly of reads

To study dN/dS, we generated and assembled RNA-seq CDS for the *G. bimaculatus* sister species *G. assimilis*. We assembled all the trimmed reads for the RNA-seq datasets of *G. assimilis* shown in Table S2. For this, the *G. assimilis* reads were *de novo* assembled into contigs using Trinity (Grabherr et al., 2011) set to default parameters using Galaxy (https://usegalaxy.org/). We then identified CDS using the PlantTribes pipeline tools (Wall et al., 2008). To assess the completeness of the assembled transcriptome, we used BUSCO 3.0.1 (Seppey et al., 2019) to reveal the percentage of the single-copy CDS that was observed in the standardized Arthropod conserved gene set, and as employed in gVolante ((Nishimura et al., 2017) 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 (Min et al., 2005), 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) (Altschul et al., 1997) 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 that were at least 150bp long and had 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 (Hibsh et al., 2015, Gongora-Castillo & Buell, 2013). Further to this point, single-end reads have even been applied for *de novo* assemblies in non-traditional model systems (Hibsh et al., 2015, Gongora-Castillo & Buell, 2013). Here, we have the additional advantage of a closely related reference genome to *G. assimilis*, namely *G. bimaculatus* (Ylla et al., 2020), to identify and confirm orthologs.

4.3.2 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) (Altschul et al., 1997). 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 (Edgar, 2004) set to default parameters in the program Mega-CC v7 (Kumar et al., 2012) 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 (Talavera & Castresana, 2007, Castresana, 2000).

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 (Yang, 2007, Yang & Nielsen, 2000), to measure dN, dS, and dN/dS (Yang, 2007) (note that dN/dS measures using Yang and Neilson 2000 (Yang & Nielsen, 2000) were strongly correlated to those using other models; e.g., values from the Pamilo and Bianchi 1993 method (Pamilo & Bianchi, 1993) had Spearman's R=0.95 $P<2X10^{-7}$). Values of dN/dS reflect the standardized rate of protein sequence evolution (dN to dS), whereby values >1, =1, and <1 suggest a prevalent history of positive selection, neutral evolution and purifying selection respectively (Yang, 2007). However, even when <1 for gene-wide measures of dN/dS, elevated values suggest greater roles of positive selection and/or relaxed purifying selection (Swanson & Vacquier, 2002, Buschiazzo et al., 2012). 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 (Castillo-Davis et al., 2004, Treangen & Rocha, 2011)), were defined as high confidence orthologs (N=7,220) between *G. bimaculatus* and *G. assimilis* for dN/dS analysis. The low dN values between these two cricket species, which were typically well below 1 as described in the Results and Discussion, should allow precise detection of orthologs, not only for single copy genes but also for duplicated genes, which can evolve rapidly (Demuth et al., 2006, Hahn et al., 2007). Overall, given the strict criteria we used for identification of high confidence orthologs, the paired alignments and dN, dS, and dN/dS measures herein are conservative.

4.4 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 (Clark et al., 2006, Hunt et al., 2011, Ghiselli et al., 2018, Yang, 2007, Nielsen et al., 2005, Torgerson et al., 2002, Swanson et al., 2001, Buschiazzo et al., 2012). For analysis of genes with dN/dS >1 (Table 4), 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 (Yang, 2007). As an outgroup species was required for this assessment, we used the recently available assembled and annotated *Laupala kohalensis* genome (Ylla et al., 2020). 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 (Talavera & Castresana, 2007, Castresana, 2000) 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<3, and with dS<3 (Mank et al., 2007) 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, branch-site positive selection was assessed on the *G. bimaculatus* branch using Chi-square values for 2XΔln Likelihood (P<0.05) between models without (null hypothesis) and with (alternate hypothesis) positive selection (Model=2, NSsites=2, omega fixed versus estimated) as described in the PAML manual (Yang, 2007). We note that our stringent approach to defining three-way orthologs, and the distance of the outgroup, 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 (Toll-Riera et al., 2011, Nozawa et al., 2009), and/or may generate false positives (Nozawa et al., 2009, Wisotsky et al., 2020), the latter likely being sensitive to the stringency of alignment. We thus aimed to control this factor by our strict approach to this assessment (excluding genes with any signs of dN or dS saturation).

4.5 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, expression was determined using its assembled CDS list (described in Text file S1 and in the Results and Discussion) and the RNA-seq data (Table S2), as was described for *G. bimaculatus*. We focused on the gonads, which had the highest number of sex-biased genes among tissues in *G. bimaculatus* (Fig. 2). 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.

4.6 Gene ontology

Gene ontology (GO) was characterized using the tool DAVID (Huang da et al., 2009). 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 (Gramates et al., 2017)), using BLASTX (https://blast.ncbi.nlm.nih.gov) (Altschul et al., 1997) and the best match (lowest e-value with cut off of $e<10^{-3}$ of *D. melanogaster*). Single direction BLASTX (Altschul et al., 1997) 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 the latter would be overly conservative between these insects from different orders for the purpose of functional characterization and analysis, and would prevent detection of putative paralogs in the crickets. *D. melanogaster* gene identifiers were input into DAVID (Huang da et al., 2009) to obtain gene putative GO functions and/or classifications. The *D. melanogaster* BLASTX searches were used solely for identification for putative orthologs to ascertain putative GO functions for our sex-biased and unbiased genes (and for putative SFP identification) and were not used for any dN/dS analysis, which was restricted to genes aligned within the crickets.

4.7 Seminal fluid proteins

As a complementary reproductive assessment in *G. bimaculatus*, we examined seminal fluid proteins (SFPs). We used *D. melanogaster* as our reference for SFP identification given this species has the most well studied insect genome, transcriptome and proteome to date, thus providing a more complete profile than the currently available smaller and likely partial SFP lists for crickets, which were obtained using transcriptomics and/or proteomics from reproductive tissues (Simmons et al., 2013, Andres et al., 2013). A recent proteome analysis of sexual structures in *D. melanogaster* confirmed functions for 134 SFPs (Sepil et al., 2019). Thus, we identified potential orthologs in *G. bimaculatus* to these SFPs in *D. melanogaster* (using single direction BLASTX as conducted for GO analysis) and assessed whether those genes had high confidence orthologs (and their dN/dS values) between *G. bimaculatus* and *G. assimilis*.

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Conflict of Interest

Authors declare no conflict of interest.

Author contributions

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

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Table 1. The dN/dS values of all female-biased brain genes and male-biased brain genes among the 7,220 genes with *G. bimaculatus* and *G. assimilis* orthologs (Fig 2B). Tissue-specific sex bias (TSSB) indicates genes that have sex-biased expression in the brain and are unbiased in all other paired tissues (shown by "*"). Gonad sex bias (GSB) indicates the gene has the same female- or male-biased expression status in the gonad as in the brain and is unbiased in other tissues ("**"). The best matching *D. melanogaster* (Dmel) ortholog is shown with identifiers and gene names from FlyBase (Gramates et al., 2017). Example GO terms are from DAVID (Huang da et al., 2009). Genes are listed by highest to lowest dN/dS values.

***G. bimaculatus* ID**	**dN/dS**	**TSSB**	**GSB**	**Matching Dmel ID**	**Dmel gene name**	**Example GO terms[a]**
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	0.5640			FBgn0035951	CG5068	Plasma membrane, Alpha/beta hydrolase fold-1
GBI_00147-RA	0.5270			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-1-2β)*	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	3.6750	*		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	0.3100	*		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	0.1300			FBgn0041713	*yellow-c*	Melanin biosynthetic process, cuticle pigmentation
GBI_00231-RA	0.1299	*		FBgn0259736	CG42390	Cell division/chromosome partitioning
GBI_08685-RA	0.1260	*		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	0.0890			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	0.0430			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] Examples of primary GO terms, and does not comprise an exhaustive list.

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*). 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 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 (Huang da et al., 2009) 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
		ATP binding	6.40E-04

Table 3. The *D. melanogaster* seminal fluid proteins (SFPs) (Sepil et al., 2019) 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 in *Gryllus***	**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 and universally unbiased expression in *G. bimaculatus* that had dN/dS>1 and their expression breadth across tissues (average number of nine tissues with expression >5 FPKM).

Gene category	N dN/dS >1	N Genes	Percent of genes	Chi^2 P[a]	Ave. exp. breadth	SE	MWU- test P (expression breadth)[a]
Ovary-biased$_{TSSB}$	19	1,858	1.02	a	6.74	0.74	a
Testis-biased$_{TSSB}$	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 the columns with P values indicate a statistically significant difference between categories with P<0.05. SE=standard error.

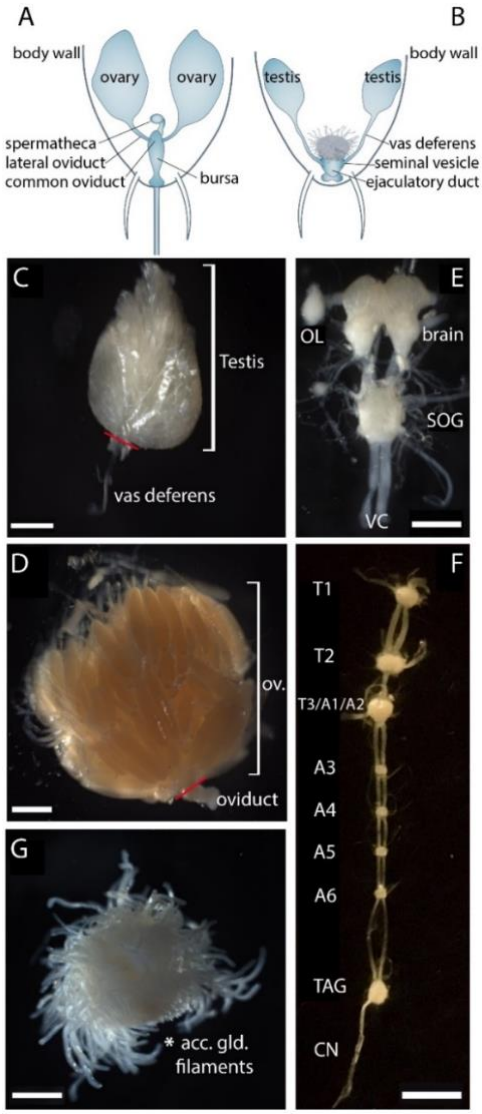

Fig. 1. *Gryllus bimaculatus* reproductive and nervous system tissues studied herein. A) Schematic diagram of the female reproductive system showing the gonads and the somatic tissues included in the somatic reproductive system under study. B) Schematic diagram for the male gonads and male somatic reproductive system. 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) (Huber, 1963, Jacob & Hedwig, 2016). 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; also shown schematically as filamentous structure in B). Scale bars: 500μm in C and E, 1000μm in D and G, 2500μm in F.

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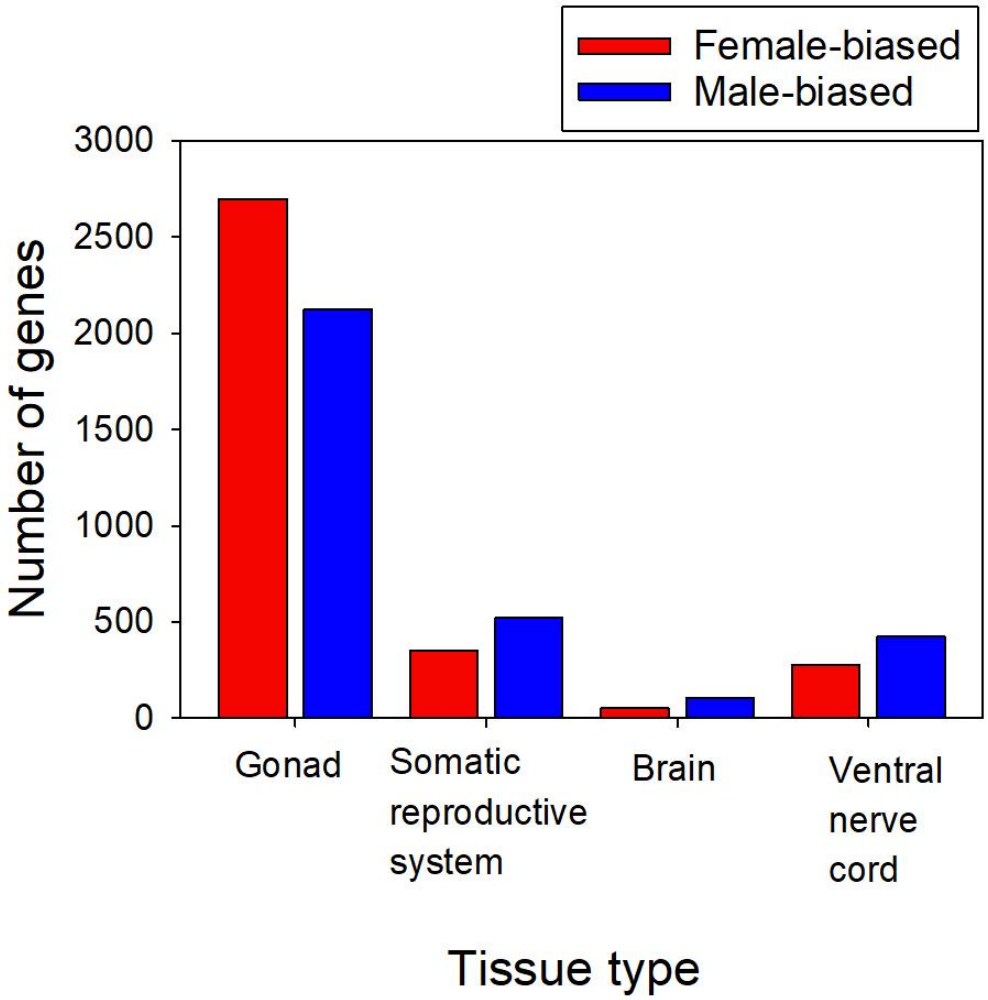

Fig. 2. The number of male-biased and female-biased genes identified in the gonad, somatic reproductive system, brain, and ventral nerve cord across all 15,539 *G. bimaculatus* genes under study (sex-biased indicates a two-fold difference in expression and P<0.05). All remaining genes not shown per tissue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

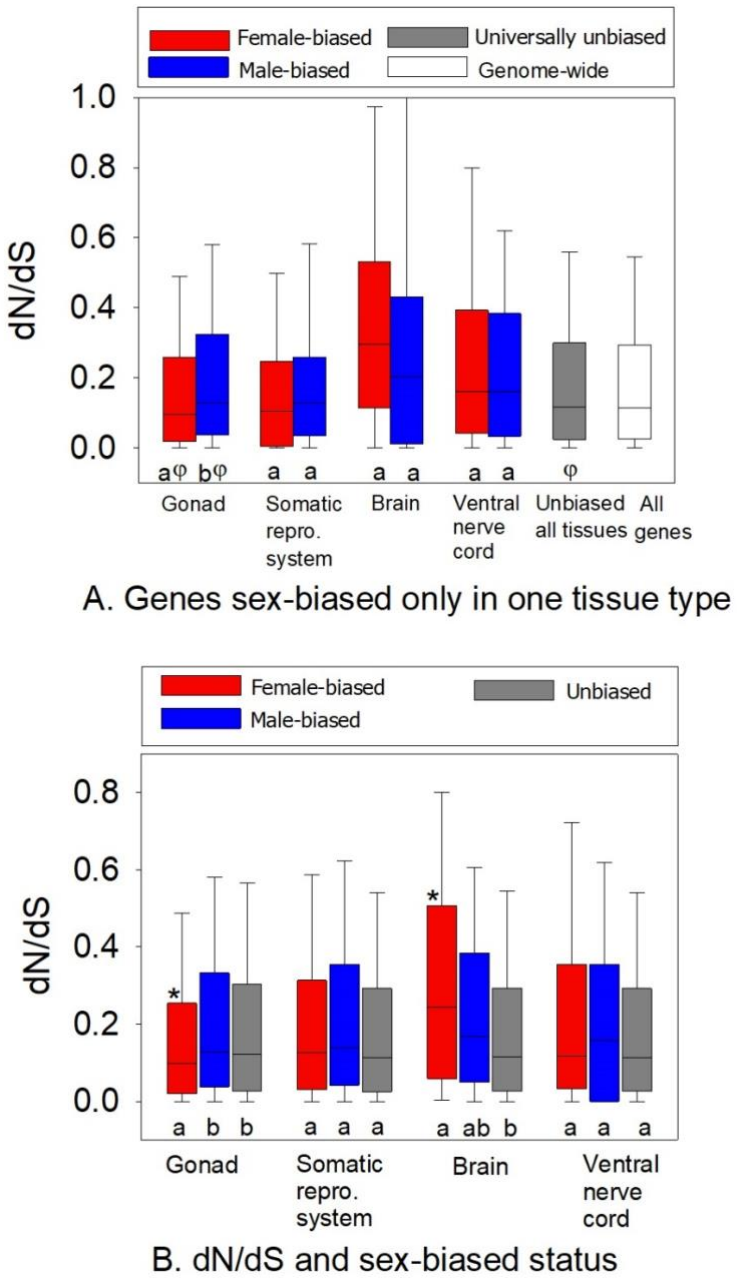

Fig. 3. The dN/dS values of genes with female- or male-biased expression in *G. bimaculatus*, and attained using the genes with high confidence orthologs its sister species *G. assimilus*. A) Genes with female- or male-biased gene expression in only one tissue type and unbiased in three remaining paired tissues, that is, with tissue-specific sex bias (TSSB). In addition, genes with universally unbiased expression in all four paired tissue types and the genome-wide dN/dS are shown. B) dN/dS of all (ALL) genes with sex-biased expression in each of four tissue types regardless of status in other tissues. In panel A, different letters (a, b) under the two bars within each tissue type indicate a statistically significant difference (MWU-test $P<0.05$), and φ indicates the difference in dN/dS in with respect to universally unbiased genes (MWU-tests $P<0.05$). For panel B, different letters among the three bars within each tissue type indicates MWU-test $P<0.05$ (note that "ab" for the brain indicates no difference of male-biased to female-biased or unbiased genes) and *indicates a difference in dN/dS between female-brain biased and ovary-biased genes. N values of genes per category are provided in Table S3. repro. = reproductive. Outliers above the 95th percentile, including dN/dS >1, were excluded from the figure to allow visualizations on the Y-axis.

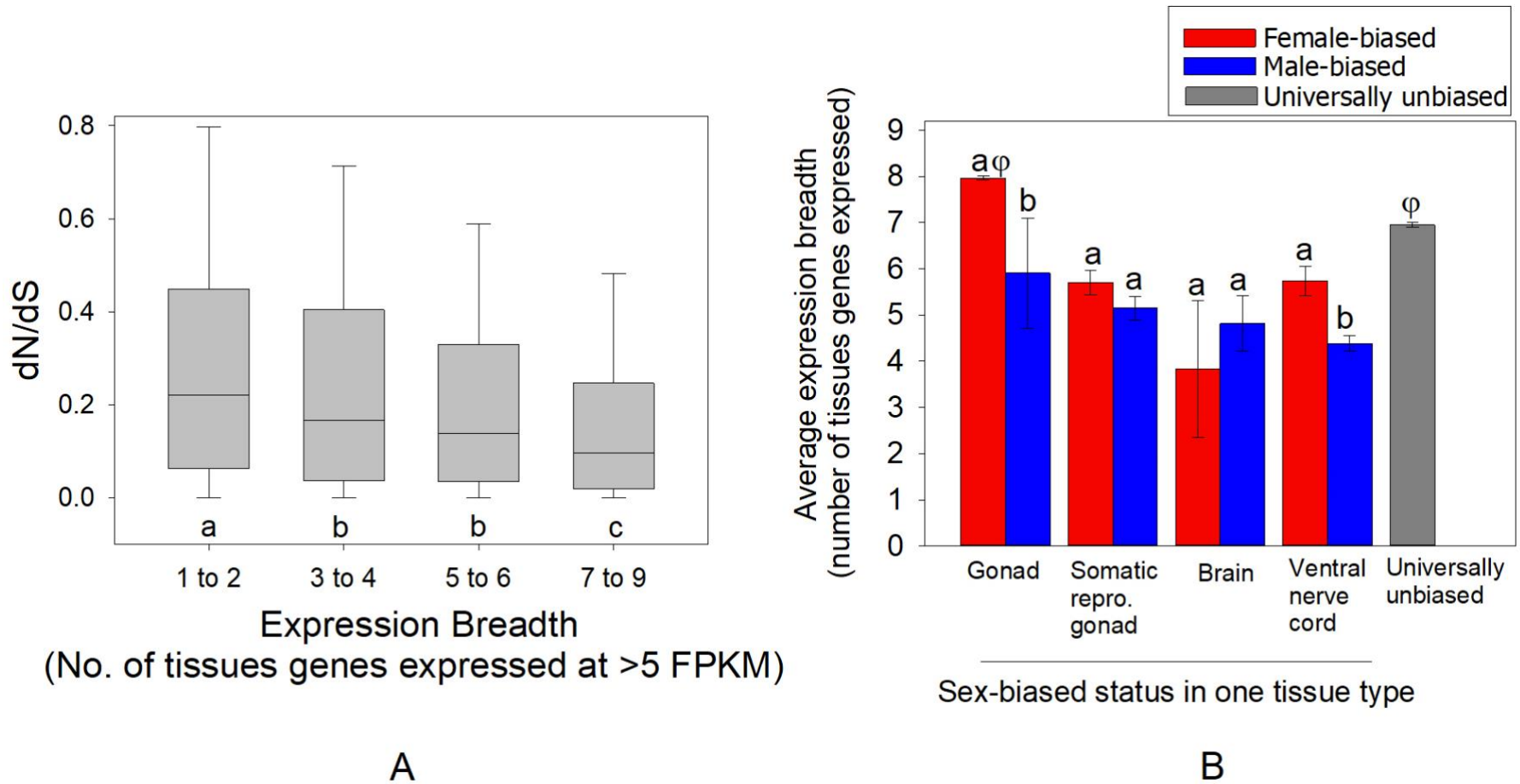

Fig. 4. A) Box and whisker plots of the dN/dS values of all studied genes with respect to their expression breadth, or pleiotropy, in *G. bimaculatus* (N=7,220 genes); B) The average expression breadth (number of tissues with expression of a gene ≥5FPKM) of genes with sex-biased expression in only one tissue type, that is, with female- or male-biased$_{TSSB}$ expression. 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 above 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. For panel A, outliers above the 95th percentile, including dN/dS>1m were excluded for visualization on the Y-axis.

SUPPORTING INFORMATION

Sex-biased genes expressed in the cricket brain evolve rapidly

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

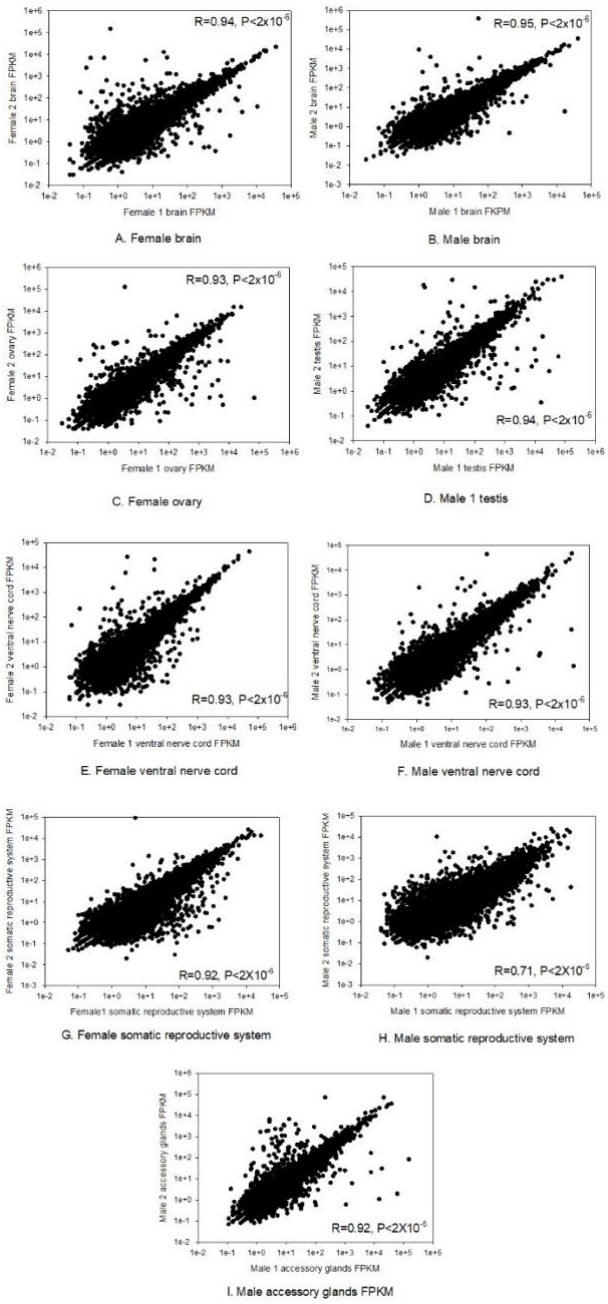

Fig. S1. The Spearman corrleation (R) in FPKM across all all 15,539 genes in *G. bimaculatus* for each of the tissues under study. A) female brain; B) male brain; C) ovary; D) testis; E) female ventral nerve cord; F) male ventral nerve cord; G) female somatic reproductive system; H) male somatic reproductive system; I) male accessory glands.

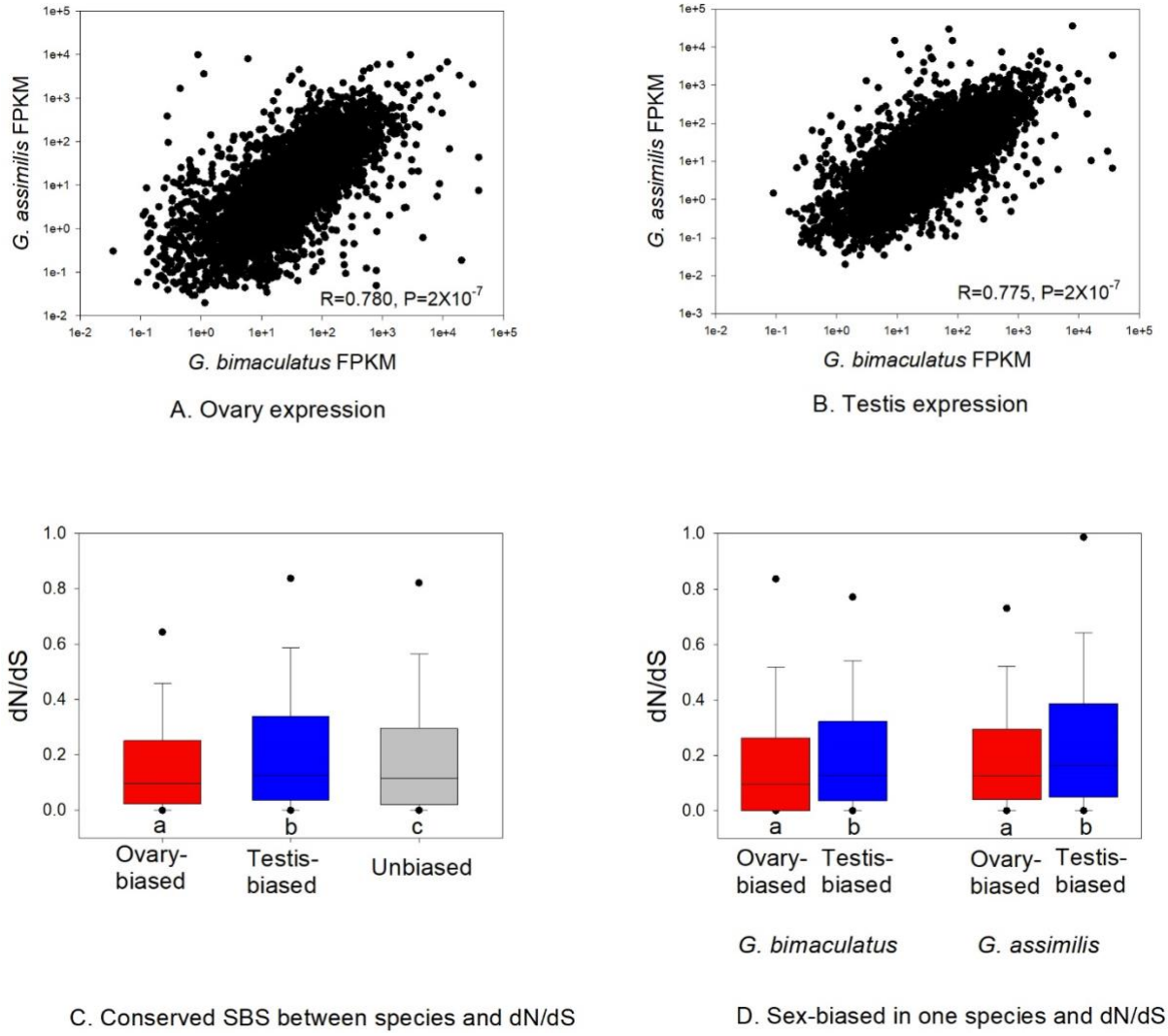

Fig. S2. Expression of genes in *G. bimaculatus* and *G. assimilis* for A) ovaries and B) testes (Spearman's R and P are shown; expression per gene is the average across samples per species). C) Box plots of dN/dS for genes have the same sex-biased status (SBS) in both *G. bimaculatus* and *G. assimilis* and; D) Box plots of dN/dS for genes that are ovary-biased or testis-biased in only one species. Different letters under bars in C and each pair of bars in D indicate MWU-P<0.05.

Table S1. The RNA-seq datasets for each of the male and female tissue types under study for *G. bimaculatus*. The number of reads (single-end) before and after trimming with BBduk (https://jgi.doe.gov/data-and-tools/bbtools/) is shown. The data are available at the Short Read Archive (SRA) under the project identifier PRJNA564136.

			No. Reads	
Sample	**Tissue**	**Sample Name**	**Before trimming**	**After trimming**
Male 1	Accessory gland	AK-28_S6.R1	8,519,999	8,455,381
	Brain	AK-25_S3.R1	10,927,264	10,543,501
	Somatic reproductive system	SHC-18_S14.R1	32,497,283	32,430,843
	Testes	SHC-17_S13.R1	19,928,912	19,751,731
	Ventral nerve cord	AK-26_S4.R1	11,488,521	11,140,299
Male 2	Accessory gland	AK-35_S13.R1	15,110,718	14,973,668
	Brain	AK-32_S10.R1	18,039,328	17,850,399
	Somatic reproductive system	AK-31_S9.R1	11,993,680	11,702,596
	Testes	AK-30_S8.R1	13,672,147	13,529,248
	Ventral nerve cord	AK-33_S11.R1	11,677,747	11,445,159
Female 1	Brain	AK-39_S17.R1	13,920,966	13,750,206
	Ovary	AK-37_S15.R1	21,725,208	21,128,416
	Somatic reproductive system	AK-38_S16.R1	13,870,827	13,718,497
	Ventral nerve cord	AK-40_S18.R1	12,599,661	12,341,413
Female 2	Brain	AK-45_S23.R1	19,312,301	19,036,974
	Ovary	AK-43_S21.R1	27,627,122	27,049,583
	Somatic reproductive system	AK-44_S22.R1	11,688,814	11,539,571
	Ventral nerve cord	AK-46_S24.R1	13,591,143	13,143,568

Table S2. The RNA-seq datasets for each of the male and female tissue types under study for *G. assimilis*. The number of reads (single-end) before and after trimming with BBduk is shown. All tissue samples were used for the RNA-seq assembly, and * indicates those samples used for gonadal expression (Exp.) analyses for comparison to *G. bimaculatus* tissue samples. The data are available at the Short Read Archive (SRA) under the project identifier PRJNA564136.

Sample	Tissue[a]	File Name	No. Reads		Exp.
			Before trimming	After trimming	
Male 1	Accessory gland	AK_1-6_S6_R1_001	7,232,269	6,927,036	
	Brain	AK_1-3_S3_R1_001	414,137	397,903	
	Carcass	AK_1-5_S5_R1_001	12,162,122	11,909,287	
	Somatic reproductive system	AK_1-2_S2_R1_001	9,786,892	9,218,317	
	Testes	AK_1-1_S1_R1_001	15,489,005	14,677,775	
	Ventral nerve cord	AK_1-4_S4_R1_001	8,085,561	7,778,520	
Male 2	Accessory gland	AK_1-12_S12_R1_001	9,684,938	9,395,491	
	Brain	AK_1-9_S9_R1_001	10,251,515	9,935,844	
	Carcass	AK_1-11_S11_R1_001	13,140,136	12,739,689	
	Somatic reproductive system	AK_1-8_S8_R1_001	9,038,153	8,700,106	
	Testes	AK_1-7_S7_R1_001	15,015,495	14,252,214	*
	Ventral nerve cord	AK_1-10_S10_R1_001	159,249,632	153,314,813[b]	
Male 3	Accessory gland	AK_1-18_S18_R1_001	9,301,332	8,771,926	
	Brain	AK_1-15_S15_R1_001	8,556,018	8,269,869	
	Carcass	AK_1-17_S17_R1_001	11,258,971	11,016,352	
	Somatic reproductive system	AK_1-14_S14_R1_001	11,468,584	11,055,961	
	Testes	AK_1-13_S13_R1_001	11,082,626	10,637,566	*
	Ventral nerve cord	AK_1-16_S16_R1_001	9,501,325	9,141,580	
Female 1	Brain	AK_1-21_S21_R1_001	12,314,902	11,529,951	
	Carcass	AK_1-23_S23_R1_001	10,318,471	9,965,655	
	Ovary	AK_1-19_S19_R1_001	12,968,675	12,330,995	*

	Somatic reproductive system	AK_1-20_S20_R1_001	20,180,007	19,613,713	
	Ventral nerve cord	AK_1-22_S22_R1_001	13,818,212	13,322,784	
Female 2	Brain	AK_1-26_S26_R1_001	10,596,275	10,191,182	
	Carcass	AK_1-28_S28_R1_001	9,471,179	8,987,504	
	Ovary	AK_1-24_S24_R1_001	14,894,350	14,584,072	*
	Somatic reproductive system	AK_1-25_S25_R1_001	10,705,738	10,183,713	
	Ventral nerve cord	AK_1-27_S27_R1_001	10,108,946	9,733,477	
Female 3	Brain	AK_1-31_S31_R1_001	9,543,257	9,388,801	
	Carcass	AK_1-33_S33_R1_001	14,562,167	14,279,995	
	Ovary	AK_1-29_S29_R1_001	10,900,114	10,725,546	
	Somatic reproductive system	AK_1-30_S30_R1_001	9,846,659	9,641,174	
	Ventral nerve cord	AK_1-32_S32_R1_001	7,954,359	7,795,480	

[a] The carcass consists of body and head (minus the legs, wings, antennae, gut and Malpighian tubules, gonad and somatic reproductive system, ventral nerve cord, brain); [b]The sample was divided into tenths and one-tenth of the reads were randomly used for assembly and expression analysis to approximately match other samples.

Table S3. The number of genes in *G. bimaculatus* under study for dN/dS (those with between-species orthologs) that had female- or male-biased expression in one (of four) paired tissue types and was unbiased in expression for the three remaining tissue types (tissue-specific sex bias, TSSB). Genes with shared sex-biased status in two of four tissues (and unbiased in two) or other types of variation in SBS are also shown.

	Number of Genes	
Sex-biased one tissue and unbiased in three tissues (TSSB, N=3,375	Female-biased	Male-biased
Gonad	1,858	1,055
Somatic reproductive system	113	126
Brain	6	16
Ventral nerve cord	82	119
Shared status in two tissues N=226	Female-biased	Male-biased
Gonad and somatic reproductive system	37	67
Gonad and brain	3	7
Gonad and ventral nerve cord	56	44
Brain and somatic reproductive system	1	6
Brain and ventral nerve cord	1	4
Other status		Other status
Universally unbiased		3,449
Variable: multiple sex-biased statuses among tissues		170
Total number of genes in all categories		7,220

Table S4. The top GO functional classifications of genes with universally unbiased expression across all four paired male-female tissue types under study. Annotation was determined in DAVID (Huang da et al., 2009) using *D. melanogaster* orthologs to *G. bimaculatus* genes. Functions are ranked by the percentage of genes matching its classification and those categories representing 5% or more of genes are shown. P-values indicate the enrichment of genes involved in each function with lower values indicating greater enrichment.

GO keyword	Percent of genes	P-value
Coiled coil	16.1	5.20E-16
Hydrolase	12.9	4.00E-03
Transferase	12.4	2.40E-21
Nucleus	11.2	8.30E-10
Phosphoprotein	11	3.40E-22
Metal-binding	10.8	6.00E-14
Nucleotide-binding	8.3	2.50E-15
Alternative splicing	8.1	4.40E-20
Cytoplasm	8	1.00E-17
ATP-binding	7	3.00E-16
Developmental protein	6.7	5.20E-11
Zinc	6.6	1.20E-09
Transport	5.1	1.70E-03
Oxidoreductase	5	4.30E-02

Table S5. The 30 genes that were expressed specifically in the male accessory glands and not in any of the eight other (male and female) tissue types in *G. bimaculatus*. The genes that had high confidence ortholog matches between *G. bimaculatus* and *G. assimilis* are shown (N=1, indicated by *), as well as those with putative orthologs identified in *D. melanogaster* (N=7), which had less strict criteria (for identification of a match for gene ontology purposes; See Methods). Gene functions were predicted using DAVID and *D. melanogaster* gene identifiers (Huang da et al., 2009).

Row	***G. bimaculatus* gene**	***D. melanogaster* match**	**Gene**	**GO putative functions**	**Expression (FPKM)**	**dN/dS**
1	GBI_11980-RA*	FBgn0035154	CG3344	Peptidase S10, serine carboxypeptidase	19.46	0.1039
2	GBI_06110-RA	FBgn0035781	CG8560	proteolysis	9.60	-
3	GBI_00239-RA	FBgn0039084	CG10175	neuron cell-cell adhesion, synaptic transmission	0.83	-
4	GBI_14669-RA	FBgn0259215	*Ionotropic receptor 93a (Ir93a)*	detection of chemical stimulus sensory perception	0.70	-
5	GBI_18443-RA	FBgn0035476	CG12766	oxidation-reduction process	0.34	-
6	GBI_06560-RA	FBgn0024288	*Sox100B*	male gonad development	0.27	-
7	GBI_04401-RA	FBgn0060296	*painless(pain)*	copulation, male courtship behavior, olfactory	0.08	-
8	GBI_08022-RA	No match	-	-	347.25	-
9	GBI_00292-RA	No match	-	-	120.78	-
10	GBI_13609-RA	No match	-	-	41.80	-
11	GBI_13241-RA	No match	-	-	20.21	-
12	GBI_14160-RA	No match	-	-	16.75	-
13	GBI_01608-RA	No match	-	-	15.96	-
14	GBI_05352-RA	No match	-	-	8.13	-
15	GBI_06938-RA	No match	-	-	5.72	-
16	GBI_21228-RA	No match	-	-	5.29	-
17	GBI_09340-RA	No match	-	-	3.41	-
18	GBI_17179-RA	No match	-	-	3.39	-
19	GBI_18175-RA	No match	-	-	1.42	-
20	GBI_03401-RA	No match	-	-	1.21	-
21	GBI_06890-RA	No match	-	-	1.15	-
22	GBI_06913-RA	No match	-	-	1.08	-

23	GBI_02473-RA	No match	-	-	0.92	-
24	GBI_02976-RA	No match	-	-	0.74	-
25	GBI_05550-RA	No match	-	-	0.6	-
26	GBI_00099-RA	No match	-	-	0.49	-
27	GBI_11938-RA	No match	-	-	0.35	-
28	GBI_16483-RA	No match	-	-	0.35	-
29	GBI_07340-RA	No match	-	-	0.29	-
30	GBI_04232-RA	No match	-	-	>0[a]	-

[a] Highly variable FPKM among individuals and thus denoted as >0 FPKM.

Table S6. The 134 seminal fluid protein (SFPs) genes for the species *D. melanogaster* from Sepil et al. 2019 (Sepil et al., 2019) and their best BLASTX matches in the 15,539 genes of *G. bimaculatus*. Due to the extended phylogenetic distance between species, the list shows all putative orthologs identified using single forward BLASTX of *G. bimaculatus* to *D. melanogaster* using BLASTX (e<0.001). Results for those contained within the 7,220 genes with high confidence orthologs between *G. bimaculatus* and *G. assimilis* are shown in Table 4 within the main text.

Row	***D. melanogaster* ID**	**SFP gene name**	**Match in *G. bimaculatus***
1	FBgn0002855	*Acp26Aa*	-
2	FBgn0002856	*Acp26Ab*	-
3	FBgn0015583	*Acp29AB*	-
4	FBgn0267327	*Acp33A*	-
5	FBgn0011559	*Acp36DE*	-
6	FBgn0034152	*Acp53C14a*	-
7	FBgn0034153	*Acp53C14b*	-
8	FBgn0053530	*Acp53C14c*	-
9	FBgn0015584	*Acp53Ea*	-
10	FBgn0020509	*Acp62F*	-
11	FBgn0015585	*Acp63F*	-
12	FBgn0015586	*Acp76A*	-
13	FBgn0003884	*alphaTub84B*	GBI_00369-RA
14	FBgn0050488	*antr*	-
15	FBgn0039598	*aqrs*	-
16	FBgn0003889	*betaTub85D*	-
17	FBgn0047334	*BG642312*	-
18	FBgn0054002	*BP1025*	-
19	FBgn0038014	CG10041	GBI_00322-RA*
20	FBgn0260766	CG42564	-
21	FBgn0038395	CG10407	GBI_00641-RA*
22	FBgn0037039	CG10587	-
23	FBgn0032853	CG10651	-
24	FBgn0032843	CG10730	-
25	FBgn0037038	CG11037	-
26	FBgn0033164	CG11112	-
27	FBgn0038067	CG11598	-
28	FBgn0038069	CG11608	-
29	FBgn0250847	CG14034	-
30	FBgn0034417	CG15117	GBI_01865-RA
31	FBgn0031617	CG15635	-
32	FBgn0030643	CG15641	-

33	FBgn0033167	CG1701	-
34	FBgn0051872	CG31872	-
35	FBgn0265264	CG17097	-
36	FBgn0250841	CG17242	-
37	FBgn0032868	CG17472	-
38	FBgn0250842	CG17575	-
39	FBgn0038919	*Qsox2*	-
40	FBgn0037433	CG17919	GBI_09042-RB
41	FBgn0034512	CG18067	-
42	FBgn0036837	CG18135	-
43	FBgn0043825	CG18284	-
44	FBgn0034753	CG2852	GBI_11684-RA
45	FBgn0050395	CG30395	-
46	FBgn0050486	CG30486	-
47	FBgn0051418	CG31418	-
48	FBgn0051419	CG31419	-
49	FBgn0051515	CG31515	-
50	FBgn0051659	CG31659	-
51	FBgn0051680	CG31680	-
52	FBgn0051704	CG31704	-
53	FBgn0032122	CG31883	-
54	FBgn0052833	CG32833	-
55	FBgn0054002	CG34002	-
56	FBgn0054033	CG34033	-
57	FBgn0054034	CG34034	-
58	FBgn0054051	CG34051	-
59	FBgn0083965	CG34129	-
60	FBgn0083966	CG34130	-
61	FBgn0260766	CG42564	-
62	FBgn0263024	CG43319	-
63	FBgn0034229	CG4847	-
64	FBgn0030828	CG5162	-
65	FBgn0036186	CG6071	-
66	FBgn0038918	*Qsox3*	-
67	FBgn0031746	CG9029	-
68	FBgn0035216	CG9168	-
69	FBgn0039597	CG9997	-
70	FBgn0004629	*Cys*	-
71	FBgn0250832	*Dup99B*	-
72	FBgn0004181	*Ebp*	-
73	FBgn0011694	*EbpII*	-
74	FBgn0000592	*Est-6*	GBI_00242-RA
75	FBgn0030932	*Ggt-1*	GBI_03406-RA

76	FBgn0041629	*Hexo2*	GBI_01177-RA
77	FBgn0040098	*lectin-29Ca*	-
78	FBgn0040097	*lectin-30A*	-
79	FBgn0040093	*lectin-46Ca*	-
80	FBgn0040092	*lectin-46Cb*	-
81	FBgn0028416	*Met75Ca*	-
82	FBgn0260745	*mfas*	GBI_04258-RA
83	FBgn0011668	*Mst57Da*	-
84	FBgn0011670	*Mst57Dc*	-
85	FBgn0053126	*NLaz*	GBI_14572-RA
86	FBgn0038198	*Npc2b*	GBI_06029-RA
87	FBgn0052190	*NUCB1*	GBI_02944-RA
88	FBgn0043539	*Obp22a*	-
89	FBgn0043530	*Obp51a*	-
90	FBgn0034471	*Obp56e*	GBI_19371-RA
91	FBgn0043533	*Obp56f*	-
92	FBgn0034474	*Obp56g*	GBI_14450-RA
93	FBgn0043532	*Obp56i*	-
94	FBgn0283509	*Phm*	GBI_06121-RA
95	FBgn0069354	*Porin2*	-
96	FBgn0030362	*regucalcin*	GBI_08029-RA
97	FBgn0033868	*S-Lap7*	-
98	FBgn0028944	*Semp1*	-
99	FBgn0037036	*Sems*	-
100	FBgn0259949	*Sfp23F*	-
101	FBgn0259951	*Sfp24Ba*	-
102	FBgn0259952	*Sfp24Bb*	-
103	FBgn0261054	*Sfp24Bc*	-
104	FBgn0259953	*Sfp24Bd*	-
105	FBgn0259956	*Sfp24C1*	-
106	FBgn0259958	*Sfp24F*	-
107	FBgn0259959	*Sfp26Ac*	-
108	FBgn0261055	*Sfp26Ad*	-
109	FBgn0259964	*Sfp33A3*	-
110	FBgn0259965	*Sfp35C*	-
111	FBgn0261058	*Sfp38D*	-
112	FBgn0259966	*Sfp51E*	-
113	FBgn0259969	*Sfp65A*	-
114	FBgn0259970	*Sfp70A4*	-
115	FBgn0261059	*Sfp78E*	-
116	FBgn0259975	*Sfp87B*	-
117	FBgn0003034	*SP*	-
118	FBgn0037038	*SP191*	-

119	FBgn0083141	*Spn28B*	-
120	FBgn0028987	*Spn28F*	GBI_00301-RB
121	FBgn0028986	*Spn38F*	GBI_05353-RD
122	FBgn0028988	*Spn42Dd*	-
123	FBgn0052203	*Spn75F*	-
124	FBgn0036969	*Spn77Bb*	-
125	FBgn0036970	*Spn77Bc*	-
126	FBgn0051413	*Qsox4*	-
127	FBgn0030589	CG9519	-
128	FBgn0085476	CG34447	-
129	FBgn0029804	CG3097	GBI_21205-RA*
130	FBgn0262621	CG43145	-
131	FBgn0053121	*Spn28Db*	-
132	FBgn0035042	CG3640	-
133	FBgn0083938	*BG642163*	-
134	FBgn0262571	CG43111	-

* In addition to the single-direction BLASTX, a reciprocal BLASTX between *G. bimaculatus* and *D. melanogaster* was also conducted for SFP genes. Each match that did not have a best hit reciprocal match (not yielding the exact same match in the top three hits are indicated by an asterisks (*)). Thus, these are lower confidence putative SFP orthologs in *G. bimaculatus*.

Table S7. The number of orthologs identified between *G. bimaculatus* (GB) and *G. assimilis* (GA) and the outgroup *L. kohalensis* (LK). Among the orthologs studied for GB-GA paired analysis, the number of genes also having an LK ortholog after three-way reciprocal BLASTX and after excluding genes with dN or dS>3 are shown (designated as high confidence). Branch-site analysis results including 2XΔL are shown for all genes studied and for sex-biased TSSB genes (or all genes for the brain) and universally unbiased genes are shown. Genes not belonging to any of these categories were excluded (Table S3).

	All genes	**Gonad-biased$_{TSSB}$**		**Somatic reproductive system-biased$_{TSSB}$**		**Ventral-biased$_{TSSB}$**		**Brain-biased$_{ALL}$**		**Universally unbiased**
Identification of three-species orthologs		Ovary-biased	Testis-biased	Female-biased	Male-biased	Female-biased	Male-biased	Female-biased	Male-biased	
N GB-GA-LK putative orthologs (BLASTX)	4,523[a]	1,300	597	62	59	49	53	15	15	2,171
N with dN and dS < 3 (High confidence)	1,933[a]	553	250	33	20	14	31	6	4	927
N branch-site test (2XΔlnL) P<0.05	220[a]	65	24	6	1	1	7	1	2	101
Percent of studied genes statistically significant	11.38%	11.75%	9.60%	18.18%	5.00%	7.14%	22.58%	NA[b]	NA[b]	10.90%

[a]The total is for all genes with orthologs, including some not belonging to any of the sub-categories. [b] Too few genes were available to study for a reliable estimate.

Text File S1

Assembly of *G. assimilis* RNA-seq data

To assess dN/dS, we compared the annotated genes in *G. bimaculatus* to the CDS list generated for *G. assimilis*. Applying Trinity and PlantTribes (see Methods) to the trimmed reads in Table S2, we obtained 33,089 non-redundant transcripts with a median and mean length of 540bp and 784.3bp respectively (standard error=30.3). The BUSCO score (Seppey et al., 2019) to the Arthropoda conserved gene set of 1,066 genes, showed 86.7% CDS had complete sequence matches, 8.6% fragmented matches, and 4.7% were missing. The latter may represent gene losses in this species, and/or genes excluded from the assembly. Thus, this suggests high efficiency of the assembly spanning a major portion of arthropod genes. From this list, we used ORF predictor with *G. bimaculatus* as a reference CDS and BLASTX to identify CDS. We found 25,128 CDS (including isoforms) with a start codon and no unknown or ambiguous nucleotides that were used for analyses. Reciprocal BLASTX of the 15,539 *G. bimaculatus* CDS to the *G. assimilis* CDS yielded 7,919 putative orthologs between the two species ($e<10^{-6}$ in both forward and reverse matches). Retaining only those putative ortholog matches that after alignment had both dN and dS values <1.5, and thus were unsaturated, yielded a total of 7,220 high confidence between-species orthologs that were used for all our dN/dS analyses.

Comparison of sex-biased gonadal expression in *G. bimaculatus* and *G. assimilis*

As described in our main text, our core target for expression analysis was *G. bimaculatus*, which has an assembled genome with complete or near complete CDS (Ylla et al., 2020), and *G. assimilis* was used primarily as a reference for assessment of protein divergence. Nonetheless, we assessed the degree of conservation of gene expression between species for the 7,220 genes with orthologs for the gonads (largest N values of all tissues, Table S3) between these two species. The results showed that gene expression in *G. assimilis* gonads was strongly correlated to that in *G. bimaculatu*s, with Spearman's R=0.780 and 0.775 ($P<2X10^{-7}$) for ovary and testis expression respectively (Fig. S2AB). In addition, 65.9 and 65.8% of all gonadally expressed genes (among the 7,220 with orthologs) that were defined as female- and male-biased in *G. bimaculatus* (ALL genes sex-biased in testis regardless of status in other tissues, N=2,043 and 1,225 respectively) had the same status in *G. assimilis*. This suggests substantial turnover in sex-biased status, a pattern observed for gonadal tissues in studied species of *Drosophila* (Assis et al., 2012, Whittle & Extavour, 2019b, Zhang et al., 2007, Harrison et al., 2015). Importantly, for genes with the same (conserved) sex-biased status in the two species, dN/dS was highest in testis-biased genes (median=0.127) and lower in unbiased (0.114), and ovary-biased (0.097) genes (MWU-tests $P<0.05$ for all paired contrasts) (Fig. S2C). Moreover, genes that were testis-biased in only in one species (*G. bimaculatus* or *G. assimilis*) and unbiased in the other species had elevated dN/dS values as compared to their ovary-biased counterparts (MWU-test $P<0.05$ for each contrast, Fig. S2D). Thus, the accelerated evolution of testis-biased genes is robust to whether the sex-biased status is observed in one species, or both species, in this taxon. All our remaining analysis is using sex-biased genes from our annotated model *G. bimaculatus*.

Assessment of expression in male accessory glands and seminal fluid proteins

We considered the evolution of genes specifically linked to the male accessory glands in *G. bimaculatus*, including those defined as putative orthologs to *D. melanogaster* seminal fluid proteins (SFPs; see below paragraph (Sepil et al., 2019)). First, we took a broad approach to study all male accessory gland-specific genes identified using our RNA-seq dataset (Table S1). Prior study of two species of crickets (*G. firmus* and *G. pennsylvanicus*) identified transcripts from the male accessory glands or SFPs, whereby some were suggested to evolve rapidly (Andres et al., 2006, Andres et al., 2013). Herein, we have the advantages of large-scale RNA-seq data from multiple tissue types, and an annotated *G. bimaculatus* genome (N=15,539 CDS) (Ylla et al., 2020), to identify male-accessory gland-specific genes in this species. We report a total of 30 genes were expressed in the male accessory glands with no expression (0 FPKM) in all eight other studied male and female tissues (Table S1).

Functional predictions of the 30 male accessory-gland specific genes using *D. melanogaster* orthologs (Table S5, e<0.001, see Methods) revealed seven genes with a match. Two of these *G. bimaculatus* genes are predicted orthologs of *painless* and *Sox100B*, which have functions in male reproduction in *D. melanogaster*; the former is involved in courtship and olfactory signalling (Table S5). Both genes were expressed at low levels (FPKM<1) in male accessory glands in *G. bimaculatus*. Only one of the 30 accessory gland specific genes had a match in the two *Gryllus* species (3.33.%, Table S5, which had very strict match criteria, see Methods). Several of the *G. bimaculatus* accessory gland genes with no *G assimilis* or *D. melanogaster* ortholog matches had relatively high expression levels (e.g., 16 to 347 FPKM; Table S5), and we speculate they could comprise orphan genes that have evolved essential male sexual functions specifically in *G. bimaculatus* (Tautz & Domazet-Loso, 2011, Whittle & Extavour, 2019a). Overall, the nearly complete lack of high confidence orthologs between *G. bimaculatus* and *G. assimilis* suggests there has been rapid evolution of male accessory gland specific genes resulting in similarity too low for ortholog detection using these methods. Alternatively, these results may reflect a history of some lineage-specific gene losses or gains of these rapidly changing genes (Tautz & Domazet-Loso, 2011, Haerty et al., 2007).

Seminal fluid proteins

Seminal fluid proteins (SFPs) play significant roles in sperm vitality, sperm storage in the female spermatheca after mating, and in fertilization (Sepil et al., 2019). In studied systems to date, which have preferentially focused on primates and *Drosophila*, genes described as SFPs have been found to evolve rapidly and/or adaptively (Haerty et al., 2007, Swanson et al., 2001, Clark & Swanson, 2005, Torgerson et al., 2002). While it may be predicted that rapid evolution of SFPs might be more pronounced in systems where females have multiple mates (such as *G. bimaculatus*) than those that are monogamous, this expected pattern was not observed for a study of 18 candidate SFPs in butterflies, where monogamy was unexpectedly linked to fast evolution of SFPs, perhaps due to relaxed selective constraints (Walters & Harrison, 2011). Research of SFPs in more diverse insects with well-described mating biology are thus needed (Walters & Harrison, 2011). *G.*

bimaculatus has high female polyandry, complete sperm mixing, and exhibits extensive pre- and post-mating female choice (Simmons, 1986, Morrow & Gage, 2001). Using the recently available list of 134 SFPs in *D. melanogaster* (shown in Table S6, (Sepil et al., 2019)), we found that only 20 genes had identifiable putative orthologs in *G. bimaculatus* genes (14.9%). This is much lower than the 64.5% of the genome-wide rate of putative ortholog detection between these two species (Chi-square with Yates's correction P<0.001). Thus, the lack of putative SFP orthologs is consistent with especially rapid evolution (Tautz & Domazet-Loso, 2011, Haerty et al., 2007) of the SFP genes following the divergence of the lineages leading to *D. melanogaster* and *G. bimaculatus*.

Among the 20 putative *G. bimaculatus* SFP genes, seven were included among the subset of 7,220 genes with between species orthologs in *Gryllus* (Table 4; note that none of these were among the 30 accessory-gland specific genes reported above). It has been inferred that SFPs tend be produced in insect accessory glands, as well as in the testis or male somatic reproductive system tissues (Sepil et al., 2019). Indeed, we found that each of these seven putative *Gryllus* SFPs exhibited expression within the testis, male somatic reproductive system, and the male accessory glands (between 0.2 to 1392.5 FPKM depending on tissue, with one exception, testis for GBI_14450-RA FPKM=0, Table 4). Significantly, for each of these seven putative cricket SFPs, we also found that the dN/dS values were consistently well above the median observed for all studied genes in the genome (which was 0.115 across all 7,220 genes, shown in Fig. 3A); specifically, the values were 0.149 (*Phm*), 0.220 (*Npc2b*), 0.230 (*Ggt-1*), 0.250 (*regucalcin*), 0.287 (*Spn28F)*, 0.344 (*Spn38F*) and 2.48 (*Obp56g*) (Table 4). Thus, the putative SFPs in the crickets studied here have evolved very rapidly, a feature shared with the SFPs that have been studied in the fellow insect *D. melanogaster* (Sepil et al., 2019, Haerty et al., 2007). It should be noted that while we consider it unlikely, we cannot exclude the possibility that some accessory gland or SFP CDS may be expressed at extremely low levels in the *G. assimilis* tissue types used for RNA-seq, causing an apparent absence of orthologs to *G. bimaculatus* in that assembly. However, we consider this unlikely given the number of tissues we assessed, including the male accessory glands (Table S2). Moreover, this would not explain the apparent paucity of *G. bimaculatus* SFP orthologs relative to those in the *D. melanogaster* genome. Thus, we suggest the absence is best explained by rapid divergence that obscures ortholog detection, and/or from gene losses or gains (Tautz & Domazet-Loso, 2011, Haerty et al., 2007).

A role of positive selection for at least one SFP gene in *Gryllus* is supported by the fact that the dN/dS value was >1 (was 2.5, Table 4) for the odorant binding SFP protein *Obp56g*. In *D. melanogaster*, *Obp56g* was first recognized as a SFP using proteomics of seminal fluid in mated females (Findlay et al., 2008), was later affirmed as a protein stored in male reproductive tissues (Takemori & Yamamoto, 2009) (which we have confirmed also express this gene in crickets: (Table 4)), and was stringently verified as an SFP by Sepil et al. 2019 (Sepil et al., 2019).

Branch-site analysis for *G. bimaculatus*

Three-way reciprocal BLASTX of *Laupala kohalensis* to *G. bimaculatus* and *G. assimillus* yielded 4,523 genes with putative orthologs. Using free-ratio branch analyses of the three species, we found dN was largely unsaturated for the *L. kohalensis* branch, with a median of 0.10. However, dS values were particularly high (median=3.3), suggesting a high mutation rate in this organism. Including only genes with dN and dS <3 yielded 1,933 genes with confidence orthologs in *L. kohalensis* (26.7% of the 7,220 genes with *G. bimaculatus* and *G. assimillus* orthologs). This conservative approach favors study of the slowly evolving genes in each sex-biased category. We found instances of positive selection at specific sites in the *G. bimaculatus* branch for sex-biased genes from all studied tissue types (2XlnL P <0.05, Table S7). For instance, we found 11.8%, 9.6% and 10.9% of studied genes exhibited positive selection for ovary-biased$_{TSSB}$, testis-biased$_{TSSB}$ and universally unbiased genes (2XlnL P per gene <0.05; Table S7). The use of conserved genes, however, biases these testis-biased estimates of positive section downward (as fast evolving genes are excluded more often: 23.7% of testis-biased genes had three-way orthologs, versus 29.8% for ovary-biased genes). Further, while the number of genes, and thus three-way orthologs, were uncommon outside the gonads (N=4-33 depending on tissue; Table S7), we found that more than three times as many female-biased than male-biased somatic reproductive system genes exhibited branch-site selection (18.2% versus 5%; but this was not statistically significant, Chi-square P=0.17, Table S7), suggesting that this narrowed level of analysis (branch-site analysis of conserved genes), may concur with the notion that some genes from the female reproductive tract and/or spermathecae, which store sperm after mating, tend to evolve adaptively due to sexual selection pressures (Swanson et al., 2004, Prokupek et al., 2008). Further studies using more closely related cricket genomes as data emerges will be needed to enhance the power of detecting branch-site positive selection using branch-site analysis.

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