Evidence of multifaceted functions of codon usage in translation within the model beetle *Tribolium castaneum*

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Running title: Codon use shapes translation in beetles

Abstract

Synonymous codon use is non-random. Codons most used in highly transcribed genes, often called optimal codons, typically have high gene counts of matching tRNA genes (tRNA abundance) and promote accurate and/or efficient translation. Non-optimal codons, those least used in highly expressed genes, may also affect translation. In multicellular organisms, codon optimality may vary among tissues. At present however, codon use remains poorly understood in multicellular organisms. Here, we studied codon usage of genes highly transcribed in germ line (testis, ovary) and somatic tissues (gonadectomized males and females) of the beetle *Tribolium castaneum*. The results demonstrate that: 1) the majority of optimal codons were organism-wide, the same in all tissues, and had numerous matching tRNA gene copies (Opt-codon$_{\uparrow tRNAs}$), consistent with translational selection; 2) some optimal codons varied among tissues, suggesting tissue-specific tRNA populations; 3) wobble tRNA were required for translation of certain optimal codons (Opt-codon$_{wobble}$), possibly allowing precise translation and/or protein folding; and 4) remarkably, some non-optimal codons had abundant tRNA genes (Nonopt-codon$_{\uparrow tRNAs}$), and genes using those codons were tightly linked to ribosomal and stress-response functions. Thus, Nonopt-codon$_{\uparrow tRNAs}$ codons may regulate translation of specific genes. Together, the evidence suggests that codon use and tRNA genes regulate multiple translational processes in *T. castaneum*.

Keywords: Optimal codons, non-optimal codons, translational selection, translation regulation, tRNA genes

1. Introduction

In protein coding genes, the synonymous codons of amino acids are not used randomly. Biases in codon usage are thought to result from selection for translational efficiency and/or accuracy.[1-9] Mutational pressures can also shape codon usage.[5,10-13] Translational selection in many organisms has been supported by findings that the highly transcribed genes preferentially use a subset of codons, often described as "optimal" codons,[2,6,12-18] and has been observed in bacteria,[5,6,17] fungi,[16,19,20] plants[2,14,21] and animals, including spiders[22] and insects (e.g., *Drosophila, Aedes, Anopheles*, *Gryllus*, *Oncopeltus,* and weakly observed in *Bombyx* [2,15,23-27]). Whole-genome data show that optimal codons typically have correspondingly high numbers of iso-accepting tRNA gene copies in the genome, reflecting an organism's relative tRNA abundance,[1,5,6,19,20,28] and is consistent with selection for translational optimization.[1,4,5,18,20,29-33] The utility of

tRNA gene number to quantify organismal tRNA abundance has been supported *in vivo* in bacteria and eukaryotes.[28,34,35] For instance, the addition of tRNA genes for a codon of a specific amino acid to the *E. coli* genome markedly improved translation rates of genes containing that amino acid.[28] In this regard, the increased use of optimal codons in highly transcribed genes,[2,5,14] and the correspondence of these codons to abundant tRNA genes,[1,4] suggest that selection may favor optimization for cost efficient and/or accurate translation.

In contrast to unicellular systems, in multicellular organisms measuring codon usage can be complicated by the plurality of tissues, as optimal codons and tRNA populations may vary among tissue types.[36-38] For instance, cellular tRNA abundances can vary among tissues or cell types for at least some codons,[37,39,40] suggesting that translational selection may differ among tissues.[37] This has also been supported by findings of some variation in codon use of genes transcribed in different tissues in the few organisms studied to date. For example, in the plant *Arabidopsis* the use of specific codons in a gene depends on the tissue type in which it is maximally expressed, suggesting this species has localized tRNA populations,[38] a pattern that has also been proposed for rice.[41] Although similar studies in metazoans have been rare, a recent investigation in *D. melanogaster* showed that codons associated with elevated expression were not universal across tissues. For example, AAT was more commonly used than AAC for Asn in some tissues (e.g., testis, hindgut), while TGT was favored over TGC for Cys in the salivary glands, that was suggested to provide evidence of tissue-specific tRNA populations.[36] Additional studies are warranted to determine the universality of distinct optimal codon identities in various tissues of an organism. In particular, the germ line and somatic tissues comprise contrasts of significant interest, as the former directly determines an organism's reproductive success and fitness and experiences haploid selection in the meiotic and sex cells, such that translational optimization may be particularly relevant to those tissues.

While much attention has been focused on optimal codons in the literature, growing experimental research, largely from single-celled models or *in vitro* systems, suggests that non-optimal codons, those codons least used in highly transcribed genes (and/or codons defined as "rare" in some studies), can also play significant regulatory roles in translation.[34,42,43] In yeast for example, it was shown that cells altered their tRNA populations under stress and had increased levels of tRNAs that matched the rare codons found in stress-response genes, thus allowing the preferential translation of those mRNAs under stressful conditions, without any change in mRNA abundance.[44] Findings in cyanobacteria have indicated that circadian rhythms are regulated post-transcriptionally based on non-optimized codon use in genes of the *kaiABC1* cluster.[45] Further, non-optimal codons have been shown to slow rates of translational elongation and to control ribosome traffic on mRNA, which allows proper co-translational protein folding and/or functionality, based on *in vitro* cell-free translation systems from *Neurospora*[7] and *Drosophila*.[9] Non-optimal codons have also been found to facilitate co-translational protein folding in various yeast models [46] These data show that the use of one or a few types of rare codon(s) in a gene may markedly affect its translation, depending on the tRNA pool, suggesting that the supply-demand relationship between non-optimal codons and their matching tRNA abundances could comprise an adaptive mechanism of translational regulation.[34,44-48] To further understand this phenomenon, genomics and molecular evolution research on codon usage patterns in animal systems should expand beyond the typical focus on optimal codons, and specifically include assessments of non-optimal codons, and their relationships to tRNA genes.

In addition to non-optimal codons *per se*, some studies have indicated that the use of codons that have no matching tRNA, and obligately require wobble codon-anticodon tRNAs (wobbly at the third nucleotide of the codon) may also influence translation.[34] For instance, an investigation in four divergent eukaryotes found that the relative translation levels of cell-cycling gene mRNAs during various stages of the cell cycle depended on the frequency of codons that had no corresponding tRNA gene copies in the genome and thus required wobble tRNA.[49] Further, experimental research in yeast, human cells, and nematodes has shown that obligatory use of wobble tRNA decelerates translational elongation by slowing ribosomal translocation on the mRNA.[34,50,51] In this regard, the use of codons that require wobble tRNA could have a significant effect on translational dynamics, particularly in slowing translation,[34] and thus should also be considered in studies of codon usage patterns in an organism.

A metazoan species providing a promising pathway for the comprehensive study of codon usage in a multicellular system is the Coleopteran rust red flour beetle *Tribolium castaneum*. *T. castaneum* is a long standing model for genetics and developmental biology, has a well characterized genome,[18,52,53] and is estimated to have diverged from the fellow insect *Drosophila* approximately 300 Mya.[54-58] While a prior pioneering study had identified a putative list of optimal codons for *T. castaneum,*[18] the approach used in that study involved correlation analyses between codon frequency and expression level. Given that this method has been thought to often be poorly suited to revealing optimal codons, defined as those most common in highly transcribed genes,[1,5,59] analyses of codon use in this taxon would benefit from being revisited with alternative methods. Optimal codons can be most readily revealed via direct contrasts of codon usage in the highest versus lowest expressed genes in the genome, also known as the contrast method.[2,13-17,21,24,59] At present, like most multicellular model organisms, a multifaceted integrative approach has not yet been applied to assessments of codon usage in this beetle taxon, including the identification of optimal and non-optimal codons in highly transcribed genes at an organism-wide level, and within the somatic versus germ line tissues, nor have assessments been available of the links been such codon usage and tRNA gene counts, wobble tRNA, and gene functionality.

In the present study, we address these outstanding issues on codon usage in *T. castaneum* using genome-wide protein-sequence datasets (CDS) and large-scale transcriptome datasets from the male and female germ lines and somatic tissues (testes, ovaries, gonadectomized (GT-) males and GT-females).[60] From these data, we rigorously study optimal and non-optimal codons in this taxon, and their relationships to tRNA abundances and gene ontology. From these analyses, we report strong evidence for organism-wide optimal codons in all four tissue types and both sexes. The majority of these optimal codons have abundant matching tRNAs ($\text{Opt}_{\uparrow\text{tRNA}}$ status), consistent with pervasive translational selection for efficient and/or accurate protein synthesis in this species. A minority of optimal codons vary among the four tissues, suggesting small, but potentially meaningful, differences in tRNA populations between tissue types. Crucially, we report that a subset of the optimal codons did not have direct tRNA matches and obligately required wobble tRNA for translation ($\text{Opt-codon}_{\text{wobble}}$), which we propose may comprise a mechanism for slowing translation for accuracy or protein-folding purposes. Finally, we find that a number of non-optimal codons unexpectedly have abundant perfectly matching tRNA gene copies ($\text{Nonopt-codon}_{\uparrow\text{tRNAs}}$) and that these rare codons are preferentially used in genes with specific functions, including ribosomal protein genes and stress response genes. Thus, we hypothesize that the use of codons with $\text{Nonopt-codon}_{\uparrow\text{tRNAs}}$ status may be a potential mechanism to ensure preferential translation of specific

109 gene mRNAs. Collectively, our results reveal the multiple roles of codon usage in this beetle, suggesting not just
110 pervasive selection for the use of specific codons in highly transcribed genes for efficient and/or accurate translation, but
111 also translational regulatory roles of wobble codons and of non-optimal codons.
112
113 **2. Materials and Methods**
114 **2.1. *T. castaneum* CDS**
115 The annotated CDS of our main target species *T. castaneum* (v.5.2) were downloaded from Ensembl Metazoa
116 (http://metazoa.ensembl.org) and are also available at BeetleBase[52,53]). The full CDS per gene (longest CDS per gene,
117 N=16,434) was used for the study of codon usage. The full genome and its descriptive GFF file was also downloaded for
118 assessments.
119 **2.2. Biological samples and RNA-seq**
120 We aimed to determine the expression level (FPKM) for each of 16,434 genes in *T. castaneum* for germ line and
121 somatic tissues. For this we used the large-scale RNA-seq datasets for the ovaries, testes, GT-females and GT-males
122 shown in Supplementary Table S1.[60] The *T. castaneum* specimens were provided by the Brown lab at KSU
123 (https://www.k-state.edu/biology/people/tenure/brown/). Samples were grown under standard conditions until adulthood
124 and tissue dissections were then performed on unmated adults (a total of 150 animals per sex per biological replicate),
125 and RNA was extracted and processed for RNA-seq, as described previously.[60]
126 **2.3. Gene expression**
127 The RNA-seq reads (76bp) per sample were trimmed of adapters and poor-quality bases using the program
128 BBduk available from the Joint Genome Initiative (https://jgi.doe.gov/data-and-tools/bbtools/) set at default parameters.
129 Gene expression level was determined for the 16,434 genes (CDS) as FPKM after mapping each RNA-seq
130 dataset per tissue to the full CDS list for each species using Geneious Read Mapper[61], which yielded highly similar
131 results as other common mappers such as BBmap (https://jgi.doe.gov/data-and-tools/bbtools/). The average FPKM across
132 samples per tissue type (Supplementary Table S1) was used to measure expression per tissue. FPKM values were highly
133 correlated between replicates of each sample type (Spearman's Ranked $R>0.9$, $P<2X10^{-7}$)
134 **2.4. Identification of optimal and non-optimal Codons**
135 For identification of the optimal codons, we measured the relative synonymous codon usage (RSCU) per codon
136 per amino acid for each gene under study using CAICal.[62] RSCU values indicate the relative usage of a codon in a
137 synonymous codon family, and values >1 and <1 indicate favored and unfavored usage as compared to that expected
138 under equal usage of all codons respectively, and greater relative RSCU values among codons indicates elevated usage.
139 For each of the 18 amino acids in the genetic code with synonymous codons (note that Trp and Met only have one codon
140 each), we identified the optimal codon using the contrast method.[13-15,17,21,24,59,63] For this, we determined the difference in
141 RSCU (ΔRSCU) per codon between genes with the highest 5% versus the lowest 5% expression. The primary optimal
142 codon for each amino acid was defined as the codon with the highest and statistically significant positive ΔRSCU value,
143 indicating preferred usage in highly transcribed genes.[13-15,17,21,24,59,63] The primary non-optimal codon per amino acid was
144 defined as the codon with the largest negative and statistically significant ΔRSCU value, indicating low usage in highly

145 transcribed genes. Statistical significance per codon was applied using a t-test between RSCU values across all genes for
146 high versus low expressed genes.

147 As the literature reflects some variation in codon use terminology among studies to date, we explicitly define the
148 term "optimal codons" herein as those codons most used in highly transcribed genes based on ΔRSCU, which infers an
149 innate advantage of the codon under high transcription. Then, we secondarily assessed each optimal codon's
150 correspondence to the number of matching (codon-anticodon) tRNA genes in order to test their role in translational
151 accuracy/efficiency[1,4,5,18,20,29-33] or to infer possible other functions (e.g., wobble codons for translational slowing). For
152 non-optimal codons a similar approach was used wherein the non-optimal codon status was identified based solely on
153 ΔRSCU, and their relationships to tRNA were then separately assessed.

154 The frequency of optimal codons (Fop) is a measure of the degree of optimal codon usage per gene.[6] Fop was
155 determined in CodonW[64] using the primary optimal codons identified herein. Fop was also determined using the primary
156 optimal codons previously identified by Williford and Demuth 2012.[18] As multiple codons per amino acid were classified
157 as optimal in that assessment, we defined each primary optimal codon from the study as that with the strongest average
158 positive correlation across tissues for measuring Fop.

159 For an additional layer stringency, we wished to exclude the possibility that expression-mediated mutational-
160 biases towards specific nucleotides, which have been observed to some extent in certain organisms to date (e.g., *E. coli*,
161 humans[65,66]), contribute towards codon differences among high and low expressed genes herein. For this, we extracted all
162 introns for every gene in the genome (those with introns) using the GFF file available (see section 2.1). Introns are
163 thought to be mostly selectively neutral,[18,67] and thus the nucleotide content should reflect any underlying mutational
164 pressures in the genome, and on the nucleotide composition of synonymous codons in an organism.[13,18,67] If mutational
165 pressures on introns are not associated with gene expression level, it will exclude this factor in causing optimal codons in
166 the highly expressed genes, and further affirm the role of selection. All introns that were >50bp were extracted as the
167 region between exons and were concatenated per gene. The association between GC content and expression level were
168 assessed using a scatter plot and Spearman's ranked R.

169 **2.5. Identification of tRNA Genes**

170 To assess whether or how the optimal and non-optimal codons were related to the tRNA gene copy number, we
171 determined the number of iso-accepting tRNA genes per codon in the genome (*T. castaneum* v. 5.2) using tRNA-scan
172 SE.[18,53,68] The list of tRNA gene numbers identified in the current genome version was identical to that reported
173 previously[18] and is shown in Table 1.

174 **2.6. GO Functions**

175 The predicted GO functions were determined using Panther[69] using the option for *T. castaneum* as species

176 **2.7. Data Availability**

177 The CDS and genome v. 5.2 for *T. castaneum* are available at Ensembl Metazoa (http://metazoa.ensembl.org).
178 RNA-seq data for all samples from *T. castaneum* described in Supplementary Table S1 are available at the SRA database
179 under Bio-project number PRJNA564136.

180

3. Results and Discussion

3.1. Optimal codons in *T. castaneum*

We first report the organism-wide, or global, optimal codon per amino acid for *T. castaneum* using ΔRSCU and the average expression levels of all annotated genes across all four studied tissue types (testis, ovary, GT-male, GT-female) in Table 1. The primary optimal codon was defined as the codon with the largest positive ΔRSCU between highly and lowly transcribed genes and with P<0.05), was found for 17 of the 18 amino acids with synonymous codons. Seven primary optimal codons ended in T, three in A, five in C and two in G. We noted that Ile had two codons with nearly identical ΔRSCU values. Further, CAC for His showed signs of optimal codon usage in several individual tissues (see following section), and including this codon yields a study-wide total of 18 optimal codons (Table 1). The range of ΔRSCU values is similar to or larger than that observed in other multicellular eukaryotes, including nematode species, *Drosophila, Populus* and *Neurospora.* [2,14-16] Thus, the patterns in Table 1 are consistent with selection pressures have favored the use of a specific subset of codons in highly expressed genes[5] (for results on non-optimal codons see section 3.4 below).

While the striking use of specific optimal codons in genes under high expression levels in Table 1 in itself provides evidence of selection on codon usage, we wished to include additional layers of stringency to affirm the role of selection in favoring these codons. First, we determined the frequency of optimal codons (Fop), a measure of the degree of optimal codon usage per gene,[6] for all studied genes in the genome (N=16,434). As shown in Fig. 1A, we found that the Fop increased from genes with low (top 5% in the genome), to moderate (5 to 95%), to high (top 5%) expression levels (Ranked ANOVA and Dunn's paired test P<0.05). As low and high expressed genes were used to identify the optimal codons, the Fop was expectedly lowest and highest in those categories of genes respectively. Importantly however, moderately expressed genes, which were not used to identify the optimal codons, showed intermediate Fop values, suggesting a genome-wide tendency for greater use of optimal codons in CDS with elevated expression. Second, as codon usage can vary with protein length in some eukaryotes,[2,5,70] we repeated the assessment in Fig. 1A using genes with similar CDS lengths, which we binned into short (<150 codons), medium (≥150, <300), and long CDS (≥300). For each of these three length categories, we found the same stepwise increase of Fop values with expression level (Ranked-ANOVAs P<0.001). Thus, the link between expression and optimal codons cannot be explained by protein length. Third, from examination of introns, wherein nucleotide content is mostly shaped by mutational pressures,[18,67,71] we found that the GC (and thus AT) content of introns was uncorrelated to gene expression level (Spearman's correlation R= -0.09, Fig. 1B),[72,73] and thus indicates an absence of expression-mediated mutational biases[12,65,66,71] in this species. Further to this point, unlike some organisms wherein optimal codons typically end in only two or three types of nucleotides,[2,14,21,24] all four nucleotides are represented at the terminal position of optimal codons of this species (Table 1); this also excludes mutational biases in shaping the optimal codons in highly transcribed genes in this taxon.[5] Taken together, while we do not exclude the possibility that non-selective (mutational) mechanisms may contribute toward codon use of genes, particularly those under low or even moderate expression,[74] our observations indicate that a history of selection pressures likely plays a significant role in shaping the codon use of the most highly transcribed genes in this organism (top 5% expression), shown in Table 1.

217 **3.2. Most, but not all, optimal codons are the same across germ line and somatic tissues**

218 In order to compare optimal codon usage among the tissues under study, we next determined the optimal codons
219 (using ΔRSCU) using genes with high versus low expression (top and lowest 5%) separately for each of the four
220 individual tissue types, ovaries, testes, GT-females and GT-males. For rigor in this assessment, we identified the subset
221 of genes in the top 5% expression class that were only in the top category for one tissue type (and were not in the top 5%
222 expression in any of the other three tissues), to discern whether or not there was a tissue effect on optimal codons. Under
223 these criteria, we identified 372, 450, 444, and 272 genes for analysis, for ovaries, testes, GT-females and GT-males
224 respectively. This allowed us to specifically assess the codon usage of genes that were maximally transcribed only in one
225 individual tissue, as it has been found that if tissue-type has an effect on codon use, this effect is most apt to be evident in
226 its highly transcribed genes[38]. The results for ΔRSCU per tissue type are shown in Table 1. We report that 15 of the 18
227 primary optimal codons (including His) from the organism-wide assessment were identified as having the same optimal
228 codon in three, or all four, of the individual tissue-types (Table 1). Thus, the vast majority of primary optimal codons
229 were the same in these divergent tissues, including male and female germ lines and somatic tissue types.

230 However, several significant differences were also observed among tissues. For example, a male-specific
231 primary optimal codon was identified for the amino acid Phe (with two synonymous codons), as the codon TTC was
232 optimal in the testes and GT-males, but not in the ovaries or GT-females (Table 1). Similarly, a GT-male-specific
233 primary optimal codon ATC was identified for Ile (with three synonymous codons), where ATT was optimal for the
234 other three tissues. In turn, an ovary-specific optimal codon was evident for Pro (with four synonymous codons), as the
235 primary optimal codon was CCC in all tissues except for the ovaries, where it was CCT. In addition, a GT-female
236 optimal codon was identified for Lys (two synonymous codons), where AAG was optimal in the ovaries, testes, and GT-
237 males, but its alternate codon AAA was optimal for GT-females. These examples show that the primary optimal codon
238 varies among tissue types in this beetle, and thus this pattern suggests that translational selection regimes, and thus
239 corresponding tRNA populations may also vary among tissues.[36] Further, it is worth noting that in some cases there may
240 be tissue-specific preferences for codons using wobble tRNA (e.g., ATC for Ile in GT-males, see section 3.3).

241 These present results are consistent with the few available studies of tissue-specific codon usages and
242 translational selection from the fellow insect *D. melanogaster*[36] and in studied plants[38,41] (note that although some
243 evidence suggests humans have tissue-specific optimal codons, this has been debated, and may largely be an effect of the
244 GC content of isochores, which exist in those organisms[75,76]). Together, while the vast majority of optimal codons are
245 shared across tissues in these beetles, non-negligible differences are observed between tissues and sexes. Direct
246 quantification of tRNAs in cells or tissues has been mostly restricted to date to lab models of bacteria, yeast or *in vitro*
247 human cell lines,[37,39,40,44,77] and the accuracy and limitations of the various approaches (based on microarrays, Northern
248 blot, quantitative PCR, RNA-seq) remains debated[40,44,78,79]. Nevertheless, the development of robust methods to sequence
249 tRNAs that are applicable to non-traditional model organisms will allow further tests of whether or how tRNA
250 expression levels vary with tissues in *T. castaneum*, as is strongly suggested by these results.[36]

251 **3.3. A majority of organism-wide optimal codons have high tRNA gene copy numbers**

252 Given the minimal differences among tissues, for our remaining analyses we focus on the organism-wide
253 optimal codon usages (Table 1). The number of tRNA gene copies in the genome has commonly been used as a measure
254 of the relative abundance of each tRNA species.[1,4,18,20,29,30,49] If optimal codon usage were consistently a result of
255 selection in response to abundant tRNAs, then the primary optimal codon per amino acid should also have high relative
256 tRNA gene frequency (Opt-codon$_{\uparrow tRNAs}$ status). When using the organism-wide optimal codon list (Table 1), we found
257 that 12 of the primary optimal codons also had the highest, or near the highest tRNA gene counts of all codons per amino
258 acid, GCT (Ala), AGA (Arg), AAC (Asn), CAA (Gln), GAA (Glu), ATT (Ile), TTG (Leu), AAG (Lys), TTC (Phe), ACT
259 (Thr), TAC (Tyr), and GTT (Val). Further, while the positive ΔRSCU of CAC for His was not statistically significant
260 using the organism-wide assessment (P=0.26), this codon was optimal when individually considered in the ovaries, GT-
261 females and GT-males (P<0.05), and had seven matching tRNA genes. Thus, when including CAC for His as a codon
262 with optimal status, yields a study-wide total of 13 of the 18 primary optimal codons that have plentiful matching tRNA
263 genes. In other words, a majority of optimal codons have Opt$_{\uparrow tRNA}$ status. These results strongly suggest translational
264 selection for accuracy and/or efficiency[1,4] across a majority of amino acids in this beetle.

265 ***Hypothesis 1: Optimal codons use wobble tRNA to resolve conflict of high translation with sequence fidelity***

266 While 13 optimal codons had a high number of direct tRNA matches as expected under selection for
267 optimization of efficient and accurate translation, for the remaining five amino acids, a much different pattern was
268 observed. Specifically, the primary optimal codon (highly used in abundant transcripts) had no direct matching tRNA-
269 genes, and a wobble tRNA (shown in Table 1) must thus be employed for translation of these codons (denoted as Opt-
270 codon$_{wobble}$). For instance, Opt-codon$_{wobble}$ status was observed for the amino acids Asp (GAT), Cys (TGT), Gly (GGT),
271 Pro (CCC) and Ser (AGT). Thus, this result shows that while these identified optimal codons are preferred in highly
272 transcribed genes, their innate benefit cannot be due to having abundant direct matching tRNA, and thus another
273 mechanism must explain their high usage. Further, as shown in Supplementary Text File 1 and Fig. S1, within the group
274 of highly transcribed genes, each of these five individual codons with Opt-codon$_{wobble}$ status showed strong associations
275 with protein length, inferring putatively significant roles of the use of these types of codons in the translation of abundant
276 mRNAs, which may vary with the length of the translated sequence.

277 Experimental studies in bacteria and eukaryotic models have shown that codons using wobble tRNA act to slow
278 translation by decelerating the translocation of ribosomes on mRNA.[34,50,51] In addition, a study of the genomes of various
279 eukaryotes (humans, yeast, *Arabidopsis*) have indicated that cell-cycle genes had high usage of codons that had no
280 matching tRNA genes in the genome, and thus must employ wobble tRNA, which inherently have lower codon-
281 anticodon binding affinity than those codons with perfect matches.[49] The differential use of codons using wobble tRNA
282 in cell-cycle genes, combined with potential oscillations in tRNA abundances, were proposed to differentially regulate
283 the translation rates of gene mRNAs during various stages of the cell cycle.[49] Further, this was speculated to possibly
284 comprise a broader evolutionarily conserved phenomenon for translational regulation in eukaryotes.[49] In addition, the
285 usage of wobble-tRNAs in a gene could have some parallel functions to the use of non-optimal codons with low tRNA
286 abundance (Nonopt-codon$_{\downarrow tRNAs}$; see Table 1 for Nonoptimal codons with few tRNAs) which can prevent jamming of
287 multiple ribosomes during the initiation of translation,[35] and/or slow or pause translation during elongation, which would

288 facilitate accurate protein-folding.[7,9,39,80] In this regard, the results from these various studies suggest that the slowing of
289 translation that is induced by wobble-tRNA[34,50,51] could comprise an evolutionarily conserved mechanism shaping
290 various aspects of translation.

291 Significantly, a key modification that mediates wobbling at the first anticodon position (position 34 of the
292 anticodon loop) is for A34, which may be enzymatically deaminated by adenosine deaminase tRNA (ADATs) to form
293 inosine (I34). The I34 can pair with mRNA 3'codon bases A, C, or U in Eukarya [81,82] (see also for an A37 ADAT
294 (*Adat1*) in *D. melanogaster*[83]). For A34 modifications in eukaryotes, available research to date suggests that deamination
295 requires the ADAT2/ADAT3 (hetADAT) enzymes, which are thought to allow A34 modifications across diverse
296 eukaryotic systems. [81,84] This modification would be essential for some codons obligately requiring wobble tRNA (those
297 with no matching tRNAs, and no matching unmodified wobble tRNAs) in the highly transcribed genes studied here,
298 including with Opt-codon$_{wobble}$ status (e.g., Pro, CCC, Table 1). Thus, in addition to wobble codons using unmodified
299 tRNAs, further functional study of ADATs is warranted in model insects, such as *T. castaneum*, including possible
300 variation in expression and activity among tissues, in order to help to further ascertain the potential consequences of use
301 of wobble codons requiring tRNA modification at A34 on translation rates and protein folding.[42,81,84]

302 Taken together, we hypothesize here that for this beetle, the use of codons with Opt-codon$_{wobble}$ status in highly
303 expressed genes comprises a mechanism to slow or pause translation at various sites, which may lead to increased
304 accuracy of translation or allow co-translational protein folding,[50]. In addition, the high frequency of five specific codons
305 with Opt-codon$_{wobble}$ status in genes with abundant mRNAs (Table 1), suggests that these codons might also play a
306 significant role in post-transcriptional differential regulation of protein levels[49] in these beetles. Additional studies of
307 protein levels of genes with high usage of codons with Opt-codon$_{wobble}$ status will be needed to further test this aspect of
308 the hypothesis.

309 **3.4. Certain non-optimal codons have abundant tRNA genes**

310 Herein, we defined the primary non-optimal codon per amino acid stringently as the codon with the largest
311 negative ΔRSCU per amino acid, rather than simply all codons that were not optimal. Using these data, we assessed
312 whether those codons with low usage in highly transcribed genes also exhibit few tRNA gene copies, as might be
313 expected if codon usage is mostly shaped by translational selection for efficient and accurate translation (i.e., for
314 adaptation of optimal codons and tRNA abundance). The organism-wide primary non-optimal codons (per amino acid)
315 are shown in Table 1.

316 The results showed that some non-optimal codons, as expected, had low numbers of matching tRNA genes
317 (Nonopt-codon$_{\downarrow tRNAs}$ status, e.g., two tRNA genes for ACG (Thr), ATA (Ile), and TTA (Leu), one for CCG (Pro)).
318 Unexpectedly, however, certain non-optimal codons had relatively moderate to high tRNA gene abundance (denoted as
319 Nonopt-codon$_{\uparrow tRNAs}$). For instance, for Arg, whilst the codon CGG had no tRNA gene copies, its sister non-optimal
320 codon CGA (ΔRSCU= -0.290 and -0.265 respectively) had four tRNA gene matches. For Gly, both the primary and
321 secondary non-optimal codons GGC and GGA (-0.104 and -0.077 respectively) had eight and 15 matching tRNA gene
322 copies respectively. For Val, the primary non-optimal codon GTA had five tRNA genes, only slightly lower than the
323 seven observed for its optimal codon GTT. We noted, that if we relaxed our definition of a non-optimal codon to

consider any codon that is not optimal, we found that some of those codons also had many corresponding tRNA genes. For example, for Pro the non-optimal codon CCA (which had a weak and nonsignificant positive ΔRSCU value, +0.029, and thus would not have satisfied our strict definition of having the largest negative ΔRSCU for this amino acid) had 13 tRNA genes, an extraordinarily high value compared with other codons. Moreover, for Asp, the (less stringently) defined non-optimal codon GAC had ten matching tRNA copies. Collectively, it is evident that codons that are not the optimal codons in this taxon are not inevitably linked to a low abundance of matching tRNA genes, and rather in some cases exhibit high matching tRNA gene counts. Thus, these patterns suggest it is possible that non-optimal codons with elevated tRNAs play a specific regulatory role for highly transcribed genes.

A recent study in yeast has indicated that stress genes may preferentially use non-optimal codons that have abundant iso-accepting tRNA genes, to increase effective gene expression by promoting their translation over other proteins rather than affecting mRNA levels.[44] Based on this notion, we hypothesize here that codons with Nonopt-codon$_{\uparrow tRNAs}$ status in *T. castaneum* may regulate the translation of abundant mRNAs of proteins with specific functions in this beetle. To further evaluate this possibility, we examined the predicted gene ontology functions of the highly transcribed genes that had relatively elevated usage of non-optimal codons with abundant matching tRNAs.

Hypothesis 2: Non-optimal codons post-transcriptionally regulate translation based on protein functions

We assessed the GO functions of highly transcribed genes (top 5% in the genome from the organism-wide analyses across all four tissues, N=822; and a cutoff of 103.3 FPKM) that had relatively elevated use of codons with Nonopt-codon$_{\uparrow tRNAs}$ status (Table 1). For this assessment, rather than assess all strictly defined non-optimal codons, we chose as examples the codons GGC for Gly, GTA for Val, and CGA for Arg. These three codons were defined as non-optimal by our strict definition (having a large negative and statistically significant ΔRSCU, Table 1) and had substantial matching tRNA gene copy counts (four to eight tRNA genes each). These codons also had negative ΔRSCU values in all four of the tissue types studied (Table 1), indicating they consistently have non-favored status in this organism.

For the amino acid Gly, we identified those highly transcribed genes that had RSCU values for GGC of >1.5. An RSCU value of one is expected for each of the four Gly codons under equal usage, and thus values of 1.5 to 4 for GGC are relatively high. Thus, while Nonopt-codon$_{\uparrow tRNAs}$ are by definition rare in highly expressed genes, this approach allowed us to specifically examine the functions of this group (of highly expressed genes) that had unusually elevated use (RSCU) of this codon with Nonopt-codon$_{\uparrow tRNAs}$ status. A total of 20.4% of the highly transcribed gene set was in this class. As shown in Table 2, these genes included those involved in oxidative stress response, such as Peroxiredoxin, and those involved in olfactory activity. Thus, we speculate that these types of genes, which use codons with Nonopt-codon$_{\uparrow tRNAs}$ status, will exhibit less tRNA competition during translation elongation than those genes that use codons with few or no matching tRNA genes, such as the fellow Gly codon GGG (with only one tRNA match), or even those genes using non-optimal codons for other amino acids, such as CCG for Pro (with one tRNA match) (Table 1). In addition, we found that genes with elevated GGC frequency encoded numerous (N=15) ribosomal proteins. Thus, this finding suggests that usage of the non-optimal codon GGC may shape translation via a second mechanism: namely, by shaping the cellular abundance of specific ribosomal proteins *per se*, which are needed for translation. In this regard, the

360 non-optimal codon usage profiles in Gly appear consistent with a hypothesis wherein the usage of GGC regulates the
361 translation of a subset of genes in this taxon, and may even regulate translation rates *per se* via effects on certain
362 ribosomal proteins.

363 In terms of Val, those genes with high expression (top 5% in the genome), very rarely used the identified
364 primary non-optimal codon GTA. In fact, only 5.1% of the 822 highly transcribed genes had GTA RSCU values >1.5, an
365 extraordinarily low frequency. Those that did exhibit RSCU values >1.5 included genes involved in cytoskeleton
366 functions and actin synthesis, such as Cofilin/actin-depolymerizing factor homolog-like protein and profilin, as well as a
367 p53-related cell death protein, and a number of uncharacterized proteins (Table 2). For Arg, which has six synonymous
368 codons, genes with RSCU values >1.5 for the non-optimal codon CGA included genes involved in olfactory signaling
369 and with cytoskeleton roles (Table 2). It is particularly noteworthy that unlike the genes with elevated RSCU for GGC
370 (Gly), which included abundant ribosomal protein genes, no ribosomal protein genes were among those with elevated
371 frequency of GTA in Val or CGA for Arg. Thus, the ribosomal proteins in particular appear to be strongly connected to
372 the usage of the non-optimal GGC Gly codon, and thus we speculate that this codon may be particularly essential to their
373 regulation.

374 As mentioned above, prior data have suggested that non-optimal codons, when combined with low tRNA
375 abundance, can play important regulatory roles by preventing the jamming of multiple ribosomes during initiation of
376 translation, or slowing translation elongation and facilitating precise protein-folding.[7,9,35,39,80] The present study,
377 however, shows an additional, and much different, plausible effect of non-optimal codons in *T. castaneum*. Specifically,
378 we show that the use of non-optimal codons with abundant tRNA genes (Nonopt-codon$_{\uparrow tRNAs}$) is tightly linked to
379 predicted gene functionality (Table 2), and thus these codons may be likely to contribute to the preferential translation of
380 mRNAs of specific types of genes. This notion agrees with recent experimental data in yeast suggesting that non-optimal
381 or rare codons in stress genes promote the preferential translation of their mRNA in cells in response to stress-induced
382 changes in tRNA pools.[44] Thus, this comprises a potential mechanism for preferential translation of specific mRNA.
383 Herein, however, given that abundant tRNA gene copies are available in the genome for codons with Nonopt-codon$_{\uparrow tRNAs}$
384 status (and thus tRNAs should be consistently abundant in cells), we speculate that the use of these non-optimal codons
385 in certain ribosomal protein and stress genes (Table 2) likely acts as a mechanism to ensure their preferential translation
386 among the various mRNAs within cells at an organism-wide level, perhaps independent of environmental or tissue-
387 specific fluctuations in tRNA levels.

388 Collectively, our data on codons with Nonopt-codon$_{\uparrow tRNAs}$ status add to the growing support for a mechanism
389 wherein non-optimal or rare codons, combined with elevated tRNA abundances, significantly shape translational
390 regulation in eukaryotes.[34,44,45,47] Further study in *T. castaneum*, possibly including assessments of protein abundance of
391 genes with elevated usage of codons with Nonopt-codon$_{\uparrow tRNAs}$ status and with high usage of non-optimal codons with
392 rare tRNA genes, will help unravel the relationships between non-optimal codon usage and translation. In addition, *in*
393 *vivo* quantification of the tRNA populations in diverse tissue types in this beetle species,[37,39,40,44,77] will help affirm
394 whether these codons consistently exhibit high tRNA abundances, which could promote their preferential translation at
395 an organism-wide level.

396 **3.5. *T. castaneum* codon usage bias in context**

397 Selection (s) on optimal codons with abundant tRNAs (defined here for those codons with Opt-codon$_{\uparrow tRNA}$
398 status, and typically denoted as under translational selection), may be influenced by factors such as effective population
399 size (Ne) and genome size. In previous studies, smaller Ne (or NeS~1; and/or shorter generation times)[85,86] or larger
400 genomes in eukaryotes have been linked to reduced selection pressures on codon use.[87] For example, using a statistic
401 aimed to quantify an organism's genome-wide selection on codon usage (using predicted selection pressures per codon
402 and tRNAs; see also[74]) to compare among species, it was reported that strong selection pressures on codon use occurs for
403 some bacteria such as *E. coli* (which also have highly skewed RSCU values (>2) for some of its codons[88]), with
404 intermediate pressure in *D. melanogaster* and weak/absent in pressure humans. The authors of that study suggested
405 that this pattern was related to their (respectively increasing) genome sizes.[87] In this context, the overall translational
406 selection pressures on optimal codon in *T. castaneum* (Table 1) may be expected to be moderate, and similar to those of
407 its fellow insect *D. melanogaster* (genome sizes of 160 and 175 MB respectively).[53,89] However, such between-taxon
408 differences on selected codon bias could also reflect weaker pressure in smaller effective population sizes, which
409 decrease respectively from bacteria, insects and humans.[85] Nonetheless, our present study is largely focused on the
410 dynamics of the most highly transcribed (top 5%) genes in the genome in *T. castaneum* (rather than all genes; Table 1),
411 and includes analyses of not only of the translational selection on optimal codons *per se* (Opt-codon$_{\uparrow tRNAs}$ status), but also
412 putative selection favouring roles of wobble and non-optimal codons (Opt-codon$_{wobble}$ and Nonopt-codon$_{\uparrow tRNAs}$ status)
413 and their relationships to tRNAs in shaping translational processes in this taxon. While our data suggest selection has
414 been a factor in shaping the frequency of each of these types of codons in highly transcribed genes in *T. castaneum*,
415 further similar studies in more multicellular organisms, including additional *Tribolium* species, will ascertain the breadth
416 of such patterns across diverse metazoans.

417 **3.6. Comparison of Present Optimal Codon List to a Prior Report**

418 On a final note, it is worthwhile to mention here that the optimal codon list we present in Table 1 differs from
419 that previously reported in *T. castaneum.*[18] The previous report used a correlation method to determine optimal codons,
420 and a comparison of the present primary optimal codon list in Table 1 (for the whole organism analyses) to those earlier
421 findings is shown in Supplementary Table S2. We found that only nine of the 18 primary optimal codons identified
422 herein, were also identified as optimal by the previous study under the correlation method[18], even when we used very
423 loose criteria for defining a match to that prior assessment (that is, considering all optimal codons that were defined at
424 any level under the correlation method, regardless of whether they were the primary, secondary, or tertiary optimal
425 codon[18], as a match to our primary optimal codon). It has been previously argued that the use of a correlation approach
426 can often yield a misleading list of optimal codons.[59] Further, the R values observed for the codons defined as optimal
427 using the prior correlation method were typically <0.1 (the highest value was 0.237, Supplementary Table S2)[18]. A range
428 of such low values, even when statistically significant, is sometimes considered a very weak or absent correlation
429 (R<0.3),[72,73] and thus may not be conducive to revealing codons most often used in highly transcribed genes, as was the
430 goal here. Moreover, we found that increased gene expression level (organism-wide expression) was not positively
431 connected to the Fop when using the optimal codons (primary optimal codon defined as strongest correlation) identified

under the prior correlation method.[18] Rather, as shown in Supplementary Fig. S2, we found only mild variation in Fop among expression classes, and Fop was reduced in low and high expressed genes as compared to moderately expressed (Ranked ANOVA and Dunn's $P<0.05$), trends inconsistent with a persistent connection between Fop and expression level. However, we did find a strong connection between expression level and Fop using the optimal codons identified herein (Table 1, Fig. 1A). The method of employing ΔRSCU between high and low expressed genes has repeatedly been shown effective for specifically revealing the optimal codons, defined as those preferentially used in the most highly transcribed genes in the genome,[14,15,17,21,24,59] as was the present objective. Thus, the optimal codons defined herein are those most often used in highly transcribed genes, and were used for all our analyses (Table 1).

3.7. Conclusions

The present study has revealed the complex dynamics of codon usage in the multicellular beetle model system *T. castaneum*. We found that the majority of optimal codons in this animal model are shared at the organism-wide level and match tRNA with abundant gene copies, supporting the presence of species-wide translational selection for efficient and/or accurate translation. However, we also showed that a non-negligible subset of optimal codons varied among the four tissue types, suggesting a likelihood of tissue- and sex-specific tRNA populations, and thus localized translational selection. Based on codon optimality status and tRNA gene copies, we propose two hypotheses. The first hypothesis suggests that the usage of codons with Opt-codon$_{wobble}$ status in highly transcribed genes in this beetle has evolved as a mechanism that slows translation, which could increase precision of translation and/or protein folding. The second hypothesis proposes that usage of codons with Nonopt-codon$_{\uparrow tRNAs}$ status is as a mechanism that promotes high translation of mRNA of genes with specific cellular functions, which we show here to include stress response and ribosomal protein genes.

Further study in *T. castneum*, including assessments of cellular protein levels of genes using codons with Opt-codon$_{wobble}$ and Nonopt-codon$_{\uparrow tRNAs}$ status in germ line and somatic tissues, will help further unravel their potential roles in translation regulation. In addition, *in vivo* quantification of the tRNA populations in various tissue types and under stressful conditions in this beetle, as this methodology improves,[37,39,40,44,77,78] will provide additional valuable insights into tRNA population stability and variation between tissues.

While our data suggest that the frequency of specific codons in *T. castaneum* obligately requiring wobble tRNA, similar to those non-optimal codons with few tRNAs, may be linked to translational slowing or protein-folding functions in highly transcribed genes, future follow-up studies should assess whether such codons cluster or show original use patterns at or near protein (folding) structural elements, which some research suggests may occur in certain organisms[7,9,46,90-92], and/or whether those codons may effectively slow or pause translation.[7,9,50] In an understudied metazoan model such as *T. castaneum,* the former may be achieved via comprehensive bioinformatics analysis of protein structural properties and codon use,[46,91] and/or the development of a cell-free translation system allowing manipulation of codon use in mRNAs such those from *Neurospora* and *Drosophila*,[7,9] while the latter may be informed by ribosomal profiling analyses during translation.[7,9,50] Population-level approaches will also be valuable to further ascertaining the selection pressures acting on codon use,[86,88,93] particularly research on the mutational spectra of codons with Opt-

467 codon$_{\uparrow tRNAs}$, Opt-codon$_{wobble}$ and Nonopt-codon$_{\uparrow tRNAs}$ status, to ascertain whether such codon mutations show signals of
468 selection favouring their fixation in highly transcribed genes of *T. castaneum*.
469 At present, most non-traditional multicellular organisms have not had as many protocols optimized for lab-based
470 experimental or transgenic research of codon optimization, including rates of translation elongation, protein folding,
471 tRNA-charging, or codon-anticodon tRNA binding, as compared to the established widely studied single-celled models
472 or *in vitro* cell lines.[7,28,34,51] We have shown here, however, using the species *T. castaneum,* that a multifaceted approach
473 using analyses of gene expression, tRNA genes, tissue-type, and gene functionality can be used to suggest how codon
474 usage shapes translational optimization and regulation in a metazoan system.
475

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481

482 **Figure Legend**

483 **Figure 1. A.** The frequency of optimal codons (Fop) across all 16,434 genes studied in *T. castaneum*. Genes are
484 categorized into low (lowest 5%, FPKM<0.013), moderate (5 to 95%) and high (top 5%) transcription (FPKM>103)
485 groups based on average expression across all four tissue types (testes, ovaries, GT-males, GT-females). Different letters
486 below bars indicate a statistically significant difference using Ranked ANOVA and Dunn's paired contrasts (P<0.05). **B.**
487 The GC content of introns with respect to the expression level per gene (Spearman's Ranked R is shown). Values are
488 shown for all genes with introns >50bp (N=5,143).
489

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Table 1. The organism-wide ΔRSCU between high versus low expressed genes (using averaged expression across all four tissue types, the ovaries, testes, GT-females, and GT-males). In addition, the ΔRSCU are shown when high and low expressed genes were determined for each of the four individual tissue types. The primary optimal (Opt.) codons are in bold and have the largest positive and statistically significant ΔRSCU (t-test P<0.05) per amino acid. For the combined four tissue assessment (organism-wide), the primary optimal (Opt.) and non-optimal codons (Non opt.) are shown with X. Cases where relatively plentiful tRNA genes match the optimal codon per amino acid are underlined and bold. The wobble anticodons for codons with zero matching tRNA copies are shown (standard anticodon/wobble anticodon shown according to classical wobble rules; see also[81,94]).

		Organism-wide RSCU & ΔRSCU (from average expression across all tissues)								**ΔRSCU per Tissue Type (from expression within each tissue)**							
Amino Acid	**Codon**	**High RSCU**	**Low RSCU**	**ΔRSCU**	**P**	**Opt.**	**Non opt.**	**tRNA No.**	**Standard/Wobble**	**ΔRSCU ovaries**	**P**	**ΔRSCU testes**	**P**	**ΔRSCU female**	**P**	**ΔRSCU male**	**P**
Ala	GCT	1.144	1.001	**+0.143**	**	**X**		**14**		+0.109		**+0.136**	*	**+0.179**	**	+0.146	**
Ala	GCC	1.238	1.203	+0.034				0	GGC/AGC	+0.023		+0.020		+0.115	*	**+0.198**	**
Ala	GCA	0.833	0.867	-0.033				2		-0.065	*[a]	-0.008		-0.110	*	-0.175	**
Ala	GCG	0.731	0.899	-0.168	**		**X**	3		-0.047		-0.128	**	-0.163	**	-0.161	**
Arg	CGT	0.919	0.830	+0.089	*			5		+0.082		+0.087	*	-0.043		+0.116	*
Arg	CGC	0.907	1.117	-0.209	**			0	GCG/ACG	-0.204	*	-0.112	**	-0.231	**	-0.091	*
Arg	CGA	0.946	1.212	-0.265	**		**X**[b]	4		-0.304	**	-0.143	**	-0.189	**	-0.282	**
Arg	CGG	0.650	0.941	-0.290	**		**X**	0	CCG/UCG	-0.235	**	-0.263	**	-0.195	**	-0.272	**
Arg	AGA	1.401	0.990	**+0.411**	**	**X**		**3**		**+0.393**	**	**+0.341**	**	**+0.415**	**	**+0.276**	**
Arg	AGG	1.096	0.801	+0.295	**			3		+0.367	**	+0.247	**	+0.250	**	+0.287	**
Asn	AAT	1.030	0.997	+0.033				0	AUU/GUU	+0.039		+0.041	*[a]	+0.012		-0.027	
Asn	AAC	0.955	0.864	**+0.091**	**	**X**		**5**		**+0.071**	*	**+0.067**	*	**+0.066**	*	**+0.115**	**
Asp	GAT	1.002	0.938	**+0.063**	*	**X**		0	AUC/GUC[e]	**+0.115**	**	**+0.084**	**	**+0.070**	*	+0.031	
Asp	GAC	0.942	0.964	-0.021			**X**[c]	10		-0.035	*[a]	-0.008		-0.026		+0.000	
Cys	TGT	0.986	0.854	**+0.131**	**	**X**		0	ACA/GCA[e]	**+0.204**	**	**+0.130**	**	**+0.155**	**	**+0.103**	**
Cys	TGC	0.802	0.827	-0.025				3		-0.026		+0.028		-0.029	*	-0.006	
Gln	CAA	1.179	1.098	**+0.081**	*	**X**		**5**		+0.043		**+0.089**	*	**+0.099**	*	**+0.064**	*[a]

Gln	CAG	0.758	0.785	-0.026				3		+0.026		+0.002		-0.053		-0.015		
Glu	GAA	1.236	1.110	**+0.125**	**	**X**		**8**		**+0.093**	*	**+0.101**	**	**+0.100**	*	**+0.086**	*
Glu	GAG	0.733	0.767	-0.034				5		-0.006		+0.002		-0.036		-0.024	
Gly	GGT	0.918	0.801	**+0.116**	**	**X**		0	ACC/GCC[e]	+0.138	*	**+0.133**	**	+0.046		**+0.063**	*
Gly	GGC	1.017	1.122	-0.104	*		**X**	8		-0.109		-0.077	*	-0.070		-0.001	
Gly	GGA	1.124	1.201	-0.077	*[a]		**X**[b]	15		-0.137	**	-0.071	**	-0.017	*	-0.070	**
Gly	GGG	0.859	0.792	+0.066				1		**+0.171**	**	+0.072	*	**+0.059**	*	+0.043	
His	CAT	0.840	0.817	+0.023				0	AUG/GUG	+0.055		+0.032		+0.016		-0.017	
His	CAC	1.014	0.978	+0.036				7		**+0.067**	*[a]	+0.054		**+0.053**	*	**+0.084**	*
Ile	ATT	1.359	1.278	**+0.081**	*	**X**[d]		**7**		**+0.121**	**	**+0.051**	*	**+0.115**	*	+0.033	*[a]
Ile	ATC	1.024	0.941	**+0.083**	*	**X**[d]		0	GAU/AAU	+0.005		+0.078		+0.012		**+0.165**	**
Ile	ATA	0.578	0.661	-0.083	*		**X**	2		-0.048		-0.057		-0.071		-0.141	**
Leu	TTA	0.999	1.127	-0.128	*		**X**	2		-0.087	*	-0.095	*	-0.121	*	-0.211	**
Leu	TTG	1.794	1.336	**+0.458**	**	**X**		**4**		**+0.409**	**	**+0.391**	**	**+0.339**	**	**+0.444**	**
Leu	CTT	0.901	0.998	-0.096	*			5		-0.139	**	-0.082	*	-0.003		-0.053	*[a]
Leu	CTC	0.877	0.926	-0.049				0	GAG/AAG	-0.081		-0.033		-0.042		+0.036	
Leu	CTA	0.492	0.561	-0.068	*			2		+0.023		-0.075	*[a]	-0.003		-0.073	*
Leu	CTG	0.900	1.008	-0.107	*			2		-0.093		-0.092	*	-0.158	**	-0.108	*
Lys	AAA	1.272	1.273	-0.000				6		-0.019		-0.005		**+0.068**	*	-0.011	
Lys	AAG	0.728	0.654	**+0.074**	*	**X**		**5**		**+0.075**	*	**+0.067**	*	-0.020		**+0.058**	*
Phe	TTT	1.058	1.042	+0.015				1		+0.073		+0.003		+0.016		-0.092	**
Phe	TTC	0.916	0.850	**+0.065**	*	**X**		**5**		+0.015		**+0.076**	*	+0.034		**+0.160**	**
Pro	CCT	0.904	0.785	+0.119	*			7		**+0.126**	*	+0.092	*	+0.097	*	+0.076	*
Pro	CCC	1.090	0.917	**+0.172**	**	**X**		0	GGG/AGG[e]	+0.064	*	**+0.131**	*	**+0.163**	*	**+0.264**	**
Pro	CCA	1.044	1.014	+0.029			**X**[c]	13		+0.166		+0.021		+0.063		-0.021	
Pro	CCG	0.889	1.102	-0.213	**		**X**	1		-0.220	**	-0.140	**	-0.249	**	-0.237	**
Ser	TCT	0.849	0.732	+0.116	*			4		+0.084		+0.073		+0.061		+0.026	
Ser	TCC	0.894	1.056	-0.162	**			0	GGA/AGA	-0.137		-0.152	**	-0.051		-0.010	
Ser	TCA	1.059	0.977	+0.082	*[a]			2		+0.114		+0.072	*	-0.017		+0.001	
Ser	TCG	1.128	1.231	-0.103	*			2		-0.066		-0.023		-0.101	*	-0.012	

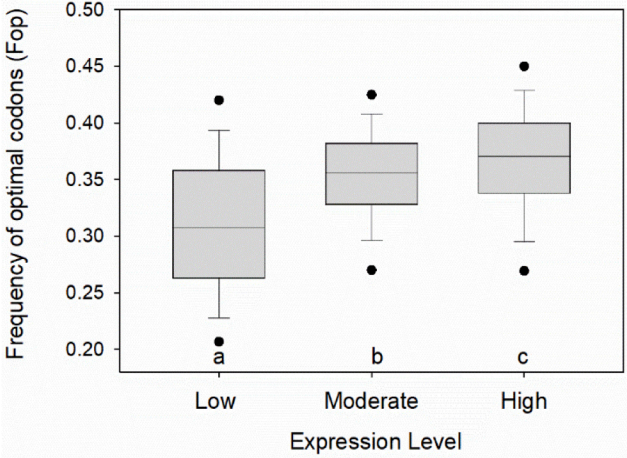
Ser	AGT	1.149	0.922	**+0.226**	**	**X**		0	ACU/GCU[e]	**+0.218**	**	**+0.197**	**	**+0.268**	**	**+0.137**	**
Ser	AGC	0.900	1.039	-0.138	*		**X**	3		-0.156	*	-0.156	**	-0.125	**	-0.105	**
Thr	ACT	1.107	0.884	**+0.222**	**	**X**		**5**		**+0.199**	**	**+0.266**	**	**+0.188**	**	**+0.207**	**
Thr	ACC	1.032	1.003	+0.029				0	GGU/AGU	+0.026		-0.026	*[a]	+0.148	*	+0.178	**
Thr	ACA	1.001	1.013	-0.012				3		-0.027		-0.059		-0.076		-0.136	**
Thr	ACG	0.812	1.006	-0.194	**		**X**	2		-0.129	*	-0.113	*	-0.213	**	-0.211	**
Tyr	TAT	0.819	0.881	-0.062	*			0	AUA/GUA	+0.040		-0.018	*	-0.041	*	-0.080	**
Tyr	TAC	1.096	0.898	**+0.197**	**	**X**		**13**		**+0.123**	*	**+0.162**	**	**+0.156**	**	**+0.210**	**
Val	GTT	1.262	1.133	**+0.129**	*	**X**		**7**		**+0.124**	*	**+0.119**	*	**+0.157**	*	**+0.101**	*
Val	GTC	0.976	0.999	-0.022				0	GAC/AAC	-0.017		-0.034	*[a]	-0.027		+0.053	
Val	GTA	0.552	0.625	-0.073	*		**X**	5		-0.047	*	-0.064	*	-0.019		-0.082	*
Val	GTG	1.156	1.140	+0.015				3		+0.020		+0.032		-0.055		-0.041	

**P<0.001, *P<0.05 and ≥0.001; [a] P-values are between 0.05 and 0.1 and thus is considered a putative optimal or non-optimal codon; [b] Secondary non-optimal codon with relatively high matching tRNA count; [c] While not having a statistically significant negative ΔRSCU, the codon is not optimal and is notable by its high tRNA count; [d] Both codons are optimal codons at nearly the same level; [e] Codon has Opt-codon$_{wobble}$ status.

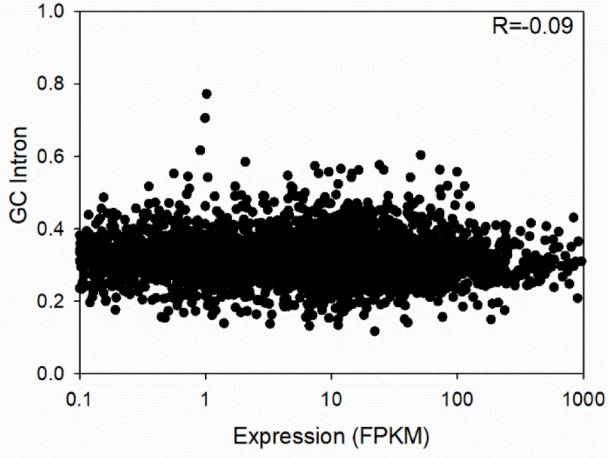
Table 2. Examples of functions of the highly transcribed genes in *T. castaneum* that have elevated use of codons with Nonopt-codon$_{\uparrow tRNAs}$ status (non-optimal codons with abundant matching tRNA genes ((≥4)). While these codons are by definition typically uncommon in highly transcribed genes (Table 1), the subset of genes with elevated use of these codons, RSCU >1.5, were identified and are shown below. These genes are candidates for translational upregulation due to the elevated use of codons with Nonopt-codon$_{\uparrow tRNAs}$ status.

High GGC Usage for Gly (with RSCU>1.5)	
Gene Functions	
Ribosomal protein genes	
TC006109	14-3-3 protein epsilon-like Protein
TC011123	40S ribosomal protein S13-like Protein
TC008667	40S ribosomal protein S20-like Protein
TC005984	40S ribosomal protein S26
TC010830	40S ribosomal protein S6
TC009214	40S ribosomal protein S7
TC014757	40S ribosomal protein S8
TC016306	60S acidic ribosomal protein P0
TC010413	60S acidic ribosomal protein P1-like Protein
TC015013	60S acidic ribosomal protein P2-like Protein
TC013536	60S ribosomal protein L17-like Protein
TC007932	60S ribosomal protein L21-like Protein
TC013168	60S ribosomal protein L4-like Protein
TC030666	60S ribosomal protein L6-like Protein
TC011182	60S ribosomal protein L7a-like Protein
Olfactory	
TC007741	Odorant binding protein 12
TC010070	Odorant binding protein C06
TC008681	Chemosensory protein 1
Stress-response	
TC004948	Peroxiredoxin 1-like Protein
TC014929	Peroxiredoxin 1-like Protein
Uncharacterized Proteins (N=50)	
High GTA usage for Val (with RSCU>1.5)	
Cytoskeletal	
TC001574	Cofilin/actin-depolymerizing factor homolog-like Protein
TC033072	profilin
p53 related	
TC034594	Cell death-inducing p53-target protein 1-like protein
Ribosomal protein genes (N=0)	
Uncharacterized Proteins (N=15)	
High CGA usage for Arg (with RSCU>1.5)	
Olfactory	
TC010070	Odorant binding protein C06; TcOBP7M ortholog
TC030421	Odorant receptor 305; Or305; ortholog
TC008681	Chemosensory protein 1; TcCSP7K; ortholog
p53 related	
TC034594	Cell death-inducing p53-target protein 1-like protein
Cytoskeletal	
TC007700	Tubulin-specific chaperone cofactor E-like protein
TC009721	Microtubule-protein RP/EB family member 1

TC012270	Troponin C, isoform 1-like Protein
TC033072	Profilin
TC001942	Putative dynactin subunit 2-like Protein (Fragment)
Ribosomal protein genes (N=0)	


Frequency of optimal codons (Fop)
0.50
0.45
0.40
0.35
0.30
0.25
0.20
a
b
c
Low
Moderate
High
Expression Level

A


R=-0.09
GC Intron
1.0
0.8
0.6
0.4
0.2
0.0
0.1
1
10
100
1000
Expression (FPKM)

B

SUPPLEMENTARY MATERIAL

Evidence of multifaceted functions of codon usage in translation within the model beetle *Tribolium castaneum*

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

Table S1. The number of RNA-seq reads for each tissue-type in the present study [1].
RNA-seq data are shown before and after adapter and quality trimming with BBDuk (https://jgi.doe.gov/data-and-tools/bbtools/). The Short Read Archive (SRA) Biosample identifiers are also shown (https://www.ncbi.nlm.nih.gov/sra).

Tissue Sample[a]	No. of Reads		SRA Biosample ID
	Before trimming	After trimming	
Tribolium castaneum			
Testes sample 1	18,006,255	17,995,655	SAMN12702873
Ovary sample 1	39,140,493	39,122,050	SAMN12702874
GT-male sample 1	25,630,261	25,609,723	SAMN12702875
GT-female sample 1	41,513,717	41,472,348	SAMN12702876
Testes sample 2	24,795,583	24,787,238	SAMN12702877
Ovary sample 2	22,306,622	22,286,961	SAMN12702878
GT-male sample 2	62,781,001	62,712,242	SAMN12702879
GT-female 2	52,275,340	52,211,149	SAMN12702880

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

Table S2. Comparison of the primary optimal codon list generated using the organism-wide analyses of high and low expressed genes in the present study (ΔRSCU) to optimal codons obtained using the correlation method in Williford and Demuth (2012), which defined up to three optimal codons per amino acid. Cases wherein the present primary optimal codon matched an optimal codon (at any level) identified under the correlation approach are indicated in the right-most column. Optimal codons defined in each study are in bold and underlined.

ΔRSCU Method Herein			Correlation Method [2]					Same optimal codon
Amino Acid	Codon	RSCU (All Tissues)	Amino Acid	Codon	Female RT	Male RT	Female & Male whole body	
Ala	**GCT**	**+0.143**	Ala	GCT	−0.028	−0.014	0.005	NO*
Ala	GCC	+0.034	Ala	**GCC**	0.176	0.16	0.184	
Ala	GCA	-0.033	Ala	GCA	−0.17	−0.15	−0.155	
Ala	GCG	-0.168	Ala	**GCG**	0.078	0.061	0.017	
Arg	CGT	+0.089	Arg	CGT	0.016	0.008	0.0002	
Arg	CGC	-0.209	Arg	**CGC**	0.068	0.075	0.052	
Arg	CGA	-0.265	Arg	CGA	−0.15	−0.154	−0.13	
Arg	CGG	-0.290	Arg	CGG	−0.012	−0.029	−0.038	
Arg	**AGA**	**+0.411**	Arg	AGA	−0.006	0.012	0.031	NO
Arg	AGG	+0.295	Arg	**AGG**	0.23	0.225	0.209	
Asn	AAT	+0.033	Asn	AAT	−0.112	−0.1	−0.135	
Asn	**AAC**	**+0.091**	Asn	**AAC**				**YES**
Asp	**GAT**	**+0.063**	Asp	GAT	−0.028	−0.023	−0.043	NO
Asp	GAC	-0.021	Asp	GAC				
Cys	**TGT**	**+0.131**	Cys	TGT	−0.014	0.009	−0.011	NO
Cys	TGC	-0.025	Cys	TGC				
Gln	**CAA**	**+0.081**	Gln	CAA	−0.101	−0.075	−0.064	NO
Gln	CAG	-0.026	Gln	**CAG**				
Glu	**GAA**	**+0.125**	Glu	GAA	−0.156	−0.137	−0.108	NO
Glu	GAG	-0.034	Glu	**GAG**				
Gly	**GGT**	**+0.116**	Gly	GGT	0.008	0.022	0.03	NO
Gly	GGC	-0.104	Gly	**GGC**	0.095	0.069	0.067	
Gly	GGA	-0.077	Gly	GGA	−0.196	−0.203	−0.145	
Gly	GGG	+0.066	Gly	**GGG**	0.196	0.205	0.157	
His	CAT	+0.023	His	CAT	−0.043	−0.054	−0.052	
His	**CAC****	+0.036	His	**CAC**				**YES****
Ile	ATT	+0.081	Ile	ATT	−0.089	−0.059	−0.087	
Ile	**ATC**	**+0.083**	Ile	ATC	0.148	0.137	0.171	**YES**
Ile	ATA	-0.083	Ile	ATA	−0.043	−0.065	−0.078	
Leu	TTA	-0.128	Leu	TTA	−0.07	−0.096	−0.121	
Leu	**TTG**	**+0.458**	Leu	**TTG**	0.182	0.237	0.188	**YES**
Leu	CTT	-0.096	Leu	CTT	−0.123	−0.111	−0.081	
Leu	CTC	-0.049	Leu	**CTC**	0.076	0.05	0.084	
Leu	CTA	-0.068	Leu	CTA	−0.013	−0.019	0.0003	
Leu	CTG	-0.107	Leu	**CTG**	0.059	0.055	0.06	
Leu	AAA	-0.090	Leu	AAA	0.194	0.161	0.158	

Ser	TCC	-0.162	Ser	TCC	0.015	−0.001	0.037	
Ser	TCA	+0.082	Ser	TCA	−0.041	−0.039	−0.048	
Ser	TCG	-0.103	Ser	**TCG**	0.103	0.09	0.073	
Ser	**AGT**	**+0.226**	Ser	**AGT**	0.071	0.108	0.075	**YES**
Ser	AGC	-0.138	Ser	**AGC**	0.056	0.045	0.06	
Thr	**ACT**	**+0.222**	Thr	ACT	0.004	0.031	0.025	NO
Thr	ACC	+0.029	Thr	**ACC**	0.111	0.097	0.142	
Thr	ACA	-0.012	Thr	ACA	−0.12	−0.119	−0.123	
Thr	ACG	-0.194	Thr	**ACG**	0.088	0.079	0.039	
Tyr	TAT	-0.062	Tyr	TAT	−0.094	−0.107	−0.124	
Tyr	**TAC**	**+0.197**	Tyr	**TAC**				**YES**
Val	**GTT**	**+0.129**	Val	GTT	−0.049	−0.031	−0.038	NO
Val	GTC	-0.022	Val	**GTC**	0.09	0.077	0.106	
Val	GTA	-0.073	Val	GTA	−0.057	−0.069	−0.058	
Val	GTG	+0.015	Val	**GTG**	0.104	0.112	0.081	

* The same optimal codon GCC was found for ovaries and testes when examined individually in the present study.

** The CAC codon is statistically significantly optimal for three of four tissues herein, but not in the summary analyses of all pooled tissues. It is included in comparison of the present optimal codons to the correlation method.

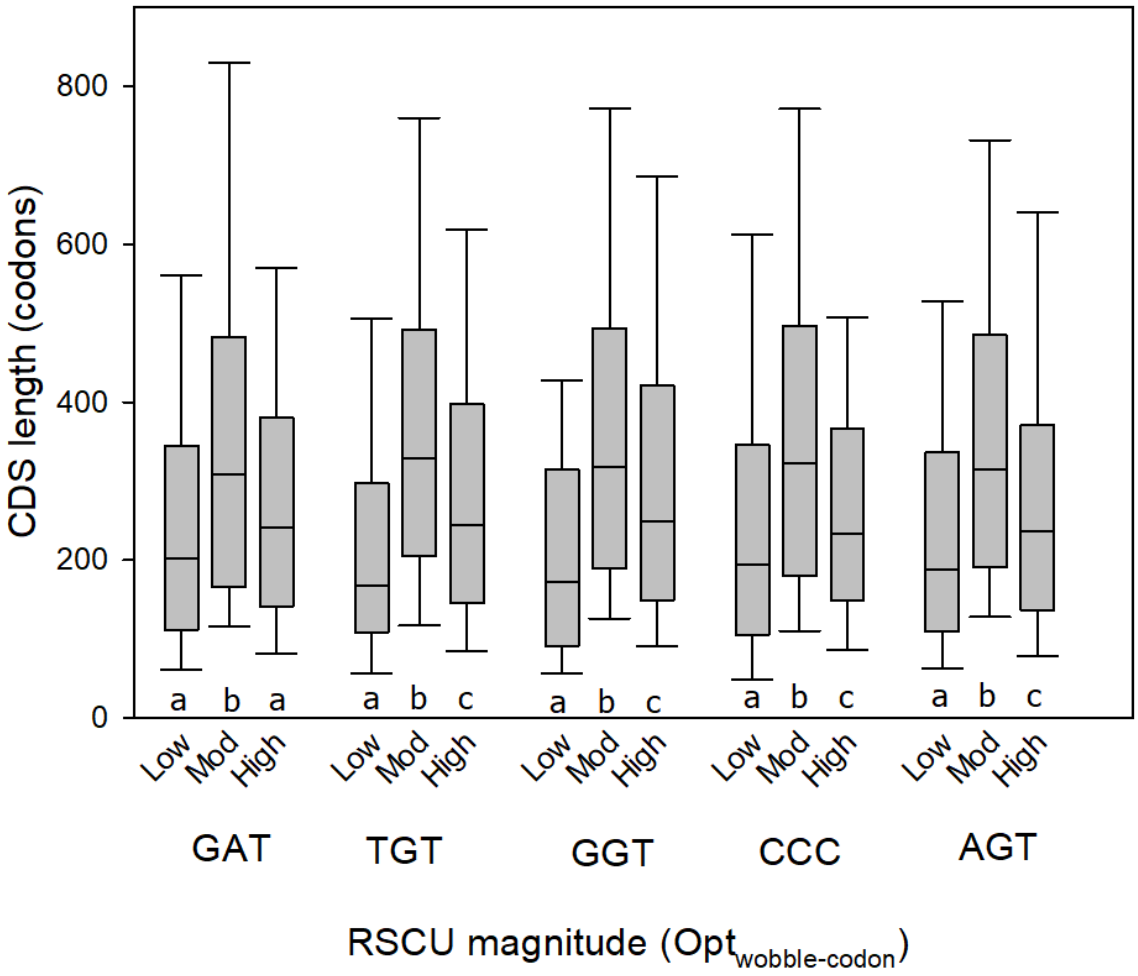


Figure S1. The relative use of codons with Opt-codon$_{wobble}$ status (GAT, TGT, GGT, CCC and AGT) in highly expressed genes with respect to CDS length. Different letters below each set of three bars (per codon) indicate a statistically significant difference using Ranked ANOVA and Dunn's paired contrasts ($P<0.05$). The 822 highly expressed genes were divided into three equal sized classes of RSCU values (low, moderate (mod), high) for each codon.

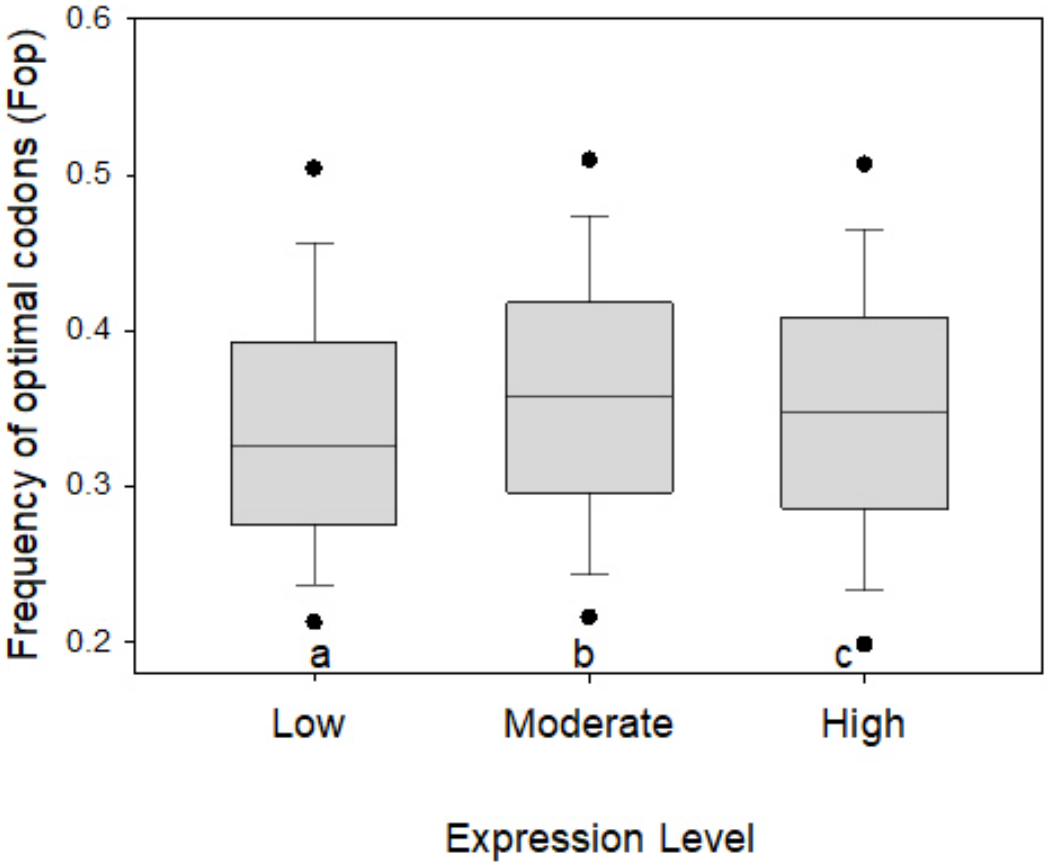


Figure S2. The frequency of optimal codons (Fop) across all genes studied in *T. castaneum* when using the primary optimal codons identified in Williford and Demuth.[2] Genes are categorized into low (lowest 5%, FPKM<0.013), moderate (5 to 95%) and high (top 5%) transcription groups (FPKM>103) based on average expression across all four tissue types (testes, ovaries, GT-males, GT-females). Different letters below bars indicate a statistically significant difference using Ranked ANOVA and Dunn's paired contrasts (<0.05).

Supplementary Text File S1: Protein length and $Opt_{\uparrow tRNA}$ status

For the beetles studied herein, we found that the use of Opt-$codon_{wobble}$ codons was connected to protein length. Specifically, for the 822 highly transcribed genes in this organism (top 5%), we ranked the RSCU for each of the five codons with Opt-$codon_{wobble}$ status, and genes were then binned into three equal sized categories (N=274 genes each) based on the relative magnitude of RSCU (low, moderate, and high). By definition as an optimal codon, each of these five codons had elevated RSCU in the highly expressed genes (as compared to low expressed genes, Table 1). However, within the highly transcribed gene set, we found that the bin containing moderate RSCU values were consistent linked to longer CDS than those with the lowest or highest RSCU values for each of the five Opt-$codon_{wobble}$ codons, namely GAT, TGT, GGT, CCC and AGT (Ranked ANOVA and Dunn's paired contrast $P<0.05$, Fig. S1). Thus, the highly transcribed CDS encoding long proteins, appear to be connected to a specific frequency of Opt-$codon_{wobble}$ codons, which may play a role in their translation. This may possibly comprise a mechanism to ensure a balance between high translation rates (ensured by moderate rather than highest usage of Opt-$codon_{wobble}$ codons) and allowing intermittent pausing during translation for accurate protein synthesis and/or protein folding (ensured by their moderate, rather than low, usage) of CDS encoding long proteins.

Supplementary References

1. Whittle, C. A., Kulkarni, A. and Extavour, C. G. 2019, Absence of a faster-X effect in beetles (Tribolium, Coleoptera). *BioRxiv; https://doi.org/10.1101/754903*

2. Williford, A. and Demuth, J. P. 2012, Gene expression levels are correlated with synonymous codon usage, amino acid composition, and gene architecture in the red flour beetle, Tribolium castaneum. *Mol Biol Evol*, **29**, 3755-3766.