Adaptation of codon and amino acid use for translational functions in highly expressed cricket genes

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Abstract

Background

For multicellular organisms, much remains unknown about the dynamics of synonymous codon and amino acid use in highly expressed genes, including whether their use varies with expression in different tissue types and sexes. Moreover, specific codons and amino acids may have translational functions in highly transcribed genes, that largely depend on their relationships to tRNA gene copies in the genome. However, these relationships and putative functions are poorly understood, particularly in multicellular systems.

Results

Here, we rigorously studied codon and amino acid use in highly expressed genes from reproductive and nervous system tissues (male and female gonad, somatic reproductive system, brain, ventral nerve cord, and male accessory glands) in the cricket *Gryllus bimaculatus*. We report an optimal codon, defined as the codon preferentially used in highly expressed genes, for each of the 18 amino acids with synonymous codons in this organism. The optimal codons were largely shaped by selection, and their identities were mostly shared among tissue types and both sexes. However, the frequency of optimal codons was highest in gonadal genes. Concordant with translational selection, a majority of the optimal codons had abundant matching tRNA gene copies in the genome, but sometimes obligately required wobble tRNAs. We suggest the latter may comprise a mechanism for slowing translation of abundant transcripts, particularly for cell-cycle genes. Non-optimal codons, defined as those least commonly used in highly transcribed genes, intriguingly often had abundant tRNAs, and had elevated use in a subset of genes with specialized functions (gametic and apoptosis genes), suggesting their use promotes the upregulation of particular mRNAs. In terms of amino acids, we found evidence suggesting that amino acid frequency, tRNA gene copy number, and amino acid biosynthetic costs (size/complexity) had all interdependently evolved in this insect model, potentially for translational optimization.

Conclusions

Collectively, the results strongly suggest that codon use in highly expressed genes, including optimal, wobble, and non-optimal codons, and their tRNAs abundances, as well as amino acid use, have been adapted for various functional roles in translation within this cricket. The effects of expression in different tissue types and the two sexes are discussed.

Keywords: Codon, amino acid, tissue-type, translational selection, regulation, tRNAs

Background

Synonymous codons in protein-coding genes are not used randomly [1]. The preferential use of synonymous codons per amino acid in highly transcribed genes, often called optimal codons, has been observed in diverse organisms including bacteria, fungi, plants and animals [2-14], including insects such as flies, mosquitoes, beetles and crickets [10, 11, 13, 15, 16]. When optimal codons co-occur with a high count of iso-accepting tRNA gene copies in the genome, which reflects an organism's tRNA abundance [3-5, 12, 17-20], it suggests a history of selection favoring translational optimization [1, 3, 5, 12, 13, 20-25]. In multicellular organisms, unlike unicellular systems, genes can be expressed at different levels among tissue types and between the two sexes [16, 26-29]. Thus, in these organisms, codon use may be more complex, given that it is plausible that optimal codons may depend on the tissue type or sex in which a gene is expressed [11, 16, 22, 30, 31], and codon use could feasibly adapt to local tissue-dependent tRNA populations [30, 32, 33]. However, only minimal data are currently available about whether and how codon use varies with high expression in different tissue types and between the two sexes in multicellular organisms.

The limited data that are available suggest that codon use varies among genes transcribed in different tissues. We recently found, for example, that some optimal codons of highly transcribed genes differed among males and females for the testis, ovaries, gonadectomized-males and gonadectomized females, which may suggest adaptation of codon use to local tRNA populations in the beetle *T. casteneum* [16]. In addition, a study in *Drosophila melanogaster* showed that certain codons were preferentially used in the testis (CAG (Gln), AAG (Lys), CCC (Pro), and CGU (Arg)) as compared to other tissues such as the midgut, ovaries, and salivary glands, a result that was taken as support for the existence of tissue-specific tRNA populations [32] (see also an analysis of codon bias by [31]). Similar patterns of tissue-related use of specific codons have been inferred in humans [33, 34] and the plants *Arabidopsis thaliana* and *Oryza sativa* [30, 35]. Given the limited scope of organisms studied to date, however, further research is needed to determine whether the codon use varies among tissues across a broader scale of organisms. Tissues that are of particular importance for research include the gonads, which are key to reproductive success, and the brain, wherein the transcribed genes are apt to regulate male and female sexual behaviors [36-38]. Translational optimization of highly transcribed genes in these tissues may be particularly significant for an organism's fitness.

While much of the focus on codon use in an organism's highly expressed genes to date has centered on optimal codons [3, 5, 7, 12, 13, 16, 21-25, 39-41], and whether they have abundant matching tRNAs that may improve translation [3, 12, 13, 20-24], growing evidence suggests that other, less well studied, types of codon statuses could also play important translational roles [42-44]. In particular, even for codons that are not optimal, the supply-demand relationship between codons and tRNA abundances may regulate translation rates, possibly affecting protein functionality and abundance [42, 45-47]. For example, *in vivo* experimental research has shown that genes using codons requiring wobble tRNAs, which imprecisely match a codon at the third nucleotide site, exhibit slowed movement of ribosomes along mRNAs [42, 48, 49]. Similarly, non-optimal codons, defined as those codons that are least commonly used in highly transcribed genes (or sometimes defined as "rare" codons), particularly those non-optimal codons with few or no tRNAs in the cellular tRNA pool [16], may decelerate translation and thereby prevent ribosomal jamming [19] and also allow proper co-translational protein folding [44, 50-53]. In this regard, wobble codons, and non-optimal codons with few matching tRNA gene copies in the genome, may have significant translational roles, including roles in slowing translation.

In contrast to non-optimal codons that have few tRNAs, some evidence has emerged suggesting non-optimal codons may sometimes have abundant tRNAs, a relationship that may act to improve translation of specific gene mRNAs [16, 45]. For instance, in yeast (*Saccharomyces cerevisiae*), rare genomic codons exhibit enhanced use in stress genes, and tRNAs matching these codons have been found to be upregulated in response to stressful conditions, allowing improvement of their translation levels without any change in transcription rates [45]. In the red flour beetle, we recently reported that some non-optimal codons have abundant matching tRNA genes in the genome [16], and these codons are concentrated in a subset of highly transcribed genes with specific, non-random biological functions (e.g., olfactory or stress roles), which may together allow preferential translation of mRNAs of those particular genes [16]. Accordingly, given these findings, further studies of codon use patterns in highly expressed genes of multicellular organisms should expand beyond the focus on optimal codons *per se* [2, 3, 7-9, 12, 21, 39, 41], and explore the use and possible translational functions of non-optimal codons, distinguishing between those that have few and plentiful tRNAs, as well as the use of wobble codons [16].

While the investigation of amino acid use remains uncommon in multicellular organisms, the available sporadic studies suggest an association between amino acid use and gene expression level [21, 54, 55]. In insects, for example, an assessment of the biosynthetic costs of amino acid synthesis (size/complexity score for each of 20 amino acids as quantified by Dufton [56]) has shown that those amino acids with low costs tend to be more commonly used in genes with high transcription levels in the beetle *T. castaneum* [21]. Further, genome-wide studies in other arthropod models such as spiders (*Parasteatoda tepidariorum*) [55], and the study of partial available transcriptomes from milkweed bugs (*Oncopeltus fasciatus*), an amphipod crustacean (*Parhyale hawaiensis*) and crickets (*Gryllus bimaculatus*, using a single ovary/embryo dataset in this system) [10], were suggestive of the hypothesis that evolution may have typically favored a balance between minimizing the amino acid costs for production of abundant proteins with the need for certain (moderate cost) amino acids to ensure proper protein function (protein stability and/or functionality) [54]. Moreover, it has been found that amino acid use is correlated to their tRNA gene copy numbers in beetles [21], and in some other eukaryotes [17], a relationship that may be stronger in highly transcribed genes [17]. Thus, these various patterns raise the possibility of adaptation of amino acid use for translational optimization in multicellular organisms [17, 21, 55]. At present, further data is needed on amino acid use in highly expressed genes in multicellular systems, that include consideration of tRNA gene number, biosynthetic costs, and expression in different tissue types.

An emerging model system that provides opportunities for further deciphering the relationships between gene expression and codon and amino acid use is the two-spotted cricket *Gryllus bimaculatus*. Within insects, *Gryllus* is a hemimetabolous genus (Order Orthoptera) and has highly diverged from the widely studied model insect genus *Drosophila* (Order Diptera) [57, 58]. *G. bimaculatus* comprises a model for investigations in genetics [59, 60], germ line formation and development [61-63] and for molecular evolutionary biology [10, 64]. In the present study, we rigorously assess codon and amino acid use in highly transcribed genes of *G. bimaculatus* using its recently available annotated genome [65] and large-scale RNA-seq data from tissues of the male and female reproductive and nervous systems [64]. From our analyses, we demonstrate that optimal codons, those preferentially used in highly expressed genes, occur in this organism, are largely shaped by selection pressures, and are nearly identical across tissues. Based on analyses of codon and tRNA gene copy relationships, we find that a majority of

optimal codons have abundant tRNAs, which is consistent with translational optimization in this species. However, some optimal codons obligately require the use of wobble tRNAs, which may act to slow translation, including for cell-cycle genes. Moreover, non-optimal codons, those codons rarely used in highly expressed genes, rather than usually having few tRNAs, often have abundant tRNAs, and thus may provide a system to upregulate the translation of specific mRNAs (for example, apoptosis gonadal genes), as has been proposed in yeast and beetles [16, 45]. Finally, with respect to amino acids, we find evidence to suggest that amino acid frequency, tRNA gene copy number, and amino acid biosynthetic costs have all interdependently evolved in this taxon, possibly for translational optimization.

Results and Discussion

For our study, codon and amino acid use in *G. bimaculatus* was assessed using genes from its recently available annotated genome [65]. We included all 15,539 *G. bimaculatus* protein-coding genes (CDS, longest CDS per gene) that had a start codon and were >150bp. Gene expression was assessed using RNA-seq data from four adult male and female tissue types, the gonad (testis for males, ovaries for females), somatic reproductive system (for males this includes the pooled vasa deferentia, seminal vesicle and ejaculatory duct and for females includes the spermathecae, common oviduct, and bursa), brain and ventral nerve cord (Additional file 1: Table S1; [64]). The male accessory glands were included for study, but were separated from the other male reproductive system to prevent overwhelming, or skewing, the types of transcripts detected in the former tissues [64]. The trimmed reads in Additional file 1: Table S1 were mapped to the 15,539 annotated G. *bimaculatus* genes independently for each of the nine tissue types under study and the expression level, or FPKM, was determined per gene.

To identify the optimal and non-optimal codons in *G. bimaculatus*, we compared codon use in highly versus lowly expressed genes [2, 7, 9, 10, 15, 16, 39, 66, 67]. For each CDS, the relative synonymous codon usage (RSCU) was determined for all codons for each amino acid with synonymous codons, whereby RSCU values >1 and <1 respectively indicate greater and lower use of a synonymous codon than that expected under equal codon use, and elevated values of codons for each amino acid indicate more frequent usage [18]. The $\Delta RSCU = RSCU_{\text{Mean Highly Expressed CDS}} - RSCU_{\text{Mean Low Expressed CDS}}$ was used to define the primary optimal codon as the codon with the largest positive and statistically significant ΔRSCU value per amino acid [2, 7, 9, 10, 15,

16, 39]. The primary non-optimal codon was defined as the codon with the largest negative and statistically significant ΔRSCU value per amino acid [16]. In the following sections, we first thoroughly describe the optimal codons identified in this cricket species, including an assessment of the variation in expression among tissue types, and the role of selection versus mutation in shaping the optimal codons. Subsequently, we thoroughly evaluate the relationships between optimal codons and the non-optimal codons and their matching tRNA gene counts in the genome to ascertain plausible functional roles. We then consider the amino acid use and tRNA relationships in highly expressed genes of this taxon.

Optimal Codons are Shared Across the Nine Distinct Tissues in *G. bimaculatus*

The organism-wide optimal codons were identified for *G. bimaculatus* using ΔRSCU for genes with the top 5% average expression levels across all nine studied tissues (cutoff was 556.2 FPKM) versus the 5% of genes with the lowest average expression levels (among all 15,539 genes under study) and are shown in Table 1. Based on ΔRSCU we report a primary optimal codon for all of the 18 amino acids with synonymous codons, each of which ended at the third position in an A (A3) or T (T3) nucleotide (boldface and underlined ΔRSCU values, Table 1). As shown in Table 2, the 777 genes in the top 5% average expression category (organism-wide analysis) were enriched for ribosomal protein genes and had mitochondrial and protein folding functions. We found that 14 of the 17 primary optimal codons (one per amino acid) that were previously identified using a partial transcriptome from one pooled tissue sample (embryos/ovaries [10]), were identical to those observed here, marking a strong concordance between studies and datasets (the differences herein were CAA for Gln, TTA for Leu, and AGA for Arg as optimal codons, and the presence of an optimal codon AAA for Lys, which had no optimal codon using previous embryonic/ovary data [10]). Thus, the present analysis using large-scale RNA-seq from nine divergent tissues (Additional file 1: Table S1) and using a complete annotated genome [65] support a strong preference for AT3 codons in this cricket.

Importantly, the expression datasets herein (Additional file 1: Table S1) allowed us to conduct an assessment of whether the identity of optimal codons varied with tissue type or sex. As certain data suggest that codon use may be influenced by the tissue in which it is maximally transcribed [16, 30], we examined those genes that exhibited maximal expression (in the top 5%) within each tissue type, that were not in the top 5% for any of the other eight remaining tissue

types [16, 30], which we refer to as Top5$_{\text{One-tissue}}$ (N values as follows, female gonad (274), male-gonad (270), female somatic reproductive system (67), male somatic reproductive system (104), female brain (24), male brain (22); female ventral nerve cord (32), male ventral nerve cord (33), and male accessory glands (162)). We found remarkable consistency among tissues, with nearly all identified optimal codons (largest positive ΔRSCU and $P<0.05$) ending in A3 and T3 in each tissue (Additional file 1: Table S2). For amino acids with two codons, the organism-wide optimal codon was always optimal across all nine tissues (Additional file 1: Table S2; with possible exceptions for the optimal codons AGG for Arg and CAG for Gln in the male brain; however this had $P>0.1$, and the N values and thus statistical power was lowest for the male brain; Additional file 1:Table S2). Nonetheless, there was some minor variation among the AT3-ending codons for amino acids with three or more synonymous codons. As an example, for the amino acid Thr, ACT was the optimal codon at the organism-wide level (Table 1) and for five tissues types (male somatic reproductive system, male brain, male ventral nerve cord, female ventral nerve cord, and male accessory glands), while the secondary organism-wide optimal codon ACA (secondary status is based on their magnitude of +ΔRSCU values) was the primary optimal codon in four other tissues (Additional file 1: Table S2). Thus, for some amino acids there is mild variation in primary and secondary status among tissues of the AT3 codons, which may reflect modest differences in the tRNA abundances among tissues [16, 32]. However, the overall patterns suggest there is remarkably high consistency in the identity of AT3 optimal codons across diverse tissues in this taxon (Additional file 1: Table S2).

While tissue-related optimal codons in multicellular organisms have only rarely been studied, the data available from fruit flies, thale cress (*Arabidopsis*), and our recent results from red flour beetles [16, 30, 32] have shown that optimal codons can vary among tissues, which suggests the existence of tissue-specific tRNA pools in those taxa [32]. The results here in *G. bimaculatus* thus differ from those in other organisms, and suggest its tRNA pools do not vary substantially with tissue or sex. Future studies using direct quantification of tRNA populations in various tissue types, which is a methodology under refinement and wherein the most effective approaches remain debated [45, 68], will help further affirm whether tRNA populations are largely similar among tissues and sex in this organism. Taken together, the results from this Top5$_{\text{One-tissue}}$ analysis, wherein the gene set for each tissue is mutually exclusive of the top 5% expressed genes in any other tissue, suggest that high transcription in even a single tissue type or

sex is enough to give rise to the optimal codons in this species. We note nonetheless that while the identity of optimal codons, and thus potentially the relative tRNA abundances, are shared among genes expressed in different tissues, the frequency of optimal codons (Fop) [22] varied among tissue types ($Top5_{One\text{-}tissue}$), suggesting the absolute levels of tRNAs may differ among tissues (see below section "*Fop varies with tissue type and sex*").

Selective pressure is a primary factor shaping optimal codons

Given that the optimal codons were highly consistent across tissues, to further investigate the potential role of selection in shaping the optimal codons we focused on the organism-wide optimal codons (Table 1). While the elevated use of the specific types of codons in highly expressed genes in Table 1 in itself provides evidence of a history of selection favoring the use of optimized codons in *G. bimaculatus* [2, 7, 9, 10, 15, 16, 66, 67, 69], the putative role of selection can be further evaluated by studying the AT (or GC) content of introns (AT-I), which are thought to largely reflect background neutral mutational pressures on genes, and thus on AT3 [16, 66, 70-74]. The *G. bimaculatus* genome contains repetitive A and T rich non-coding DNA [65], including in the introns. Nonetheless, to decipher whether any additional insights might be gained from the introns in *G. bimaculatus* we extracted the introns from the genome and found that 90.5% (N=14,071) of the 15,539 annotated genes had introns suitable for study (≥50bp after trimming). The AT-I content across all genes in this taxon had a median of 0.637, indicating a substantial background compositional nucleotide bias, and differing from the whole gene CDS (median AT for CDS across all sites=0.525). Introns (longest per gene) were nearly two- fold shorter for the most highly (top 5% organism-wide) than lowly (lowest 5%) expressed genes (1.91 fold longer in low than high expressed genes, medians were 5,183 and 2,694bp respectively, MWU-test $P<0.05$). We speculate that the shorter introns under high expression may comprise a mechanism to minimize transcriptional costs of abundantly produced transcripts in this cricket, as has been suggested in some other species including humans and nematodes [75], and may indicate a history of some non-neutral evolutionary pressures on the length of introns.

To further distinguish the role of mutation from selection in shaping AT3 in this cricket, we evaluated the relationship between gene expression (FPKM) and AT-I and AT3. We found that AT-I was positively correlated to gene expression level, with Spearman's $R=0.354$, $P<2X10^{-}$

$^{-7}$ (across all 14,071 annotated genes with introns). Thus, assuming intron nucleotide content is largely selectively neutral, this may suggest a degree of expression-linked mutational-bias [76, 77] in this organism favoring AT mutations in introns of highly transcribed genes (or conversely, elevated GC mutations at low expression levels, see below in this section). However, this correlation was markedly weaker than that observed between AT3 of protein-coding genes and expression across these same genes ($R=0.534$, $P<2X10^{-7}$), thus providing evidence that selection is a significant factor shaping AT3 [8].

For additional rigor in verifying the role of selection as compared to mutation in favoring AT3 codons (Table 1), genes from the top 5% and lowest 5% gene expression categories were placed into one of five narrow bins based on their AT-I content, specifically ≤0.5, >0.5-0.6, >0.6-0.7, >0.7-0.8, and >0.8. As shown in Fig. 1, for each AT-I bin, we found that AT3 of the top 5% expressed genes was statistically significantly higher than that of lowly expressed genes (MWU-tests P between 0.01 and <0.001). No differences in AT-I between highly and lowly expressed genes were observed per bin (MWU-test $P>0.30$ in all bins, with one exception of a minimal median AT-I difference of 0.019 for category 3, $P<0.05$, Fig. 1). Thus, this explicitly demonstrates that within genes that have a similar background intron nucleotide composition (that is, genes contained in one narrow bin of AT-I values), AT3 codons exhibit significantly greater use in highly transcribed than in lowly transcribed genes. This pattern further supports the interpretation that selection substantially shapes optimal codon use in *G. bimaculatus*.

As an additional assessment, we also considered whether the lower AT3 content of lowly expressed genes (as indicated by ΔRSCU in Table 1, and in Fig. 1) could be related to biased-gene conversion, which acts to enhance GC content [74, 78], in Additional File 1: Text File S1. We conclude that while BGC may influence GC (and thus AT) content to some extent in this taxon, it is not a major factor shaping codon use of highly versus lowly expressed genes (Table 1, Additional file 1: Table S2), thereby further supporting a substantive role of selection in shaping AT3 optimal codon use patterns in Table 1 and Fig. 1.

Fop varies with tissue type and sex

While the types of optimal codons identified herein were largely shared among tissues (Additional file 1: Table S2), the frequency of use of these codons (Fop) varied markedly with tissue type and sex in *G. bimaculatatus*. In particular, Fop was markedly higher in $Top5_{One\text{-}tissue}$

genes from the testes and ovaries and the male accessory glands, than in all other six tissue types (MWU-tests $P<0.05$, Fig. 2). Thus, this suggests that genes linked to these fundamental sexual structures and functions are prone to elevated optimal codon use that could, in principle, be due to their essential roles in reproduction and fitness, and cost-efficient translation may be particularly beneficial in the contained haploid meiotic cells [16]. Moreover, we found that the $Top5_{One\text{-}tissue}$ genes from the female somatic reproductive system had markedly higher Fop than their male counterparts (MWU-test $P<0.05$, Fig. 2). We speculate that this may reflect the essential and fitness-related roles of genes involved in the insect female structures since they transport and house the male sex cells and seminal fluids after mating [79, 80], possibly making translational optimization more consequential to reproductive success for the female than male genes. In contrast, no differences in Fop were observed with respect to sex for the brain or ventral nerve cord, and the relatively low Fop values for these tissues suggest weakened selective constraint on codon use of genes as compared to the gonads and to the male accessory glands (MWU- tests $P<0.05$ for the latter tissues versus the former, Fig. 2). In this regard, the data show striking differences in frequency of use of the optimal codons among tissue types (Fig. 2) while the identities of optimal codons themselves are largely conserved (Additional file 1: Table S2). These patterns are consistent with a hypothesis that selection for translational optimization has been higher for genes involved in the gonads and male accessory glands, than those from the nervous system.

While few comparable data on multi-tissue expression and Fop are available, and especially with respect to sex, a study of the male-female gonads and gonadectomized tissues in *D. melanogaster* indicated that codon usage bias was lower in male than female genes [31]. This pattern may be due to Hill-Robertson interference arising from adaptive evolution at linked amino acid sites in the males, dragging slightly deleterious codon mutations to fixation [31]. However, we found an opposite pattern in the mosquito *Aedes aegypti* where optimal codon use was higher in male than in female gonads [11]. Our results here, using four discrete paired male-female tissue types, suggest that the only sex-related difference in Fop for *G. bimaculatus* is for the somatic reproductive system (where male genes had lower Fop than female genes, Fig. 2). Thus, outside the somatic reproductive system, our data show that tissue type of maximal expression plays the predominant role in shaping Fop in this cricket model, rather than sex. Moreover, the low Fop observed in the brain (Fig. 2) suggests that Hill-Robertson effects may be

greatest in this tissue type, a notion that is consistent with recent observations of a rapid rate of protein sequence evolution of sex-biased brain genes in this species [64]. It is worth noting that the finding that the degree of optimal codon use is particularly pronounced for genes transcribed in the gonads in Fig. 2 may suggest greater absolute (but not relative) tRNA abundances of the optimal codons in those reproductive tissues, which are essential for formation of the sex cells.

Functional Roles of Optimal and Non-Optimal Codons Inferred by their Relationships to tRNA Gene Copies

The hypothesis of translational selection for efficient and/or accurate translation in an organism has been thought to be substantiated by associations between optimal codon use in highly expressed genes and their matching tRNA gene copy numbers in the genome [3, 5, 12, 13, 16, 20-25] In some organisms however, the correspondence between optimal codon use in highly expressed genes and the matching tRNA abundance has bean weak [21], or not observed for some (outlier) codons [81], that has been interpreted as limited support for adaptation of tRNA abundance and optimal codon use [21]. However, growing evidence suggests that there is a complex supply-demand relationship between codons and tRNAs that may affect multiple aspects of translation [42-44, 82], such that a universal connection between optimal codons and matching tRNA gene copy numbers may not always be expected [16, 42, 44]. For instance, some optimal codons may obligately require wobble tRNAs (no direct matching tRNAs) [16], which act to allow slow translation [48, 49], and thus a positive relationship between codon use in highly expressed genes and high tRNA abundance would not be expected for those codons. In turn, while non-optimal (or rare) codons may have few tRNAs, and thus act to slow translation [44], in some cases they may have numerous matching tRNAs, which could conceivably allow for translational upregulation of gene mRNAs using those codons [16, 45]. Given this context, to allow a precise interpretation of the codon-tRNA relationships in Table 1, and given some variation in terminology in the literature, we explicitly describe the codons using their ΔRSCU status and their tRNA abundances as follows: Opt-codon$_{\uparrow tRNAs}$ are those optimal codons (elevated use in highly expressed genes) that have relatively high tRNA gene copy numbers, Opt-codon$_{wobble,}$ include those optimal codons obligately requiring the use of wobble tRNAs, Nonopt-codon$_{\downarrow tRNAs}$ are the non-optimal codons (least used in highly expressed genes) with few

tRNAs, and Nonopt-codon$_{\uparrow tRNAs,}$ represents non-optimal codons with abundant tRNA gene copies [16].

To assess the relationships between the codon use and tRNA gene numbers for each amino acid in Table 1, we first determined the number of tRNA genes per amino acid in the *G. bimaculatus* genome using a recently updated version of the program tRNA-scan-SE (v. 2.0.5, see Methods) [83, 84]. We report 1,391 putative tRNAs for the *G. bimaculatus* genome (Table 1). To evaluate the propensity for translational selection *per se*, defined as a strong relationship between optimal codon use in highly expressed genes and tRNAs [5, 12, 18, 21], we compared the 18 primary optimal codons to the number of tRNAs per gene. We found that for 11 of 18 amino acids, the primary optimal codon had the highest or near highest matching number of tRNAs gene copies (≥18 tRNA copies) among the synonymous codons (Table 1), or Opt-codon$_{\uparrow tRNAs}$ status. Thus, this concurs with a model of translational selection for accurate and/or efficient translation for a majority of optimal codons in this cricket (Table 1) [5, 12, 16, 18, 21]. However, some optimal codons obligately required a wobble tRNA, or had Opt-codon$_{wobble}$, status, which we suggest may also serve important functional roles.

Some optimal codons require wobble tRNAs

Seven of the 18 identified optimal codons in Table 1 had Opt-codon$_{wobble}$ status, and had no exact matching tRNAs in the genome. These included the codons AAT (Asn), GAT (Asp), TGT (Cys), GGT (Gly), CAT (His), TTT (Phe), and TAT (Tyr) (Table 1). Thus, the elevated use of codons with Opt-codon$_{wobble}$ status in highly transcribed genes cannot be ascribed to translational selection *per se*. We suggested in a recent report for *T. castaneum*, that optimal codons obligately using wobble tRNAs may likely be employed in highly expressed genes as a mechanism to slow translation, perhaps for protein folding purposes [16]. Indeed, experimental research in yeast, human cells, and nematodes has shown that ribosomal translocation along the mRNA is slowed by codons requiring wobble tRNAs [42, 48, 49], and thus may allow co-translational protein folding. The inefficiency of wobble interactions between codons and tRNAs, including chemically modified wobble tRNAs (e.g., adenosine to inosine, I34 in the anticodon loop [85, 86], appears to act as a mechanism to decelerate translation as compared to codons with exact tRNA matches [42, 43]. In this regard, wobble codons in highly expressed genes studied here, may serve a similar function to non-optimal codons (those that have few

tRNAs, see below section), which growing studies suggest may regulate the rate, or rhythm, of translation to allow co-translational protein folding [44, 50-53]. Notably, we found the highly transcribed genes in *G. bimaculatus* were preferentially involved in protein folding as shown in Table 2, and thus this comprises a primary active process within the tissues/cells under study. In this regard, our collective results suggest a hypothesis that wobble codons in highly transcribed genes may slow translation and effectively assist in the process of protein folding.

To further study the possible roles of wobble codons, we assessed the gene ontology (GO) functions of the four codons with Opt-codon$_{wobble}$ status that had the highest ΔRSCU values (GGT, GAT, CAT and TAT with ΔRSCU values of +0.610, +0.520, +0.511 and +0.430 respectively (Table 1)) to determine if genes using these codons tended to be involved in particular processes. For this, we examined the subset of highly expressed genes that were especially enriched for each wobble codon (had RSCU≥1.5, where a value of 1 indicates equal use of the codon per codon family, and thus ≥1.5 indicates a substantial elevation in use) in the organism-wide dataset (Table 1), and for the genes with Top5$_{One\text{-}tissue}$ status in the gonads (Additional file 1: Table S2), which had the largest N values of any tissue type (Additional file 1: Table S2; gene ontology determined from putative orthologs to *D. melanogaster* ($e<10^{-3}$, BLASTX [87]) and the program DAVID [88] and Flybase.org [89], see Methods). The results are shown in Additional file 1: Table S3. The functions of the organism-wide highly expressed genes with especially elevated use of the Opt-codon$_{wobble}$ codons included ribosomal protein genes, and genes involved in mitochondrion functions (Additional file 1: Table S3), thereby specifically affirming that high use of these codons are apt to serve functions in these types of genes. For the gonads, we found that the top GO clusters for genes with high use of GAT in the ovaries (with Top5$_{One\text{-}tissue}$ status) and of TAT in the testes (with Top5$_{One\text{-}tissue}$ status) were involved in mitosis and cell cycle functions (Additional file 1: Table S3). Thus, this pattern for highly expressed gonadal genes in this cricket is in agreement with a prior experimental study that suggested the use of wobble codons in genes in cultured human and yeast cells might regulate the cell cycle, by controlling translation of cell-cycle genes [90]. Taken together, our results are suggestive that the use of Opt-codon$_{wobble}$ codons in highly expressed cricket genes may act to slow translation as a means to regulate the level of cellular proteins, and to ensure proper co-translational folding, particularly affecting genes involved in the cell-cycle (Additional file 1: Table S3) and ribosomal and mitochondrial proteins (Table 2).

425

426 ***Non-optimal codons may have different functions that depend on tRNA abundance***

427 The primary non-optimal codon per amino acid was defined as the codon with the largest
428 negative ΔRSCU with a statistically significant P value [16]. With respect to the identified non-
429 optimal codons, we found striking patterns with respect to tRNAs that concur with two possible
430 functional roles, that include firstly, slowing translation, and secondly, regulating differential
431 translation of cellular mRNAs. With respect to the former case, we found two amino acids had a
432 primary non-optimal codon with Nonopt-codon$_{\downarrow tRNAs}$, status, that included CGC (Arg), ATC (Ile)
433 (Table 1). This suggests their infrequent use in highly expressed genes may be due to the rarity
434 or absence of matching tRNAs in the cellular tRNA pools. Moreover, these codons were not only
435 non-optimal, and thus by definition are rare in highly transcribed genes, but their exact matching
436 tRNAs were absent in the genome, and thus require wobble tRNAs, a combination that would in
437 theory make them especially prone to slowing down translation. The use of non-optimal codons
438 has been suggested to decelerate translation, which may prevent ribosomal jamming [19], and/or
439 permit proper protein folding [44, 50, 51, 91], while, as described above, the use of codons
440 requiring wobble tRNAs may also slow translation [42, 48, 49]. Thus, we propose the use of
441 these two codons in genes that have Nonopt-codon$_{\downarrow tRNAs}$, status, and require wobble tRNAs,
442 could play significant roles in slowing translation in highly expressed genes in *G. bimaculatus*.
443 Importantly however, the other non-optimal codons in Table 1 had tRNA counts
444 markedly higher than zero (≥15 gene copies; Nonopt-codon$_{\uparrow tRNAs}$ status). Thus, the infrequent
445 use of those non-optimal codons in the highly expressed genes is not likely to be due to a role in
446 slowing translation. In fact, the use of these codons combined with high tRNA abundance
447 suggests the potential for a high supply : demand ratio [16, 42, 45-47], a relationship that may
448 give rise to preferential translation of any highly expressed genes that contain unusually elevated
449 Nonopt-codon$_{\uparrow tRNAs}$ codons [16]. This proposed mechanism of up-translation using non-optimal
450 (or rare) codons has been recently suggested for stress genes in yeast [45], and for highly
451 expressed genes in the red flour beetle, wherein genes with an elevated frequency of Nonopt-
452 codon$_{\uparrow tRNAs}$ status codons were linked to specific biological functions [16], suggesting their
453 mRNAs may be preferentially translated. In this regard, the Nonopt-codon$_{\uparrow tRNAs}$ status codons in
454 *G. bimaculatus* could also have significant biological roles in up-regulation of specific cellular
455 mRNAs in this cricket model.

To further evaluable this possibility for *G. bimaculatus*, we studied as examples the Nonopt-codon$_{\uparrow tRNAs}$ codon GTG for Val ,which had an organism-wide ΔRSCU of -0.484 and 40 tRNAs, the codon GGC for Gly with respective values of -0.709 and 41 tRNAs (note both Val and Gly are four-fold degenerate), and CTG for the six-fold degenerate Leu with a ΔRSCU of -0.692 and 30 matching putative tRNAs (Table 1). These were chosen as examples due to their relatively high putative tRNA counts (as compared to other Nonopt-codon$_{\uparrow tRNAs}$ codons from amino acids with the same degeneracy level). For each of these codons, we examined those Top5$_{\text{One tissue}}$ genes (only in the top 5% expression in one tissue type) in the gonads that had RSCU value ≥1.5, indicating enhanced use. The results are shown in Table 3. We found that genes preferentially using Nonopt-codon$_{\uparrow tRNA}$ codons were associated with a diverse range of functions. For example, for the ovaries, the highly expressed genes that preferentially used the Nonopt-codon$_{\uparrow tRNAs}$ codon GTG (for Val) included a match to *Bicaudal C* (*BicC*), which is involved in oogenesis [92]. Remarkably, this ovary gene also had elevated use of the wobble codons GGC ad CTG (Table 1). Similarly, for the ovaries, an ortholog of *santa-maria*, which has been associated with phototransduction [93] and apoptosis [94], had elevated use of each of the wobble codons GTG, GGC and CTG. The fact that both *BicC* and *santa-maria* each have high use of all three of these Nonopt-codon$_{\uparrow tRNAs}$ codons, which by definition have abundant matching tRNA genes, suggests their gene transcripts are preferentially translated in the ovary as compared to other transcripts in the transcript pool. For CTG (Leu), the Top5$_{\text{One-tissue}}$ genes in the ovaries preferentially using this codon with Nonopt-codon$_{\uparrow tRNAs}$ status included another apoptosis gene, *apoptosis inducing factor* (*AIF*) [95], which also had elevated use of GGC for Gly, suggesting these codons may facilitate apoptosis in the female gonad cells. With respect to the testis, GTG (Val) was preferentially used in genes such as *belle*, which is involved in male germ-line stem cell development [96, 97] and *no child left behind* (*nclb*), involved in male gonad development [98], suggesting that use of this non-optimal codon may promote translation of these particular transcripts in the male gonadal mRNA pools. Enhanced use of GGC and CTG in testes genes matching *Dual-specificity tyrosine phosphorylation-regulated kinase 2* (*Dyrk2*), which is involved in apoptosis and sensory roles [99, 100], and *short spindle 3 (ssp3)*, involved in male meiosis [101] (Table 3), infers that these two codons may promote translation of apoptosis and meiotic proteins in the testes. When taken together, these patterns in *G. bimaculatus*, similar to recent findings in *T. castaneum* [16], suggest that the combination of elevated use of non-optimal

487 codons and a high supply of tRNAs may plausibly be involved in preferential translation of the
488 transcripts of specific genes in this system, particularly for apoptosis genes and genes with
489 female and male gonadal functions (Table 3).
490
491 **Amino Acid Use, Biosynthesis Costs, and tRNA Gene Copies have Interdependently**
492 **Evolved**
493 Next, we asked whether amino acid use in the highly expressed genes in *G. bimaculatus*
494 (top 5% using the organism-wide assessment) varied with their size/complexity (S/C) scores,
495 which were developed to quantify the relative biosynthesis costs of different amino acids [56],
496 hydropathy, or with their broad role in protein folding properties [102, 103] (Additional file 1:
497 Table S4). As shown in Fig. 3, for highly expressed genes the amino acid usage (across all 20
498 amino acids) was not correlated to hydropathy (Spearman's correlation across all 777 organism-
499 wide highly expressed genes $P>0.60$) and showed no broad relationship to specific protein
500 folding properties (ranked ANOVA $P>0.05$ between groups, Fig. 3BC). However, a very strong
501 negative correlation was observed between amino acid use and S/C scores across the 20 amino
502 acids (Spearman's $R=-0.87$, $P<2X10^{-7}$, Fig. 3A, Table 4; see also [10]). An inverse relationship
503 between S/C score and the frequency of the 20 amino acids was also observed across all 15,539
504 studied *G. bimaculatus* genes irrespective of expression level (for all genes $R=-0.70$, $P=4X10^{-4}$,
505 Additional file 1: Fig. S1), but the correlation was stronger in the subset of highly expressed
506 genes, suggesting that the connection between amino acid use and S/C scores is ameliorated with
507 elevated transcription. Thus, these patterns both at the genome-wide level and using highly
508 expressed genes measured across nine tissue types, indicate preferential use of low-cost amino
509 acids in genes producing abundant mRNAs.
510 To further decipher this relationship, we compared amino acid usage using the organism-
511 wide highest and lowest expressed genes (top and lowest 5%, averaged across nine tissues). As
512 shown in Table 4, we found that 19 of 20 amino acids had a statistically different frequency
513 between the most and least transcribed genes in the genome (t-tests $P<0.05$), with the only
514 exception being Thr. The amino acids with the largest increase in frequency in highly expressed
515 genes (as compared to lowly expressed) were Ile (S/C score=16.04; with 49.0% greater use under
516 high expression) and Lys (30.14; 49.1% greater use under high expression), suggesting that
517 enhanced use of these amino acids with intermediate S/C scores may be more crucial to efficient

translation or function of abundant transcripts, than the use of those with the lowest possible S/C scores in this taxon. We note this is consistent with an earlier analysis based on a partial transcriptome from one pooled ovary/embryo sample and without tRNA data in that study, where amino acids with intermediate S/C scores Glu, Asp, and Asn were preferred [10], that all had >22% increased use under high transcription here. This type of complex relationship between S/C score and amino acid use has also been suggested in spiders [55].

Under a null hypothesis of equal usage of each of 20 amino acids, we would assume a frequency of 5% for every amino acid per gene, with values above and below this threshold indicating favored and unfavored usage respectively. In this context, we observed that for the five highest cost amino acids (Tyr, Cys, His, Met and Trp, S/C scores of 57.00 to 73.00), the average usage was less than 5% (between 1.18 and 3.10%) in both the highly and lowly expressed genes (Table 4), indicating these biochemically costly amino acids are consistently rarely used in this taxon. Taken together, organism-wide highly expressed genes in *G. bimaculatus* exhibit a pattern of elevated use of amino acids with low S/C scores (Fig. 3A), and also exhibit elevated use of specific amino acids with intermediate S/C scores (Table 4), and very low use of the highest cost amino acids. We speculate that the pattern of favored use of some intermediate cost amino acids may be due to the roles of these amino acids in protein folding (e.g., beta and alpha folding respectively, Additional file 1: Table S4) and thus their use may ensure proper function of abundantly produced gene products.

With respect to tRNA abundances, we found that amino acid frequencies in Table 4 were positively correlated to the tRNA gene counts per amino acid (the tRNA counts included all those matching any of synonymous codons per amino acid) in *G. bimaculatus*. The correlation was observed both for the highly and for the lowly expressed genes (Spearman's Ranked $R=0.65$ and 0.75, $P<0.05$, Table 4). Thus, this suggests the frequency of amino acid use within genes is connected to its tRNA abundance in this organism. However, despite being correlated in both groups (high and low expressed genes) in this cricket species, we suggest that the relationship is apt to be most beneficial to the organism by reducing the translational costs of genes that are highly transcribed, as these genes should presumably be most commonly translated.

We next asked whether tRNA abundance, or gene copy number, was connected to S/C scores in *G. bimaculatus*. Indeed, the 20 amino acids showed a striking tendency to be inversely connected to the total tRNA counts per amino acid in the organism-wide highly expressed genes

(Spearman's R=-0.52, P=0.02, Fig. 4). Thus, the abundance of tRNAs in the genome is directly connected to how biochemically costly an amino acid is to produce by the organism. While comparable studies of relationships between biosynthetic amino acid costs and tRNAs are uncommon, a similar negative pattern has been observed in a study from beetles [21], suggesting this phenomenon may be shared among diverse insects. Taking all our results in combination, it is evident that amino acid frequency is positively correlated to the matching tRNA gene counts (Table 4), and negatively correlated to S/C scores (Fig. 3A, Additional file 1: Fig. S1), and that tRNA gene counts per amino acid are negatively related to S/C scores (Fig. 4). In other words, genes exhibit a tendency for preferred use of low cost amino acids that have abundant tRNAs. We therefore suggest the hypothesis that all three parameters, amino acid frequency, tRNA genes in the genome, and biochemical costs, have evolved interdependently for translational optimization in *G. bimaculatus.*

It should be noted that while we specify herein that our tRNAs counts obtained from tRNA-scan-SE (v. 2.0.5) [83, 84] from the recently available cricket genome [65] are considered preliminary predictions in this study (see Methods, Table 1), the accuracy of this list is substantiated by the marked correlation of tRNA gene counts with S/C scores (Fig. 4) and with amino acid frequency (Table 4). In this regard, we consider the relative tRNA counts apt to provide an appropriate and accurate profile for *G. bimaculatus*.

Variation in amino acid use with respect to sex and tissue type

Finally, we determined whether amino acid frequency per gene varied among tissue type or sex for those genes with $Top5_{One\text{-}tissue}$ status. The results for amino acid frequency are shown in Additional file 1: Table S5, and correlations between use for each sex per tissue type are provided in Additional file 1: Table S6. For each sex, we found strong correlations in the frequency of amino acid use (across 20 amino acids) for all paired contrasts of tissues, with Spearman R values between 0.861 and 0.98 ($P<2X10^{-6}$). This suggests the relative amino acid use is largely consistent among highly expressed genes from all tissue types. However, the R values were weakest (R<0.9) for contrasts of the male gonad to all other tissues, suggesting a possible testis-effect on amino acid use. In terms of differences between sexes, we determined the percent difference in frequency of amino acid use between females and males for each tissue type (Additional file 1: Table S5). We found that amino acid use varied between the sexes, with

between two to six amino acids per tissue type (gonad, somatic reproductive system, brain, ventral nerve cord) exhibiting statistically significant differences between sexes. As an example, for the Top5$_{\text{One-tissue}}$ genes from the brain which had six amino acids with statistically significant differences between males and females, we found that some amino acids, namely Arg and Tyr, had in excess of 21% difference in their use between the sexes in *G. bimaculatus* (t-test $P<0.05$; Additional file 1: Table S5), thus revealing particularly marked variation for this tissue. In this regard, there are non-negligible differences in amino acid use between the sexes, particularly for the brain, suggesting that high expression in a particular sex may be a significant factor contributing to amino acid use.

Conclusions

Our collective results herein strongly suggest a model whereby codon use and amino acid use have adapted to facilitate multiple functions in highly expressed genes the cricket *G. bimaculatus*. Specifically, we showed that optimal codons are largely shared across diverse tissue types and both sexes in this organism, and are likely shaped by selective pressures (Table 1, Fig. 1, Additional file 1: Table S2). Further, we revealed that a majority of optimal codons have abundant tRNA gene copies (Table 1), which is concordant with functional roles in translational optimization [12, 22]. Importantly however, we found that a substantial subset of optimal codons obligately require wobble tRNAs (Table 1), suggesting their use may have evolved as a mechanism to slow translation in highly transcribed genes, a notion that is supported by available experimental *in vivo* translation research [42, 48, 49]. These wobble codons may facilitate protein folding and/or be involved in regulation of genes such as cell-cycle genes (Additional file 1: Table S3). In turn, we demonstrated that non-optimal codons, particularly those that have few or no matching tRNAs gene copies in the genome (Table 1), may also act to slow translation, concurring with the notion that non-optimal codons may limit ribosome jamming or protein folding [19, 44, 50-53]. Crucially however, our data revealed that not all non-optimal codons are apt to have this putative function. Rather, we find that many non-optimal codons have abundant directly matching tRNA genes (Table 1), and are linked to specific types of gene ontology functions such as apoptosis and gonadal functions (Table 3). Thus, the non-optimal codons with abundant tRNAs likely provide a major organismal mechanism to promote the upregulation of specific mRNAs in the cellular mRNA pool, agreeing

611 with a model proposed in some recent studies [16, 45]. Finally with respect to amino acid use,
612 our data suggest a hypothesis that amino acid use, biochemical costs of amino acids [56], and
613 tRNA gene counts in the genome (Fig. 3A, Fig. 4, Table 4) have interdependently evolved as a
614 mechanism for translational optimization of highly expressed genes in *G. bimaculatus*.
615 Future research should include the direct quantification of tRNAs in different tissue
616 types, a method that remains under development and debate [33, 45, 68, 104], to assess whether
617 those results add support to the conclusion of similar relative tRNA abundances among amino
618 acids across tissue types and sexes in this cricket. Moreover, further studies should be conducted
619 of the frequencies of optimal, as well as non-optimal, codons and their relationships to tRNA
620 abundances and gene functionalities, in a wider range of multicellular organisms. Such research
621 will reveal whether the phenomena observed herein are shared across divergent systems.
622
623

Materials and Methods

Biological Samples and RNA-seq

Gene expression level was determined for all 15,539 *G. bimaculatus* protein-coding genes (CDS, longest CDS per gene) [65] that had a start codon and were >150bp. RNA-seq was obtained for four adult male and female tissue types, the gonad (testis for males, ovaries for females), somatic reproductive system, brain and ventral nerve cord and for the male accessory glands (Additional file 1: Table S1) as described previously [64]. The expression level of each *G. bimaculatus* gene was determined by mapping reads per RNA-seq dataset per tissue to the complete CDS list using Geneious Read Mapper [105], to determine FPKM per gene. FPKM was robust to mapping programs, and other common mappers including BBmap (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbmap-guide/) and Bowtie2 [106] yielded similar results [64].

Optimal codons for the organism-wide analysis (averaged expression across all nine tissues) and ΔRSCU is described in the "Results and Discussion". For each codon, using $\Delta RSCU = RSCU_{\text{Mean Highly Expressed CDS}} - RSCU_{\text{Mean Low Expressed CDS}}$, t-tests were conducted between highly and lowly expressed genes to assess statistical significance. To isolate the effect of each individual tissue type, the optimal codons were determined separately for each of the nine tissues under study (males and females for each tissue type, and male accessory glands). It has been suggested that optimal codon use in a gene largely depends on the tissue type in which it is maximally transcribed [16, 30]. Accordingly, to identify optimal codons for each tissue type, we examined those genes that were in the top 5% expression in that one tissue type and not in the top 5% expression for any of the remaining eight tissues (denoted as $Top5_{\text{One-tissue}}$) versus those with the lowest 5% expression (or all those tied with the FPKM cutoff of the lowest 5% [16]). Using these highly and lowly expressed genes per tissue, the ΔRSCU was determined as described for the organism-wide optimal codons.

The frequency of optimal codons (Fop) [4] for each gene under study was determined, using the identified optimal codons, in the program CodonW (Peden 1999). Fop was then compared for genes with high transcription in the various tissue types and two sexes in *G. bimaculatus*.

Intron Analysis

We compared the AT (or GC) content of introns, which are thought to largely reflect the innate mutational pressures on the nucleotide content of genes [74, 107, 108], to the AT3 content (third nucleotide position) of CDS of highly and lowly expressed genes for the *G. bimaculatus* organism-wide optimal codons [16]. For this, using the genomic data for *G. bimaculatus*, we extracted the introns for all genes (with introns), and retained those >50bp after trimming of 10bp from the 5' and 3' ends which may contain regulatory/conserved regions [74] (and studied the longest intron per gene). For additional stringency, given that highly transcribed genes have been suggested to exhibit mutational biases (e.g., C to T) within a small number of organisms (e.g., *E. coli*, humans [76, 77]), we tested whether there was a correlation between gene expression and intron AT content in *G. bimaculatus*. To further assess the role of selection, as compared to mutation, in favoring AT3 codons (Table 1), genes from the top 5% and lowest 5% gene expression categories were placed into one of five bins based on their AT-I content as shown in Fig. 1.

tRNA Gene Copies

The number of tRNA genes per amino acid in the *G. bimaculatus* genome was determined using the recently updated version of tRNA-scan-SE (v. 2.0.5) [83, 84]. The Eukaryotic filer called EukHighConfidenceFilter was used, which was designed to narrow the tRNA-scan output to a conservative high confidence tRNA [83] (used at default settings with the exception of ml -1). We note that since the rigor of the updated program has not been explicitly tested in insects outside *Drosophila* (P. Chan, personal communication), we consider the tRNA predictions preliminary, and focus on the relative values of tRNAs among codons and amino acids. The accuracy of the predictions, however, is strongly supported by the correlations between tRNA gene copy numbers and amino acid costs and amino acid frequency (see section "*Amino Acid Use, Biosynthesis Costs, and tRNA Gene Copies have Interdependently Evolved*"). The filter acted to reduced the absolute counts of tRNAs per amino acid to the high confidence dataset. Nonetheless, the tRNA counts with and without the filter were strongly correlated across amino acids (Spearman's Ranked $R = 0.90$, $P < 2X10^{-7}$), and thus relative gene counts remain intact using both measures.

Amino Acid Use

686 The frequency of each of the 20 amino acids in protein-coding genes in an organism may
687 be influenced by factors such as their size/complexity Dufton scores (which range from 1 to 73
688 depending on the amino acid, [56]), as well as hydropathy (where positive hydrophobicity values
689 indicate hydrophobic nature, while negative suggest a hydrophilic amino acid [102, 103]), and/or
690 their role in protein folding structures (alpha helices, beta sheets, or breakers used to affect
691 bonding in helices) [103]. We thus aimed to study each of these parameters, using established
692 values per amino acid shown in Table S4. We evaluated whether amino acid frequency in
693 proteins of highly transcribed genes at an organism-wide level in *G. bimaculatus* (top 5%
694 average expression across all eight male and female tissue) was correlated to S/C score [56], as
695 well as hydropathy and protein folding characteristics [56, 102, 103]. In addition, we assessed
696 and compared amino acid use per tissue type/sex by examining genes with $Top5_{One\text{-}tissue}$ status per
697 tissue type.
698
699 **Gene Ontology**
700 For gene ontology functions, we used the gene ontology from the fly *D. melanogaster*,
701 which comprises the most well studied insect genome to date [89]. For this, we conducted a
702 BLAST search of the full. *G. bimaculatus* CDS list under study to *D. melanogaster* CDS list
703 (version 6.29 [89]) using BLASTX [87], applying a cutoff of $e<10^{-3}$. For those genes having
704 matches within these criteria, the *D. melanogaster* gene identifiers of were then input into the
705 program DAVID [88] for gene ontology analyses and searched in FlyBase [89].

706 # List of abbreviations

707 $Top5_{One\text{-}tissue}$, genes with an expression level in the top 5% in one tissue type only, and not in the
708 other eight tissues

709 FPKM, frequency per kilobase million

710 MWU-test, Mann-Whitney U-test

711

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

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

Competing interests

The authors declare they have no competing interests.

Funding

This work was supported by funds from Harvard University.

Acknowledgements

The authors thank Dr. Guillem Ylla for providing early access to the assembled *G. bimaculatus* genome and members of the Extavour lab for discussions. The services of the Bauer core sequencing facility at Harvard University are appreciated.

Authors' contributions

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

Additional Files

Additional File 1: The file contains the Supplementary Tables, Figures and Text which are denoted and Tables S1 to S6, Figure S1, and Text File S1.

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Table 1. The organism-wide ΔRSCU values determined using genes with the top 5% expression level (when averaged across all nine tissues) and lowest 5% expression level (*P<0.05 **P<0.001). The number of putative tRNAs as determined using tRNA-scan and Euk filter [83] are shown. The primary optimal codon per amino acid and its ΔRSCU value are in bold and underlined. An optimal codon that has a relatively high number of tRNAs (≥18, Opt-codon$_{\uparrow tRNAs}$) and those with no tRNAs, and thus obligately requiring the use of wobble tRNAs (Opt-codon$_{wobble}$), are shown, as well as the putative wobble anticodon. The primary non-optimal codons that have matching tRNA gene numbers substantially in excess of 0 (≥15, Nonopt-codon$_{\uparrow tRNAs}$) and those with few/no tRNAs (Nonopt-codon$_{\downarrow tRNAs}$) are indicated. Codons not having primary optimal or non-optimal status are indicated by "--".

Amino acid	**Codon (DNA)**	**Standard anticodon**	**ΔRSCU**	**P**	**No. tRNAs**	**Optimal and non-optimal status**	**Wobble anticodon (optimal)**
Ala	**GCT**	**AGC**	**+0.871**	**	35	Opt-codon$_{\uparrow tRNAs}$	
Ala	GCC	GGC	-0.344	**	0	--	
Ala	GCA	UGC	+0.518	**	18	--	
Ala	GCG	CGC	-1.039	**	22	Nonopt-codon$_{\uparrow tRNAs}$	
Arg	CGT	ACG	+0.463	**	40	--	
Arg	CGC	GCG	-1.053	**	0	Nonopt-codon$_{\downarrow tRNAs}$	
Arg	CGA	UCG	+0.185	**	39	--	
Arg	CGG	CCG	-0.548	**	2	--	
Arg	**AGA**	**UCU**	**+0.881**	**	18	Opt-codon$_{\uparrow tRNAs}$	
Arg	AGG	CCU	+0.047		26	--	
Asn	**AAT**	**AUU**	**+0.416**	**	0	Opt-codon$_{wobble}$	GUU
Asn	AAC	GUU	-0.244	**	37	Nonopt-codon$_{\uparrow tRNAs}$	
Asp	**GAT**	**AUC**	**+0.520**	**	0	Opt-codon$_{wobble}$	GUC
Asp	GAC	GUC	-0.482	**	31	Nonopt-codon$_{\uparrow tRNAs}$	
Cys	**TGT**	**ACA**	**+0.368**	**	0	Opt-codon$_{wobble}$	GCA
Cys	TGC	GCA	-0.365	**	38	Nonopt-codon$_{\uparrow tRNAs}$	
Gln	**CAA**	**UUG**	**+0.254**	**	39	Opt-codon$_{\uparrow tRNAs}$	
Gln	CAG	CUG	-0.218	**	37	Nonopt-codon$_{\uparrow tRNAs}$	
Glu	**GAA**	**UUC**	**+0.496**	**	31	Opt-codon$_{\uparrow tRNAs}$	
Glu	GAG	CUC	-0.480	**	18	Nonopt-codon$_{\uparrow tRNAs}$	
Gly	**GGT**	**ACC**	**+0.610**	**	0	Opt-codon$_{wobble}$	GCC
Gly	GGC	GCC	-0.709	**	41	Nonopt-codon$_{\uparrow tRNAs}$	
Gly	**GGA**	**UCC**	+0.483	**	19	--	
Gly	GGG	CCC	-0.383	**	11	--	
His	**CAT**	**AUG**	**+0.511**	**	0	Opt-codon$_{wobble}$	GUG
His	CAC	GUG	-0.452	**	37	Nonopt-codon$_{\uparrow tRNAs}$	
Ile	**ATT**	**AAU**	**+0.603**	**	22	Opt-codon$_{\uparrow tRNAs}$	
Ile	ATC	GAU	-0.452	**	0	Nonopt-codon$_{\downarrow tRNAs}$	

Ile	ATA	UAU	+0.045		19	--	
Leu	**TTA**	**UAA**	**+0.537**	**	28	Opt-codon$_{\uparrow tRNAs}$	
Leu	TTG	CAA	+0.383	**	16	--	
Leu	CTT	AAG	+0.409	**	39	--	
Leu	CTC	GAG	-0.629	**	0	--	
Leu	CTA	UAG	+0.007		28	--	
Leu	CTG	CAG	-0.692	**	30	Nonopt-codon$_{\uparrow tRNAs}$	
Lys	**AAA**	**UUU**	**+0.263**	**	20	Opt-codon$_{\uparrow tRNAs}$	
Lys	AAG	CUU	-0.160	**	50	Nonopt-codon$_{\uparrow tRNAs}$	
Phe	**TTT**	**AAA**	**+0.407**	**	0	Opt-codon$_{wobble}$	GAA
Phe	TTC	GAA	-0.265	**	48	Nonopt-codon$_{\uparrow tRNAs}$	
Pro	**CCT**	**AGG**	**+0.749**	**	36	Opt-codon$_{\uparrow tRNAs}$	
Pro	CCC	GGG	-0.359	**	0	--	
Pro	CCA	UGG	+0.483	**	31	--	
Pro	CCG	CGG	-0.843	**	36	Nonopt-codon$_{\uparrow tRNAs}$	
Ser	**TCT**	**AGA**	**+0.731**	**	36	Opt-codon$_{\uparrow tRNAs}$	
Ser	TCC	GGA	-0.208	**	0	--	
Ser	TCA	UGA	+0.493	**	21	--	
Ser	TCG	CGA	-0.723	**	15	Nonopt-codon$_{\uparrow tRNAs}$	
Ser	AGT	ACU	+0.325	**	0	--	
Ser	AGC	GCU	-0.619	**	60	--	
Thr	**ACT**	**AGU**	**+0.644**	**	35	Opt-codon$_{\uparrow tRNAs}$	
Thr	ACC	GGU	-0.223	**	0	--	
Thr	ACA	UGU	+0.493	**	37	--	
Thr	ACG	CGU	-0.873	**	31	Nonopt-codon$_{\uparrow tRNAs}$	
Tyr	**TAT**	**AUA**	**+0.430**	**	0	Opt-codon$_{wobble}$	GUA
Tyr	TAC	GUA	-0.186	**	43	Nonopt-codon$_{\uparrow tRNAs}$	
Val	**GTT**	**AAC**	**+0.600**	**	26	Opt-codon$_{\uparrow tRNAs}$	
Val	GTC	GAC	-0.394	**	0	--	
Val	GTA	UAC	+0.314	**	30	--	
Val	GTG	CAC	-0.484	**	40	Nonopt-codon$_{\uparrow tRNAs}$	
Amino acids with one codon							
Met	ATG	CAU			43	--	
Trp	TGG	CCA			32	--	
Total tRNAs					1,391		

Table 2. Top predicted GO functional groups for organism-wide highly expressed genes (top 5% expression levels when averaged FPKM across all nine tissues). The top five clusters with the greatest enrichment (abundance) scores are shown. P-values are derived from a modified Fisher's test, where lower values indicate greater enrichment. Data is from DAVID software [88] using those *G. bimaculatus* genes with *D. melanogaster* orthologs (BLASTX e $<10^{-3}$ [87]).

Enrichment Score: 18.88	P-value
Ribosomal protein	7.30E-31
Cytosolic ribosome	9.00E-11
Enrichment Score: 12.49	
Mitochondrion	3.50E-17
Enrichment Score: 8.98	
Transit peptide: Mitochondrion	4.30E-04
Enrichment Score: 8.39	
Electron transport	1.90E-10
Enrichment Score: 6.49	
Protein folding	2.40E-10

Table 3. Examples of genes that exhibit the top 5% expression levels in the ovaries and top 5% expression levels in the testes (but are not in the top 5% of any other tissue type, $Top5_{One\ tissue}$) in *G. bimaculatus* that have elevated use of a non-optimal codon with high tRNAs counts ($Nonopt\text{-}codon_{\uparrow tRNAs}$ status; elevated use in this table indicates the RSCU in a gene is ≥1.5). The codons include GTG for Val, GGC for Gly, and CTG for Leu (RSCU values ≥1.5). Genes are listed that have an identified putative *D. melanogater* (Dmel) ortholog (best match BLASTX e $<10^{-3}$ [87] and a known gene name at FlyBase [89].

GB ID	Dmel ID	Gene Name
Ovaries- GTG for Val (RSCU≥1.5)		
GBI_17906-RA	FBgn0039889	*ADP ribosylation factor-like 4* (*Arl4*)
GBI_01735-RA	FBgn0261788	*Ankyrin 2* (*Ank2*)
GBI_16610-RA	FBgn0024227	*aurora B* (*aurB*)
GBI_20301-RA	FBgn0000182	*Bicaudal C* (*BicC*)
GBI_10942-RA	FBgn0024491	*Bicoid interacting protein 1* (*Bin1*)
GBI_05907-RA	FBgn0000337	*cinnabar* (*cn*)
GBI_11302-RA	FBgn0030608	*Lipid storage droplet-2* (*Lsd-2*)
GBI_09650-RA	FBgn0031145	*Nuclear transport factor-2* (*Ntf-2*)
GBI_06633-RB	FBgn0031530	*Polypeptide GalNAc transferase 2* (*Pgant2*)
GBI_13292-RA	FBgn0039214	*puffyeye* (*puf*)
GBI_11680-RC	FBgn0004855	*RNA polymerase II 15kD subunit (RpII15*)
GBI_13051-RB	FBgn0025697	*scavenger receptor acting in neural tissue and majority of rhodopsin is absent* (*santa-maria*)
GBI_03901-RD	FBgn0003312	*shadow* (*sad*)
GBI_03557-RA	FBgn0037802	*Sirtuin 6* (*Sirt6*)
GBI_00841-RB	FBgn0003714	*technical knockout* (*tko*)
Testes- GTG for Val (RSCU≥1.5)		
GBI_00920-RA	FBgn0038984	*Adiponectin receptor* (*AdipoR*)
GBI_00615-RA	FBgn0263231	*belle* (*bel*)
GBI_03558-RA	FBgn0032820	*fructose-1,6-bisphosphatase* (*fbp*)
GBI_04579-RA	FBgn0030268	*Kinesin-like protein at 10A* (*Klp10A*)
GBI_09377-RA	FBgn0015754	*Lissencephaly-1* (*Lis-1*)
GBI_12141-RA	FBgn0038167	*Lkb1 kinase* (*Lkb1*)
GBI_02406-RA	FBgn0263510	*No child left behind* (*nclb*)
GBI_09426-RA	FBgn0261588	*pou domain motif 3* (*pdm3*)
GBI_08602-RA	FBgn0036257	*Rho GTPase activating protein at 68F* (*RhoGAP68F*)
GBI_05329-RA	FBgn0032723	*short spindle 3* (*ssp3*)
Ovaries- GGC for Gly (RSCU≥1.5)		
GBI_17906-RA	FBgn0039889	*ADP ribosylation factor-like 4(Arl4)*

GBI_06216-RA	FBgn0031392	*Apoptosis inducing factor (AIF)*
GBI_20301-RA	FBgn0000182	*Bicaudal C (BicC)*
GBI_11302-RA	FBgn0030608	*Lipid storage droplet-2 (Lsd-2)*
GBI_05398-RA	FBgn0029687	*VAMP-associated protein of 33kDa ortholog A(Vap-33A)*
GBI_09822-RD	FBgn0261458	*capulet (capt)*
GBI_01828-RA	FBgn0011296	*lethal (2) essential for life (l(2)efl)*
GBI_10179-RA	FBgn0024841	*pterin-4a-carbinolamine dehydratase (pcd)*
GBI_13051-RB	FBgn0025697	*santa-maria*

Testes- GGC for Gly (RSCU≥1.5)

GBI_15155-RA	FBgn0016930	*Dual-specificity tyrosine phosphorylation-regulated kinase 2* (*Dyrk2*)
GBI_09377-RA	FBgn0015754	*Lissencephaly-1(Lis-1)*
GBI_00388-RA	FBgn0010288	*Ubiquitin carboxy-terminal hydrolase (Uch)*
GBI_09426-RA	FBgn0261588	*Pou domain motif 3 (pdm3)*
GBI_05329-RA	FBgn0032723	*short spindle 3 (ssp3)*

Ovaries- CTG for Leu (RSCU≥1.5)

GBI_17906-RA	FBgn0039889	*ADP ribosylation factor-like 4* (*Arl4*)
GBI_01735-RA	FBgn0261788	*Ankyrin 2* (*Ank2*)
GBI_06216-RA	FBgn0031392	*Apoptosis inducing factor* (*AIF*)
GBI_07513-RA	FBgn0005666	*bent* (*bt*)
GBI_20301-RA	FBgn0000182	*Bicaudal C* (*BicC*)
GBI_05907-RA	FBgn0000337	*cinnabar* (*cn*)
GBI_11302-RA	FBgn0030608	*Lipid storage droplet-2* (*Lsd-2*)
GBI_16524-RA	FBgn0027786	*Mitochondrial carrier homolog 1* (*Mtch*)
GBI_09650-RA	FBgn0031145	*Nuclear transport factor-2* (*Ntf-2*)
GBI_05851-RA	FBgn0003074	*Phosphoglucose isomerase* (*Pgi*)
GBI_06633-RB	FBgn0031530	*Polypeptide GalNAc transferase 2* (*pgant2*)
GBI_09582-RA	FBgn0036187	*RIO kinase 1* (*RIOK1*)
GBI_13051-RB	FBgn0025697	*santa-maria*
GBI_03901-RD	FBgn0003312	*shadow* (*sad*)

Testes- CTG for Leu (RSCU≥1.5)

GBI_00369-RA	FBgn0003884	*Alpha-Tubulin at 84B* (*alphaTub84B*)
GBI_15155-RA	FBgn0016930	*Dyrk2*
GBI_03558-RA	FBgn0032820	*fructose-1,6-bisphosphatase* (*fbp*)
GBI_10438-RA	FBgn0038923	*mitochondrial ribosomal protein L35* (*mRpL35*)
GBI_09426-RA	FBgn0261588	*Pou domain motif 3* (*pdm3*)
GBI_08602-RA	FBgn0036257	*Rho GTPase activating protein at 68F* (*RhoGAP68F*)
GBI_05329-RA	FBgn0032723	*short spindle 3* (*ssp3*)

GBI_00450-RA	FBgn0024289	*Sorbitol dehydrogenase 1* (*Sodh-1*)
GBI_14282-RA	FBgn0029763	*Ubiquitin specific protease 16/45* (*Usp16-45*)

Table 4. The average amino acid use of the top 5% expressed genes ($Top5_{One\text{-}tissue}$) in *G. bimaculatus* and 5% lowest expressed genes for the organism-wide analyses (using average expreession across all nine tissue types). **indicates P<0.05 using a two tailed t-test. The number of predicted tRNAs in the genome per amino acid are shown. SE- standard error.

Amino acid (AA)	**S/C Score**	**AA Freq. High exp**	**SE**	**AA Freq. Low exp**	**SE**	**Percent Diff.**	**P**	**tRNAs**
Gly	1	6.66	0.21	8.71	0.13	**-30.70**	**	71
Ala	4.76	7.32	0.24	11.54	0.14	**-57.72**	**	75
Val	12.28	6.73	0.19	6.27	0.08	**+6.80**	**	96
Ile	16.04	5.70	0.15	2.91	0.04	**+49.01**	**	41
Leu	16.04	9.07	0.26	8.13	0.10	**+10.31**	**	141
Ser	17.86	6.75	0.21	7.63	0.11	**-12.94**	**	132
Thr	21.62	5.16	0.15	5.08	0.07	+1.69		103
Lys	30.14	6.93	0.18	3.53	0.06	**+49.08**	**	70
Pro	31.8	4.62	0.15	6.95	0.11	**-50.40**	**	103
Asp	32.72	5.08	0.16	3.83	0.06	**+24.64**	**	31
Asn	33.72	4.30	0.13	2.68	0.04	**+37.70**	**	37
Glu	36.48	6.53	0.22	5.09	0.07	**+22.08**	**	49
Gln	37.48	3.75	0.15	3.49	0.05	**+6.92**	**	76
Phe	44	4.10	0.10	2.70	0.04	**+34.20**	**	48
Arg	56.34	5.61	0.15	10.04	0.12	**-78.95**	**	125
Tyr	57	3.10	0.08	1.87	0.05	**+39.53**	**	43
Cys	57.16	2.08	0.06	2.51	0.03	**-20.75**	**	38
His	58.7	2.24	0.07	2.53	0.04	**-12.99**	**	37
Met	64.68	2.61	0.06	2.32	0.02	**+10.93**	**	43
Trp	73	1.18	0.03	1.48	0.02	**-25.80**	**	32

Notes: A negative correlation between S/C score and the frequency of amino acids was observed for highly and lowly expressed genes (Spearman's Ranked R=-0.87 and -0.75, $P<10^{-7}$). Further, a positive correlation between the frequency of amino acids and tRNA counts was observed for highly and lowly expressed genes (Spearman's Ranked R=0.65 and 0.74, P<0.05). Percent Diff.= percent difference.

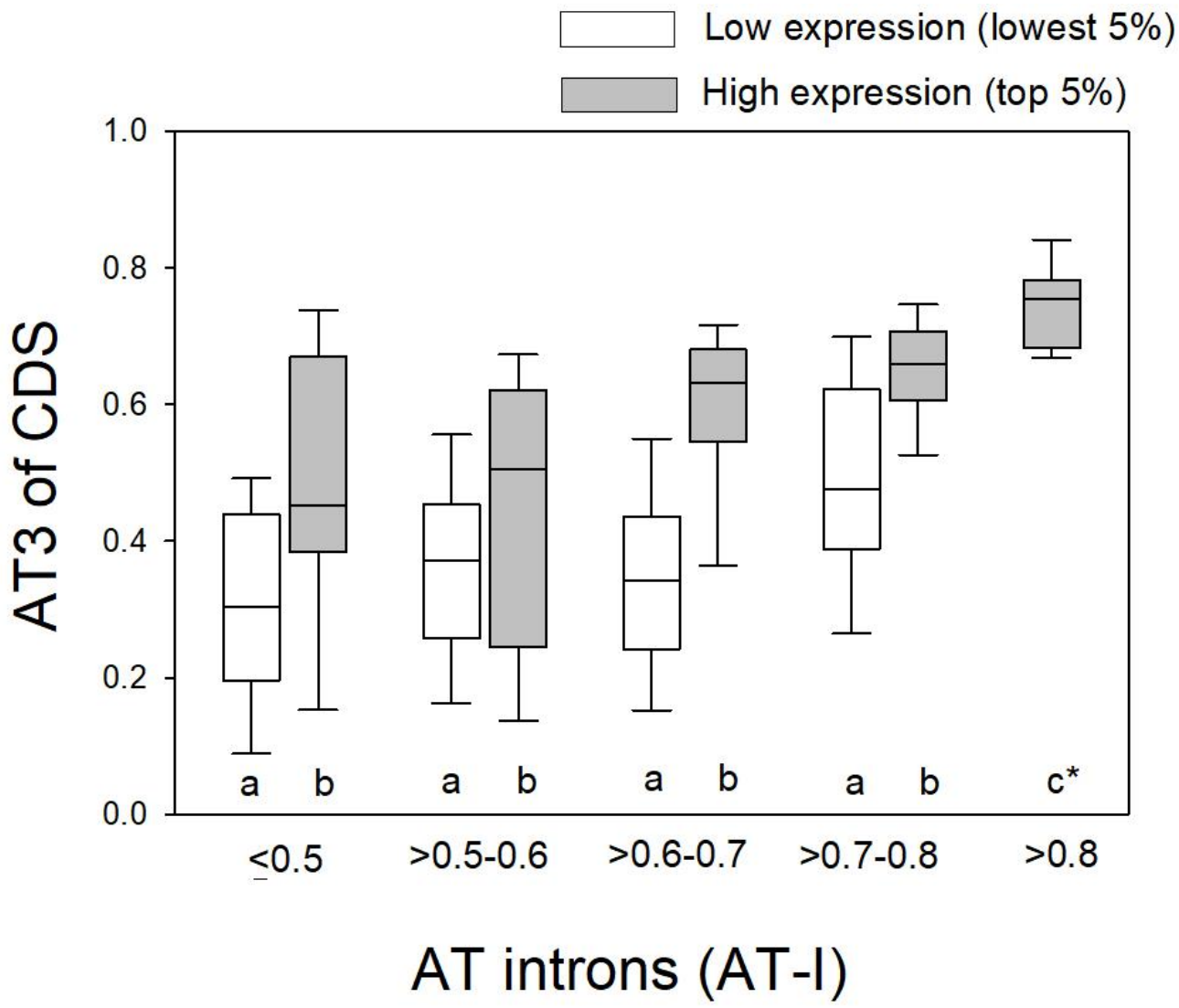


Fig. 1. Box plots of the AT3 of codons of lowly and highly expressed genes within narrow bins of AT-I, and thus presumably having similar background mutational pressures. Genes were binned into categories with similar AT-I content to ascertain differences in AT3 attributable to non-mutational (selection) pressures in highly transcribed genes. Different letters in each pair of bars indicates $P<0.05$ using MWU-tests. No statistically significant differences in AT-I were observed between highly and lowly expressed genes for any bins (MWU-test $P > 0.30$; with the exception of a minor AT-I difference in medians of 0.019 for category 3 (0.6-0.7)). *AT3 for this bar is statistically significant from all other bars. Only one gene had AT-I >0.8 for lowly expressed genes and thus the bar for this category was excluded.

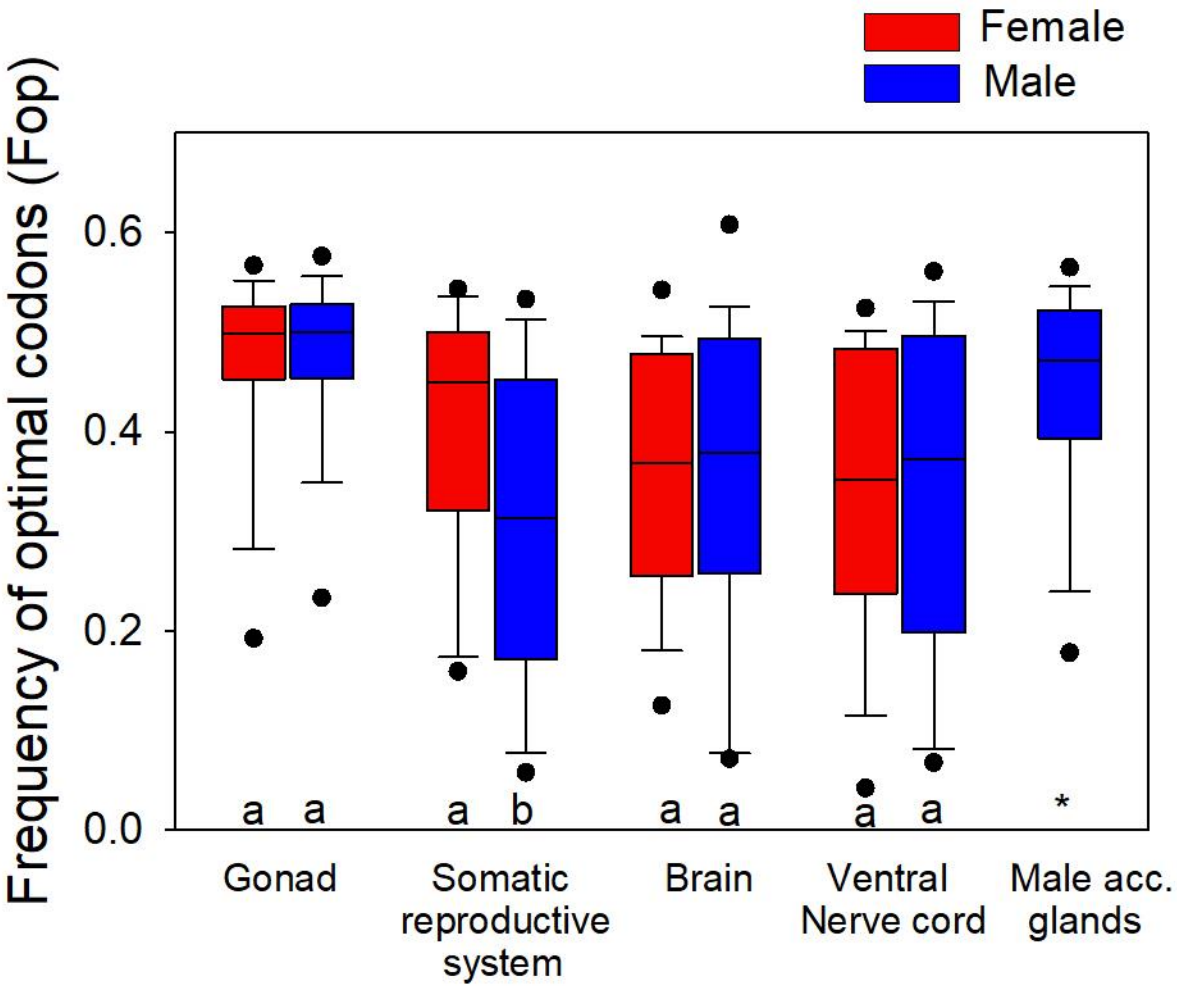
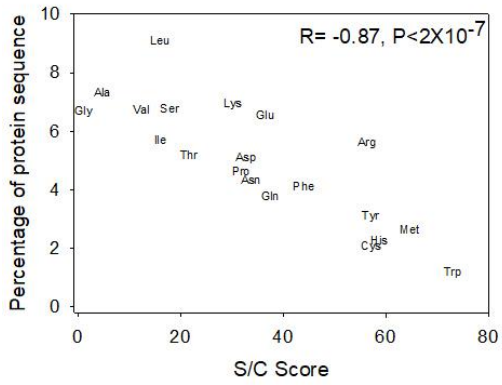
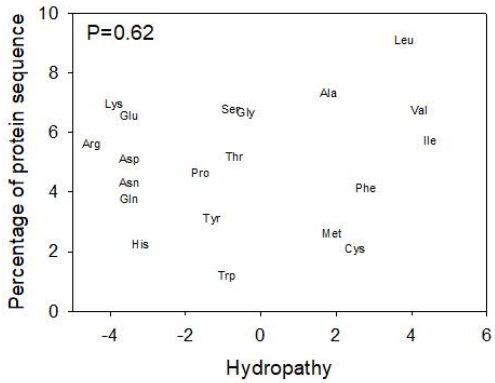


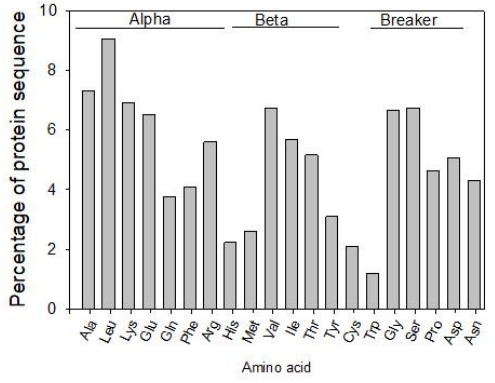
Fig. 2. The frequency of optimal codons (Fop) for genes with expression in the top 5% in one tissue type and not in any other tissues (Top5$_{\text{One-tissue}}$) for *G. bimaculatus*. Different letters within each pair of bars indicates a statistically significant difference (MWU-test $P<0.05$). Note that the gonad genes had higher Fop values than all other categories (MWU-tests $P<0.05$). *Indicates a difference of male accessory (acc.) gland genes from all other bars.



A. S/C score, highly expressed genes



B. Hydropathy, highly expressed genes



C. Alpha, beta, breaker; highly expressed genes

Fig. 3. The relationship between amino acid properties and amino acid use (percent per gene, averaged across genes) in the organism-wide highly expressed genes. A) size/complexity (S/C) score; B) hydropathy, and C) folding properties. For A and B Spearman's R and/or P values are shown, and for C no differences were detected between groups (alpha, beta, and breaker, Ranked ANOVA P>0.05).

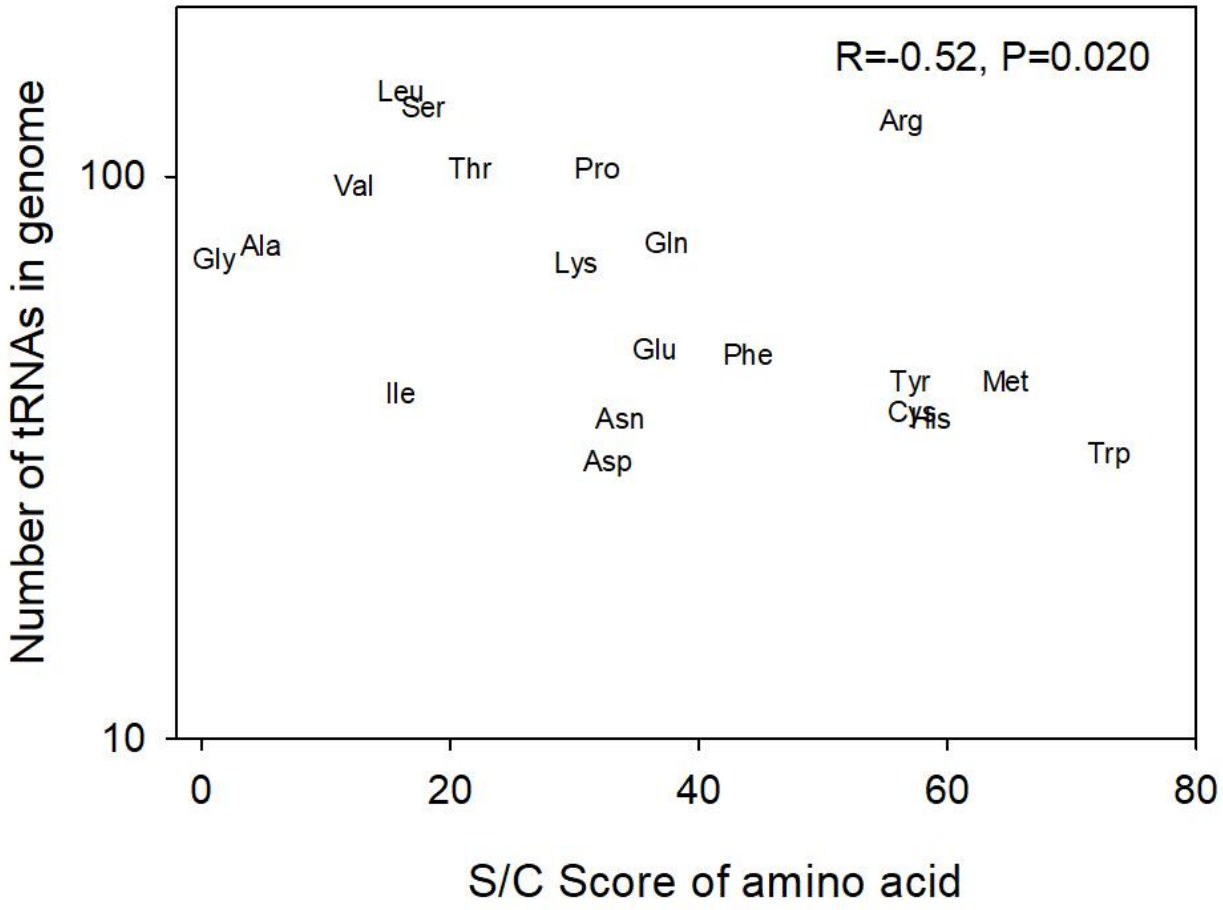


Fig. 4. The predicted gene counts of tRNAs in the *G. bimaculatus* genome and the S/C score of each of 20 amino acids [56]. The Spearman R correlation and P value is shown.