# Evidence for non-optimal codon choice in highly transcribed sex-biased genes of *Drosophila melanogaster*

**Authors:** Carrie A. Whittle[1], Cassandra G. Extavour[1,2]

[1]Department of Organismic and Evolutionary Biology, Harvard University, 16 Divinity Avenue, Cambridge MA 02138, USA

[2]Department of Molecular and Cellular Biology, Harvard University, 16 Divinity Avenue, Cambridge MA 02138, USA

Corresponding author: Cassandra G. Extavour

Email: extavour@oeb.harvard.edu

Phone: (617)-496-1935

Fax: (617)-496-9507

**ORCIDs**

C.A. Whittle 0000-0002-9331-0520

C.G. Extavour 0000-0003-2922-5855

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## Abstract

Biases in synonymous codon use occur across multicellular organisms. Optimal codons, defined as those most commonly used in highly transcribed genes, are thought to arise from selection for cost-efficient translation, which would favor codons with abundant matching tRNAs. Such presumed selection is described as optimal codon choice. Non-optimal codons, defined as those least commonly used in highly transcribed genes, may in principle also play important roles, including regulating protein folding or mRNA translation. However, the dynamics of non-optimal codon use remain understudied. Here we rigorously examine non-optimal codon use, using sex-biased genes expressed in the gonads of *Drosophila melanogaster* as a case study. Using this model, we show that as genes evolve sex-biased expression, they tend to evolve a preference for non-optimal codon use, and that this preference is stronger for testis-biased genes than for ovary-biased genes. Further, from thorough analyses of each of 18 degenerate amino acids, we found evidence that the use of non-optimal codons is not random. Instead, specific non-optimal codons are favored over their synonymous non-optimal codons, an effect again especially pronounced in testis-biased genes. Moreover, we found that non-optimal codon use is positively linked to elevated disorder of the encoded proteins, a pattern most consequential in testis-biased genes. Remarkably, all 18 degenerate amino acids were associated with higher disorder when they were encoded by the identified primary non-optimal codon, than when encoded by its sister optimal codon. We hypothesize that selection may have acted to promote non-optimal codon choice to regulate translation. We discuss the putative roles of tRNA gene copy numbers, pleiotropy, and sex-biased expression in the evolution of this level of gene regulation.

52 ## Introduction

53 The genetic code contains 18 amino acids encoded by synonymous codons (1-5). Optimal codons
54 are defined as those codons most frequently used for amino acids in abundant transcripts, and are thought
55 to promote cost efficient or more accurate translation, a phenomenon often described as optimal codon
56 choice (1, 6). To date, optimal codons have been identified in unicellular (1, 7, 8) and some multicellular
57 organisms, including insects such as flies, beetles, crickets and mosquitoes (3, 7, 9-16). The optimization
58 of codons in highly expressed genes is thought to have resulted from adaptation of the cDNA to favor use
59 of the most common tRNAs in the cells, often measured as the number of matching tRNA genes in the
60 genome, which gives rise to a high ratio of tRNA supply to codon demand (8, 17-22). However, a number
61 of studies have found that some optimal codons have no tRNAs (wobble codons) in certain taxa, a scenario
62 that may slow translation (11, 12, 23). While studies in multicellular taxa have largely focused on optimal
63 codons (1, 6, 16, 19, 24-29), non-optimal codons, defined as those least commonly used in highly
64 expressed genes, may also have translational roles (4, 11, 12, 30-33). When non-optimal codons have few
65 or no matching tRNA genes, some data suggest that their roles could include targeted slowing of
66 translation for co-translational protein folding or to prevent ribosome jamming (4, 30, 33-37). In addition,
67 under a somewhat paradoxical scenario, some non-optimal codons have been found to have abundant
68 tRNAs, and yet are not used as optimal codons. In these cases, this high tRNA abundance to codon use
69 ratio could lead to rapid and preferential translation of specific mRNAs, namely, those mRNAs with
70 unusually high use of the non-optimal codons (11, 12, 38, 39). At present however, studies on non-optimal
71 codon use and its potential molecular consequences remain sparse. Key outstanding questions include
72 whether some non-optimal codons may be favored over others, that is, whether non-optimal codons may
73 exhibit non-random use, and if so, whether favored non-optimal codons may be linked to specific
74 properties of the proteins whose transcripts deploy them.

75 An attractive model system to study codon use, particularly non-optimal codon use, is sex-biased
76 genes expressed in the gonads of multicellular organisms. Because codon use in such genes is especially
77 apt to affect reproductive success and fitness, highly expressed gonad-biased genes are expected to be
78 subject to heritable translation-related selection on codon use. Indeed, pioneering studies in the insect *D.*
79 *melanogaster* and in certain plants have shown that optimal codon use differs between sexes, and is lower
80 in male- than in female-biased reproductive genes (13, 27, 40). Thus, gonadal genes exhibit sufficient
81 selection differences in codon use to be detectable on a gene-wide level (e.g., Fop, or frequency of optimal
82 codons (41) (13, 27, 40)), and thus provide an informative system to fill gaps in our knowledge on the

83 dynamics of non-optimal codon use. *D. melanogaster* as a study system is especially valuable given its
84 vast genomic and gene expression resources available for study (42-44).

85 Some previous observations suggest that the dynamics of non-optimal codon use in genes may be
86 dependent on protein-folding properties (4, 34, 45). For example, studies of the circadian clock proteins
87 FQR (in *Neurospor*a) and PER in *D. melanogaster* have found that experimentally changing non-optimal
88 codons to optimal codons in disordered regions impaired protein form and function, a pattern not found
89 upon optimization of codons in structurally ordered regions (α-helixes) (30, 31, 34). Thus, non-optional
90 codons in those two genes may contribute to proper protein structure, particularly for disordered regions
91 (30, 34). Further, changes in a single synonymous codon, including non-optimal codons, have been linked
92 to altered protein folding and disease phenotypes (31, 36, 46-48). Thus, the extent and accuracy of protein
93 folding may be a factor linked to a gene's overall non-optimal codon use (30), or potentially to the use of
94 specific non-optimal codons. One approach to quantify the contribution to protein folding of individual
95 amino acid sites (and thus, on a codon-by-codon level) and also on a gene-wide basis, is to assess the
96 relative solvent accessibility (RSA). The RSA is an index that quantifies the exposed surface area of an
97 amino acid within the protein structure (that reflects secondary and tertiary structures), relative to its
98 maximum possible surface area in Angstroms (49, 50). Low RSA values typically indicate deeply buried
99 hydrophobic amino acids or protein regions, while exposed residues at the protein surface are typically
100 hydrophilic with relatively high RSA values. RSA analyses have proven effective in revealing links
101 between protein folding and the rate of protein sequence evolution, including slow protein evolution in
102 highly ordered (low RSA) regions (51-56). In this regard, it is reasonable to infer that RSA may also
103 provide an effective means to study protein folding properties with respect to optimal and non-optimal
104 codon use.

105 Here, from the study of highly expressed sex-biased genes in *D. melanogaster*, we provide
106 evidence of non-random non-optimal codon use, that is, selection for the use of non-optimal codons.
107 Specifically, we report a stepwise increase in non-optimal codon use from highly transcribed sexually
108 unbiased, to ovary-biased, to testis-biased genes. We show that certain non-optimal codons are markedly
109 preferred over other sister non-optimal codons, especially in the testis-biased genes. In addition, genes
110 with the highest frequency of non-optimal codon use (>60% of amino acids) show significantly elevated
111 gene wide RSA, and this pattern is most prevalent in testis-biased genes. We further reveal that for a single
112 amino acid, the most strongly favored non-optimal codon per amino acid is consistently linked to elevated
113 RSA as compared to its sister optimal codon, a pattern observed for 18 of 18 degenerate amino acids in

the genetic code. The pronounced accumulation of non-optimal codons in testis-biased genes includes both codons with few and with plentiful matching tRNA gene copy numbers. This suggests that selection for non-optimal codons in testis-biased genes could enhance at least two potential functions, namely slowed translation and targeted up-translation (4, 11, 35)) We hypothesize that the accumulation of favored non-optimal codons in sex-biased genes, and especially in male-biased genes, may be mediated by very low cross-tissue gene pleiotropy (57-59), and by weak or absent potential for sexual conflict (60, 61).

## Results and Discussion

### *A Priori* Classifications of Organismal Optimal and Non-optimal codons in *D. melanogaster*

Prior to analyses of sex-biased gene sets and codon use, we first aimed to define the organism-wide optimal and non-optimal codon status for *D. melanogaster* for each of the 59 codons encoding the 18 degenerate amino acids. For this, we compared the relative synonymous codon usage (RSCU) (62) of a set of highly expressed (ribosomal protein genes) versus all genes in the genome, and defined this value as the $\Delta RSCU_{Ribosome\text{-}All}$ (see Materials and Methods (63)). All codon classifications obtained with this method are provided in Table S1. We identified optimal codons as universally G3 and C3 codons, as previously suggested for *D. melanogaster* (3, 13, 28, 64). This pattern of codon use is not explainable by mutational biases, but rather likely arose from selection pressures (3, 28, 64). Herein, for our study, for each of the 18 degenerate amino acids we explicitly defined the (primary) optimal codon (11, 12) as that with the largest positive $\Delta RSCU_{Ribosome\text{-}All}$ value compared with its sister codons (Table S1). All other codons for a given amino acid (a total of 41) were classed as non-optimal codons. These typically had a negative or near-zero ΔRSCU value (and an RSCU value 1.5-fold to six-fold smaller than that of the optimal codon). We then focused on further analysis of these non-optimal codons, which have been minimally considered or excluded in codon use studies to date (3, 13, 28, 64)(see Supplementary Text File S1 for details).

We classified all optimal and non-optimal codons based on the number of exact matching tRNA genes in the *D. melanogaster* genome (Table S1, GtRNA database (65)), because tRNA abundances, often well reflected by gene copy numbers, underlie purported translational functions (8, 11, 17-20, 23, 35)). 12 of the 18 optimal codons had the most abundant perfectly matching tRNA genes among its sister codons (e.g., GCC for Gly with 14 tRNAs; denoted as $Opt_{high\text{-}tRNAs}$ status; high/low tRNAs were demarcated by

145 the median of 5 tRNA genes). The other six optimal codons had no matching tRNAs, and were classed as
146 wobble codons ($Opt_{wobble}$; e.g. Pro-CCC). In turn, 26 of the 41 non-optimal codons had low tRNA numbers
147 (Non-$opt_{low\text{-}tRNAs}$, status; e.g., ATA for Ile) and the remaining 15 had abundant tRNA genes (Non-$opt_{high\text{-}}$
148 $_{tRNAs}$ status; e.g., ACT for Thr) (Table S1). Hereafter, we focus on the study of sex-biased genes and their
149 patterns of optimal and non-optimal and optimal codon use (Table S1). When pertinent, we also consider
150 statuses of their tRNA gene abundances.
151
152 **Identification and Characterization of the Sex-Biased Gene Sets**
153 We identified highly expressed testis-biased (N=916), ovary-biased (N=258) and unbiased
154 (N=605) genes in *D. melanogaster* (Fig. S1A) using strict criteria of at least five-fold-bias and ≥100
155 RPKM in at one sex versus the other (testis versus ovaries (mated)) based on transcriptome data from
156 modEncode (Table S2, (42, 44) see Materials and Methods). For stringency, we also compared gene
157 expression in the male accessory glands and virgin ovaries (Table S2). We found that 86.9% of the
158 identified testis-biased genes were also male-biased in accessory glands (using a cutoff of ≥ 5 fold sex-
159 bias), and that 77.1% of the identified ovary-biased genes were also female-biased in virgin ovaries,
160 indicating that strong male-female transcriptional differences were also present in those alternate
161 reproductive tissue types. The predicted functions of these genes based on GO analysis showed testis-
162 biased genes were strongly linked to testicular roles such as spermatogenesis, and ovary-biased genes
163 were associated with female reproductive process roles such as the chorion and oogenesis. In contrast,
164 unbiased genes were preferentially involved in the ribosome and other housekeeping functions (Table S3;
165 chromosomal locations of genes are discussed in Supplementary Text File S1). In terms of characteristics
166 of these genes with sex-biased expression, the CDS lengths were shorter for testis-biased (median=273
167 codons) and unbiased genes (median=269 codons) than for the ovary biased genes (median=498 codons;
168 MWU tests $P<0.05$) (Fig. S1B). We then determined the *tau* value, an index of gene expression specificity
169 with values from 0 to 1 (59), across 59 tissue stages in *D. melanogaster* (Table S2). *tau* exhibited a
170 stepwise decline from testis-biased genes (median=0.95) to ovary-biased genes (median=0.83) to unbiased
171 genes (median=0.74) (ranked ANOVA and Dunn's $P<0.05$ for each paired contrast; median genome-wide
172 *tau*=0.85, Fig. S1C). Thus, the testis-biased genes exhibited higher specificity, and lower cross-tissue
173 pleiotropy (58, 66, 67), than ovary-biased and unbiased genes.
174 We assessed the relationships between tRNA gene numbers, amino acid use, and amino acid size
175 complexity (S/C) scores, which are thought to reflect biosynthetic costs of amino acids (provided in Table

176 S4 (68). The tRNA gene copy number per amino acid (pooled across all codons) was strongly positively
177 correlated to the amino acid use (percentage of each amino acid relative to all concatenated amino acids
178 per gene set) for all genes (testis-biased genes: Spearman's R=0.91, $P<10^{-7}$; ovary-biased genes: R=0.85,
179 $P<10^{-7}$; unbiased genes: R=0.83, $P<10^{-7}$; Fig. S2A-C). Moreover, the S/C score per amino acid (68) was
180 inversely correlated to the organismal tRNA gene numbers (R=-0.59,$P<10^{-7}$, Fig. S2D). In sum, the
181 patterns suggest that all three gene sets have a history of selection on codon use (8, 11, 18), further
182 affirming their utility as a model to study dynamics of codon use in this taxon.

183 By examining the codon adaptation index (CAI), an index that reflects the gene-wide codon
184 frequencies relative to a highly expressed reference gene set (here, ribosomal genes) (62, 63), we found a
185 stepwise decrease in CAI from unbiased, to ovary-biased, to testis-biased genes (ranked ANOVA and
186 Dunn's P<0.05, Fig. S3A; note that CAI was highly correlated to Fop across the genome, Spearman's
187 R=0.967, $P<10^{-7}$). In other words, as genes evolved increased gonad-biased expression, they also evolved
188 increases non-optimal codon use, with the highest non-optimal codon use in testis-biased genes (see
189 Supplementary Text File S1). Further, we found that the expression level (two classes, ≥200 RPKM, and
190 ≥100-200 RPKM) and CDS length (short and long CDS lengths, with long defined as ≥272 codons; cutoff
191 at the 33$^{rd}$ percentile of all gene lengths) were linked to CAI within each sex-biased gene set (Fig. S3B).
192 The differences in CAI between gene sets (Fig. S3A) were robust to both transcription level and length
193 cutoffs (Fig. S3A,B; see also Supplementary Text File S1). The high non-optimal codon use in testis-
194 biased genes is not due to enhanced genetic interference, as those processes would be expected to
195 ameliorate non-optimal codon use in longer rather than shorter genes, a pattern that was absent from our
196 data (Fig. S3) (13, 69-71). The testis-biased genes herein were short (Fig. S1B), and even within the testis-
197 biased gene set (Fig. S3B), shorter genes (<272 codons) had greater non-optimal codon use (lower CAI)
198 than longer genes for both expression classes ((MWU-tests P<0.05, Fig. S3B; see Supplementary Text
199 File S1). Thus, other factors must explain the stepwise increase in non-optimal codon use from unbiased,
200 to ovary-biased to testis-biased genes.

201

202 **Non-optimal Codon Use is Non-random in *D. melanogaster* Sex-biased Genes**

203 To further characterize codon use in unbiased and sex-biased genes, we assessed codon use on a
204 codon-by-codon basis for each gene set described above. We determined the ΔRSCU between testis-
205 biased genes versus ovary-biased genes ($\Delta RSCU_{Testis\text{-}Ovary}$), and between testis-biased genes versus
206 unbiased genes ($\Delta RSCU_{Testis\text{-}Unbiased}$), for each of the 59 codons with synonymous codons (*cf.* (27)). In

these contrasts, a positive ΔRSCU value indicates higher use of a codon in the testis-biased genes, and a negative value denotes a reduction in its use within testis-biased genes (and thus elevated use in the respective ovary or unbiased genes, see Materials and Methods). The $\Delta RSCU_{Testis\text{-}Ovary}$ and $\Delta RSCU_{Testis\text{-}Unbiased}$ values per codon are shown in Table S5. Remarkably, we found that testis-biased genes reduced their use of optimal codons, and this pattern was universal to all of the degenerate amino acids. Specifically, 16 of the 18 optimal codons in *D. melanogaster* had a statistically significant negative $\Delta RSCU_{Testis\text{-}Ovary}$ ($P<0.05$, only exceptions were for Ser and Thr $P>0.05$), and 18 of 18 optimal codons had a negative value for $\Delta RSCU_{Testis\text{-}Unbiased}$. The magnitude of negative ΔRSCU values was consistently larger for the $\Delta RSCU_{Testis\text{-}Unbiased}$ contrast than for the $\Delta RSCU_{Testis\text{-}Ovary}$ contrast, consistent with a stepwise decline in the use of optimal codons from unbiased genes, to ovary-biased genes to testis-biased genes (Table S5, Fig. S3). This suggests the possibility that there are weaker translational selection pressures for optimal codons in both types of sex-biased genes, and especially in the testis-biased genes. However, another significant hypothesis worthy of consideration here is that the patterns may reflect a non-random displacement of optimal codons by the favored use of non-optimal codons in testis-biased genes. In other words, it could be the case that testis-biased genes reduce their use of optimal codons not arbitrarily, but rather in favor of preferential use of non-optimal codons.

To test this hypothesis, we asked whether there was any pattern to the non-optimal codons that were used in testis-biased genes. We found that for the nine amino acids encoded by two synonymous codons (Asn, Asp, Cys, Gln, Glu, His. Lys, Phe, Tyr), the universal reduction in use of the optimal codon in testis-biased genes was compensated by increased use of the sister non-optimal codon (as indicated by positive $\Delta RSCU_{Testis\text{-}Unbiased}$ and $-\Delta RSCU_{Testis\text{-}Ovary}$ values, Table S5), consistent with a hypothesis of preferential use of the non-optimal codons in testis-biased genes. Nonetheless, the two-fold degenerate amino acids cannot be used to fully differentiate whether there may also be codon choice among the non-optimal codons. Thus, we examined the nine amino acids that were encoded by three, four or six synonymous codons (Ala, Arg, Gly, Ile, Leu, Pro, Ser, Thr, Val). If the low use of optimal codons in testis-biased genes were solely due to weaker selection for optimal codons, then one would expect a similar degree of enhanced use of all the synonymous non-optimal codons per amino acid in testis-biased genes (under a neutral model). In contrast, however, we found that the elevated use of non-optimal codons in testis-biased genes was non-arbitrary (Fig. 1A, Table S5). As an example, for the four-fold degenerate amino acid Thr, a neutral model would predict similar and increased use of the three non-optimal codons (ACG, ACA, ACT) under reduced use of the optimal codon (ACC). However, we found that only ACT

238 (Non-opt$_{high\text{-}tRNAs}$) had elevated use in testis-biased genes versus ovary-biased genes ($\Delta RSCU_{Testis\text{-}Ovary}$
239 =+0.166; t-test P<0.05. Fig. 1B), while the other two non-optimal codons showed no elevation in use. This
240 is not consistent with a neutral model of non-optimal codon use, and instead suggests a profound
241 preference for one specific non-optimal codon over the others (Fig. 1B). The same preference for ACT
242 was also observed using the $\Delta RSCU_{Testis\text{-}Unbiased}$ comparison (ΔRSCU=+0.201, Table S5), a codon that
243 was detected even though the ovary-biased and unbiased genes were from fully non-overlapping gene
244 sets, indicating that the preference for this non-optimal codon in the testis-biased genes is robust to the
245 two contrasts. Another example is Ile (three-fold degenerate) where the reduced frequency of the optimal
246 codon ATC in testis-biased versus unbiased genes contrast was accompanied by a strong preferential use
247 of just one of the two non-optimal codons (ATA; $\Delta RSCU_{Testis\text{-}Ovary}$ =+0.114; 9.5 fold higher than that of
248 its synonymous non-optimal codon ATT; Table S5), and the same non-optimal codon preference was also
249 identified using the $\Delta RSCU_{Testis\text{-}Unbiased}$ contrast (value was +0.266. t-test P<0.05, Fig. 1C, Table S5).
250 While the same primary non-optimal codons (largest positive ΔRSCU) tended to occur in both the
251 $\Delta RSCU_{Testis\text{-}Ovary}$ and the $\Delta RSCU_{Testis\text{-}Unbiased}$ contrasts, the latter were more often statistically significant
252 (Fig. 1, Table S5). This likely reflects not only the larger ΔRSCU values in the latter contrasts, but also
253 the larger N values and thus power for the unbiased gene set (N=605 for unbiased, and 258 for ovary-
254 biased). Overall, the pattern of the highly favored use of a specific non-optimal codon over others per
255 synonymous codon family in testis-biased genes was also observed for Gly (GGA, Fig. 1D), Ala (GCA),
256 Leu (TTG), Ser (AGT) and Val (GTA as primary non-optimal codon, GTT as secondary), Pro (CCT,
257 particularly $\Delta RSCU_{Testis\text{-}Ovary}$ contrast) and Arg (AGG as primary, AGA as a close secondary) (Table S5,
258 Fig. 1A). All primary non-optimal codons ended in A3, T3 or G3 (Table S5), thus excluding a role of
259 mutational biases for a particular nucleotide at the 3$^{rd}$ "silent" position, and suggesting instead that
260 selective processes have shaped the use of those codons. Together, these patterns show that testis-biased
261 genes show little use of use optimal codons, but instead preferentially use specific non-optimal codons,
262 rather than randomly choosing among the available non-optimal codons.

263 Notably, the primary non-optimal codon per amino acid (Table S5, Fig. 1A) in testis-biased genes
264 was not predicted by tRNA status. Specifically, for Arg, Leu and Ser, the primary non-optimal codon had
265 low tRNA abundance (Non-opt$_{low\text{-}tRNAs}$ status), while others such as Gly, Pro and Thr favored the use of
266 codons with highly abundant tRNAs (Non-opt$_{high\text{-}tRNAs}$ status (Table S5)). We speculate that this pattern
267 suggests that selection of non-optimal codons with few tRNAs (Arg, Leu and Ser) may typically be
268 involved in slowing translation for protein folding or to limit ribosome jamming, while those with

269 abundant tRNAs (Gly, Pro and Thr) may often be involved in ensuring up-translation of mRNAs carrying
270 those codons. These predictions are based on presumed supply-demand ratios of tRNAs:codons (11, 30,
271 32, 35, 72). Thus, the primary non-optimal codons identified here may regulate gene function via specific
272 translational roles.

273 We determined the percentage of genes in each gene set under study that exhibited extreme use of
274 the primary non-optimal codons (defined as RSCU values ≥1.5). As shown in Fig. S4A, a larger
275 percentage of testis-biased genes exhibited extreme use of the primary non-optimal codons than did the
276 comparable ovary-biased and/or unbiased genes (Chi-square $P<0.05$; shown as examples are Thr-ACT,
277 Gly-GGA, Ile-ATA and Val-GTA; see Supplementary Text File S1 for parallel data for optimal codons,
278 Fig. S4B). It is worth noting that the percent of genes with extreme use of optimal codons with plentiful
279 tRNAs differed from those optimal codons with wobble status (Fig. S4B; in all gene sets, $P<0.05$). This
280 observation is important as it allows us to infer that these two classes of optimal codons may not be
281 equivalent in functions (see Supplementary Text File S1), and thus in future studies should not be treated
282 as equal optimal codons, nor studied without including tRNA gene data.

283

284 **Amino Acids Using Non-optimal Codons Have High Surface Exposure and Disorder**

285 To determine whether these patterns of non-optimal codon use were linked to specific biophysical
286 properties of the encoded proteins, we first assessed the solvent accessibility RSA value for every amino
287 acid (and its associated codon) in the testis-biased genes (N=299,817 amino acids), ovary-biased genes
288 (155,282 amino acids) and unbiased genes (218,580 amino acids). We found that RSA value was strongly
289 correlated to the disorder probability scores (from NetsurfP (73)) in all three datasets (Spearman's $R \geq 0.68$
290 for each of three datasets, $P<10^{-7}$). Thus, higher RSA reflects both surface exposure and intrinsic disorder
291 (74-76). In terms of major secondary structures of proteins (α-helices, β-strands and random coils (73)),
292 a majority of amino acids in the proteins of all three gene sets were located within random coils (60.6%
293 for testis-biased, 57.2% of unbiased, and 70.8% for ovary-biased genes, Chi-square tests $P<0.05$). α-
294 helices were the next most common structures in all three gene sets (31.1, 22.4 and 31.5%, respectively),
295 and β-strands the least common (11.2, 8.3 and 6.8%, Fig. S5A). The lowest RSA values occurred for
296 amino acids located in β-strands, while these values were intermediate for α-helices and highest for
297 random coils for all three gene sets (Fig. S5B, median RSA values across all residues for testis-biased
298 genes were 0.13, 0.34 and 0.53, for ovary-biased genes were 0.11, 0.33 and 0.60, and for unbiased genes
299 were 0.11, 0.30 and 0.57 for β-strands, α-helices and random coils respectively; MWU-tests $P<0.05$ for

300 all contrasts per gene set). These patterns are consistent with disordered regions having the greatest surface
301 exposure.

302 For each gene under study, we then determined the percent of amino acids encoded by non-optimal
303 codons, as a gene-wide direct measure of non-optimal codon use (denoted as Percent-Non-opt). The genes
304 were placed into bins of low (≤50%), moderate (>50 to ≤60%) and high Percent-Non-opt codon use
305 (>60%; note that we excluded Met and Typ from this analysis, discussed in Supplementary Text File S1).
306 We found the average RSA per gene increased from the lowest Percent-Non-opt bin (median RSA=0.40),
307 to the moderate (median=0.43) to high (median=0.47) bins (MWU-tests each $P<0.05$) in testis-biased
308 genes (Fig. 2A). In other words, the elevated non-optimal codon use in these testis-biased genes was linked
309 to greater disorder in their encoded proteins, whereas genes using more optimal codons tended to be more
310 well-folded (Fig. 2A). For ovary-biased genes and unbiased genes, no statistically significant differences
311 were observed in RSA between the highest Percent-Non-opt bin and the moderate and low bins ($P>0.05$,
312 Fig. 2B,C), likely because the highest non-optimal codon class was so uncommon in those gene sets for
313 testing (N≤23 genes in each case). Nonetheless, the moderate Percent-Non-Opt bins had elevated average
314 RSA per gene as compared to the low bins for both the ovary-biased and the unbiased genes (MWU-tests
315 $P<0.05$). Overall, elevated non-optimal codon use is associated with greater protein surface exposure in
316 testis-biased, ovary-biased and unbiased genes, with the most striking stepwise correlation observed for
317 the testis-biased genes (Fig. 2A-C).

318 Importantly, of the 255 genes (out of 1,779 highly expressed genes under study; Fig. S1A) within
319 the highest Percent-Non-opt class (>60% non-optimal codons, Fig. 2D), 83% were testis-biased (N=212),
320 while only 9.0% were ovary-biased (N=23) and 7.8% were unbiased (N=20; Fig. 2D). Thus, the highest
321 degree of non-optimal codon use, and elevated protein RSA and disorder, are core and novel features of a
322 major fraction of the testis-biased genes, but these features are not prevalent among ovary-biased or
323 unbiased genes. Even when taking into account that there were more testis-biased genes than ovary-biased
324 or unbiased genes, we find that 23.1% (212 of 916) of the testis-biased gene set belongs to the highest
325 class bin of non-optimal codon use, versus only 8.9% (23 of 258) and 3.3% (20 of 605) of the ovary-
326 biased and unbiased gene sets respectively. In terms of genes in the moderate Percent-Non-opt bin (Fig.
327 2E), testis-biased genes were most common in absolute numbers (N=325; that 35.4% of all testis-biased
328 genes), with less than a third as many ovary-biased genes (N=99 of 258, 38.3% of the ovary genes), and
329 even fewer unbiased genes (N=78; 12.9% of unbiased genes). In the lowest Percent-Non-opt bin (with the
330 highest optimal codon use), unbiased genes were most strongly represented with 509 genes (84.1% of the

331 unbiased gene set), while 389 were testis-biased (42.5% of testis-biased gene set), and 136 were ovary-
332 biased genes (52.7% of the ovary-biased gene set; Fig. 2F). Overall, it is evident that a major portion of
333 testis-biased genes were linked to the highest non-optimal codon use bin (Percent-Non-opt>60%), a group
334 that also had very high protein-wide RSA (Fig. 2A-C). These RSA patterns add another layer of support
335 (in addition to the results in Fig. 1) to the conclusion that the high non-optimal codon use in *D.*
336 *melanogaster* genes is non-random, given that it is also negatively associated with the degree of protein
337 folding in all gene sets under study (Fig. 2A-C), and that that the highest degree of non-optimal codon use
338 (and thus high RSA) is especially common in testis-biased genes (Fig. 2A,D).

339 In Figs. 2G-I, we provide examples of the predicted structures of a testis-biased gene from the
340 highest Percent-Non-opt bin, an ovary-biased gene from the moderate Percent-Non-opt gene bin, and an
341 unbiased gene from the low Percent-Non-opt bin. The testis-biased sperm-related gene protein Mst57Da
342 (FBgn0259139) encodes 73.0% of its amino acids with non-optimal codons, and is predicted to be highly
343 unstructured (Fig.2G) with an average CDS-wide RSA of 0.72±0.09. The ovary-biased gene protein Jabba
344 (FBgn0259682), that is involved in early embryogenesis (42), has 55.1% of its amino acids encoded by
345 non-optimal codons, and shows more helical structures and a lower RSA value (0.50 ±0.01; Fig. 2H) than
346 the sperm protein (Fig. 2G). The unbiased gene encoding ribosomal protein RpL3 has 73.2% optimal
347 codon use an average RSA of 0.36 ±0.01 (Fig. 2I), and high levels of helical and beta-sheets illustrating
348 its extensive degree of folding (NetsurfP predictions for all three proteins are in Fig. S6). In sum, these
349 patterns demonstrate the marked positive associations in non-optimal codon use and RSA.

350 Functional clustering shows that the 212 testis-biased genes in the highest Percent-Non-opt bin
351 (>60% non-optimal codons, Fig. 2D) are enriched in genes involved in sperm-related functions (Table
352 S6). Examples include genes encoding accessory gland proteins Acp54A1*,* Acp26Aa and Acp36DE*,* the
353 testis-enriched gene *RpL10Aa,* the seminal fluid protein Sfp87B*,* and spermatogenesis genes *soti*, *Mst89B*,
354 M*st57Da*, and other Mst genes (Table S7; gene functions from DAVID and FlyBase (42, 77)). Thus, these
355 testis-biased genes with the highest percentage of non-optimal codons were highly specialized to testicular
356 functions, including spermatogenesis.

357

358 ***tau suggests a mechanism to accumulate non-optimal codons***

359 Analyses of gene expression specificity across 59 tissues/stages in *D. melanogaster* (Table S2),
360 revealed that testis-biased genes had exceptionally elevated *tau* values, with a median near the maximum
361 possible value of 1 for genes in the high, moderate and low Percent-Non-opt bins (median values≥0.96,

362 Fig. 2D-F; see Materials and Methods). Thus, the expression of these genes is highly specific to the testis.
363 In turn, ovary-biased genes, had intermediate *tau* values (median between 0.83 to 0.87) among low,
364 moderate and high Percent-Non-opt bins, while unbiased genes had the lowest *tau* (0.71 to 0.77) (Fig. 2D-
365 F). Assuming expression breadth reflects a gene's cross-tissue pleiotropy (58, 66, 67), then testis-biased
366 genes comprise the least pleiotropic gene set under study. The low pleiotropy of these testis-biased genes
367 may allow them to be under less severe functional constraint than those involved in a wide range of tissues
368 or pathways (57, 66, 78, 79), possibly allowing them the flexibility to evolve the observed high non-
369 optimal codon use (Figs. 1, 2). Moreover, sex-biased gene expression, including sex-specific expression
370 (79.0% of testis-biased genes were sex-specific herein, Fig. S1 legend), may act as a means to limit sexual
371 conflict (60, 61, 80). Thus, we predict that non-optimal codon use in the testis-biased genes may typically
372 be of little or no fitness consequence to the ovaries or other tissues, facilitating accumulation of non-
373 optimal codons in testis genes to regulate gene expression for specialized testicular functions.
374

375 **All 18 Degenerate Amino Acids have Higher RSA Values When Encoded by the Primary Non-**
376 **optimal Codon than by the Optimal Codon**

377 Finally, we assessed the RSA value for each individual amino acid when encoded by the primary
378 non-optimal codon (Table S5) versus by the optimal codon (Tale S1) in the testis-biased genes. Our
379 analyses of all amino acids encoded by testis-biased genes showed that for all 18 degenerate amino acids,
380 the primary non-optimal codon was linked to a higher average RSA than the primary optimal codon (Fig.
381 3; sign test $P<0.001$; averages and standard errors are in Table S8). Thus, the elevated RSA of non-optimal
382 codons included every degenerate amino acid, regardless of its maximum solvent accessibility and its
383 polarity (which vary among amino acids (50)). When encoded by a non-optimal codon, residues were
384 consistently located in less deeply folded protein regions, than when that same amino acid was encoded
385 by its optimal codon. This result includes the primary non-optimal codons Thr-ACT, Ile-ATA, and Gly-
386 GGA (Fig. 2B-D) and spans all two-fold, three-fold, four-fold and six-fold degenerate amino acids (Table
387 S5). Thus, the favored use of the primary non-optimal codons versus the optimal codon in more disordered
388 regions was universal across all 18 degenerate amino acids.

389 In addition, the primary non-optimal codons, which all exhibited elevated RSA compared to
390 optimal codons, included those with few and those with plentiful matching tRNA genes (i.e. with Non-
391 $opt_{low\text{-}tRNAs}$ and Non-$opt_{high\text{-}tRNAs}$ statuses (Fig. 3, Table 1, Table S5; optimal codons included both $Opt_{high\text{-}}$
392 $_{tRNAs}$ and $Opt_{wobble}$ statuses). The rarity of exact matching tRNAs for Non-$opt_{low\text{-}tRNAs}$ codons (Table 1,

393 Table S5, Fig. 3) may make these types of codons poised to slow translation, and allow proper co-
394 translational protein folding (32, 35), including in disordered regions (30). However, the high ratio of
395 tRNA genes to codon use for Non-opt$_{high\text{-}tRNAs}$ codons suggests that this class of codons likely has different
396 functions, possibly in the rapid translation of mRNA segments containing the codons, or the up-translation
397 of any mRNAs with high use of these (otherwise) rare codons within a cellular mRNA pool (11, 12, 39).
398 Thus, despite potentially having distinct functions in slowing translation (Non-opt$_{low\text{-}tRNAs}$) or in targeted
399 enhanced translation of mRNAs (Non-opt$_{high\text{-}tRNAs)}$) (4, 11, 30-32, 35, 72), the two types of non-optimal
400 codons (Table 1), are each consistently favored over optimal codons in less-structured protein segments
401 (Fig. 3). Based on these findings, we speculate that the combined use of primary Non-opt$_{low\text{-}tRNAs}$ and Non-
402 opt$_{high\text{-}tRNAs}$ codons (Fig. 3) may respectively act to slow down and speed up local rates of translation
403 within specific types of mRNAs (for instance, in highly transcribed mRNAs that have ameliorated use
404 these otherwise rare codons, such as testis and sperm genes, Table S5, Fig. 3), particularly within the
405 disordered regions, thereby providing a means to control translational pacing and potentially allowing
406 proper protein conformation and functionality.
407
408 **Conclusions and Future Directions**
409 The collective evidence herein supports a model of selection for non-optimal codon use. Non-
410 optimal codons are non-arbitrarily used in sex-related genes, and certain non-optimal codons are favored
411 over others, a pattern particularly prevalent in testis-biased genes, and with likely functions in regulating
412 translation.
413 Future studies should assess the prevalence and evidence for selection for non-optimal codon use
414 in other multicellular organisms, including a system whereby non-optimal codons are more common in
415 the ovaries (15) or in other specific organ systems. In addition, investigation of the placement of all four
416 categories of codons studied herein, namely Opt$_{high\text{-}tRNAs}$, Opt$_{wobble,}$ Non-opt$_{low\text{-}tRNAs}$ and Non-opt$_{high\text{-}tRNAs}$,
417 with respect to the starting ramp of translation, versus middle and terminal CDS regions, and especially
418 with respect to the RSA in disordered regions, will help reveal whether these codons may cause localized
419 pacing (fast/slow) of translation (35, 81, 82) in *D. melanogaster* and other multicellular models.
420 Experimental studies to quantify translation rates and protein folding of testis genes with extreme use of
421 Non-opt$_{high\text{-}tRNAs}$ codons (Fig. S4, Table S5; cf. (30, 83)) may further reveal their putative roles in the rapid
422 translation of specific mRNAs in a cellular mRNA pool or of mRNA regions (11, 39), while investigations
423 of those genes with extreme use of Non-opt$_{low\text{-}tRNAs}$ codons (Fig. S4) may inform on their function in the

424 slowed translation and precise protein conformation in testis-involved genes, as inferred for circadian
425 genes (4, 30, 31). Additional data on the tRNA populations in the testes and ovaries in this species as the
426 technology improves (38) (discussed in Supplementary Text File S1), will also help determine whether or
427 how there is variation in tRNA populations among the gonads, beyond that predicted by tRNA gene
428 frequency (Fig. 2). Research should also evaluate whether younger testis-biased genes have greater
429 flexibility to evolve a set of preferred non-optimal codons with functional roles (*cf.*(83)). Finally, *in vitro*
430 or *in vivo* studies that replace non-optimal codons with optimal codons (30, 31, 83), and vice-versa, to
431 evaluate their effects on protein structure and functions for testis-biased and ovary-biased genes studied
432 here, may provide further insights into the potential specific protein biophysical roles of the non-optimal
433 codons in this species. As part of such analyses, an evaluation of altered Non-opt$_{low\text{-}tRNAs}$ and Non-opt$_{high\text{-}}$
434 $_{tRNAs}$ statuses within disordered protein regions may reveal their individual roles on the pacing of
435 translation, protein conformation and protein functionality.
436

437 ## Materials and Methods

438

439 **Genomic Data and Expression Analyses**

440 We downloaded the genome of *D. melanogaster* (13,986 protein coding genes) version 6.50 (42).
441 For our analysis, we extracted the longest CDS per gene (11, 12, 84). To identify sex-biased genes, we
442 used the modEncode database that contains expression data (42, 44) for a wide range of *D. melanogaster*
443 tissue types and developmental stages (N=59 tissues/stages studied herein, Table S2 (42-44)), and that we
444 also used to measure *tau* (59) across all tissues. We identified highly expressed sex-biased genes as those
445 with ≥100RPKM and ≥5 fold sex-bias in one sex versus the other in the contrast of 4-day mated testes
446 versus 4-day mated ovaries, and unbiased genes as those with <5 fold bias (and ≥100RPKM in at least
447 one gonad). For additional rigor, we also identified sex-biased genes using male accessory glands versus
448 virgin ovaries (Table S2). Further details on identification of sex-biased genes and calculations of
449 expression specificity using *tau* ((59), described previously in (85) using the data in Table S2, are provided
450 in Supplementary Text File S1.
451

452 **Codon Use and ΔRSCU**

453 The organism-wide RSCU values (Table S1) for *D. melanogaster* were obtained from the Codon
454 Statistics Database (CSD) (63). From these, we calculated the $\Delta RSCU_{Ribosome\text{-}All} = RSCU_{Ribosomal\ genes}$ -

455 $RSCU_{All\ genes}$ (using the concatenated genes per dataset), which is a more conservative version of the
456 approach using the highest and least expressed genes (25-28, 86). We identified the largest positive
457 $\Delta RSCU_{Ribosome\text{-}All}$ values as the optimal codon (and each was also identified as optimal at the CSD, with
458 P-values<0.05). All other codons per amino acid were classed as non-optimal, given their large disparity
459 in use from the optimal codon (see Supplementary Text File S1 section "Genome Datasets and Gonadal
460 Expression Analyses"). The tRNA gene counts in the *D. melanogaster* genome were determined using the
461 GtRNA database ((http://gtrnadb.ucsc.edu) (65) which utilizes tRNAscan-SE to extract tRNAs from the
462 genome (87, 88). CAI values that reflect adaption similarity of codon use to the highly expressed
463 ribosomal gene set, and the Fop values, were obtained from the CSD (63).
464 For the study of differences codon use among sex-biased genes, we determined herein the RSCU
465 values and the codon raw counts per codon for every testis-biased, ovary-biased and unbiased gene using
466 Codon W (89). For each of the studied 59 codons encoding a degenerate amino acid, the RSCU values
467 were used to calculate $\Delta RSCU_{Testis\text{-}Ovary}$=average $RSCU_{Testis\text{-}biased\ genes}$ – average $RSCU_{Ovary\text{-}biased\ genes}$ and
468 the $\Delta RSCU_{Testis\text{-}Unbiased}$=average $RSCU_{Testis\text{-}biased\ genes}$ – average $RSCU_{Unbiased\ genes}$. The RSCU values per
469 codon were compared across all genes per contrast using t-tests to obtain P-values (27). The codon counts
470 per gene under study (for each testis-biased, ovary-biased and unbiased gene) were used to calculate the
471 Percent-Non-opt codon use per gene (relative to all optimal and non-optimal codons per gene), based on
472 the non-optimal and optimal codon identities that were defined in Table S1 (see also Supplementary Text
473 File S1).
474
475 **Protein Folding, Relative Solvent Accessibility and Codon Use in the Sex-biased Genes**
476 To determine the RSA per amino acid we used NetsurfP v3.0 set to default parameters (73). This
477 program is a machine-learning based tool proven effective for determining RSA and other structural data
478 of proteins (73, 90). NetsurfP v3.0 has been widely used to characterize protein folding and structures (73,
479 90), and its RSA calculations have been shown to be equivalent to those predicted by other top predictor
480 tools such as SPOT-1D-Single (73). We also found strong correlations using protein structures from the
481 Protein Data Base and RSA from GETAREA (49) (data not shown). We determined the type of secondary
482 structure that each amino acid was located within, namely a helix, string (β-string/sheet) or a random coil,
483 and the disorder scores per amino acid, using NetsurfP 3.0 (73).
484 Protein folding predictions for the visualization of structures in Fig. 2G-I were determined using
485 Alphafold2 (91), available for the *D. melanogaster* proteins (Uniprot proteome Identity UP000000803,

taxon identity= 7227) from the AlphaFold2 database (https://alphafold.ebi.ac.uk) (92). Visualization was conducted using Geneious Prime (93).

**GO Functions**

Gene ontology and functional analysis was performed using DAVID (77) .

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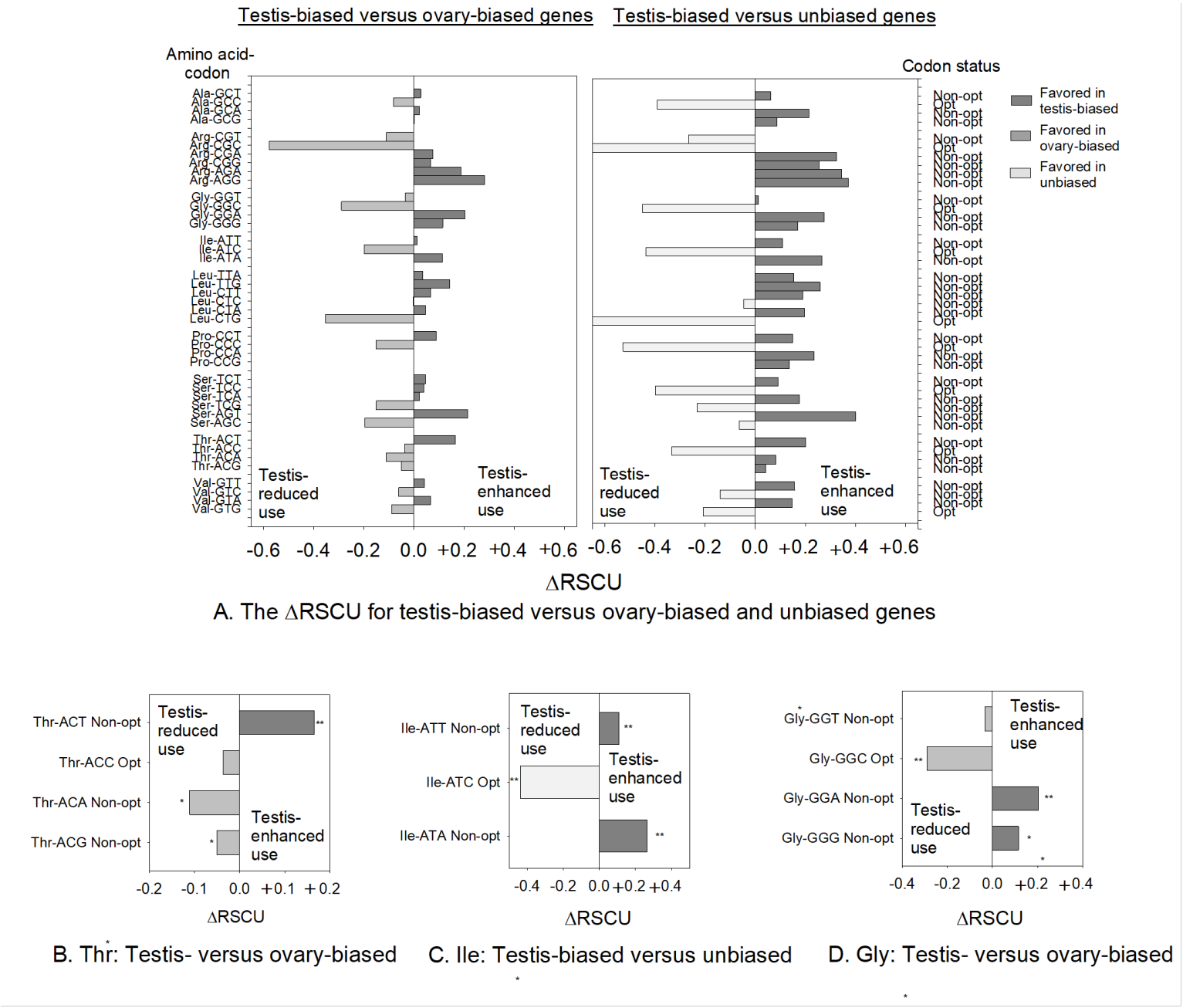


Fig. 1. A) The values for $\Delta RSCU_{Testis\text{-}Ovary}$ (left panel) and $\Delta RSCU_{Testis\text{-}Unbiased}$ (right panel) per codon for amino acids with three or more synonymous codons (P-values in Table S5). The amino acid-codon and the codon status (left) with respect to tRNAs (right) are each indicated. Close-up examples of the ΔRSCU for B) all four codons for Thr; C) all three codons for Ile; and D) all four codons for Gly (* indicates P<0.05 in B-D). In panel A, $\Delta RSCU_{Testis\text{-}Unbiased}$ for Leu-CTG $Opt_{high\text{-}tRNAs}$ =0.818 and Arg-CGC $Opt_{wobble}$=1.11 and were outside the shown limit of X-axis limit.

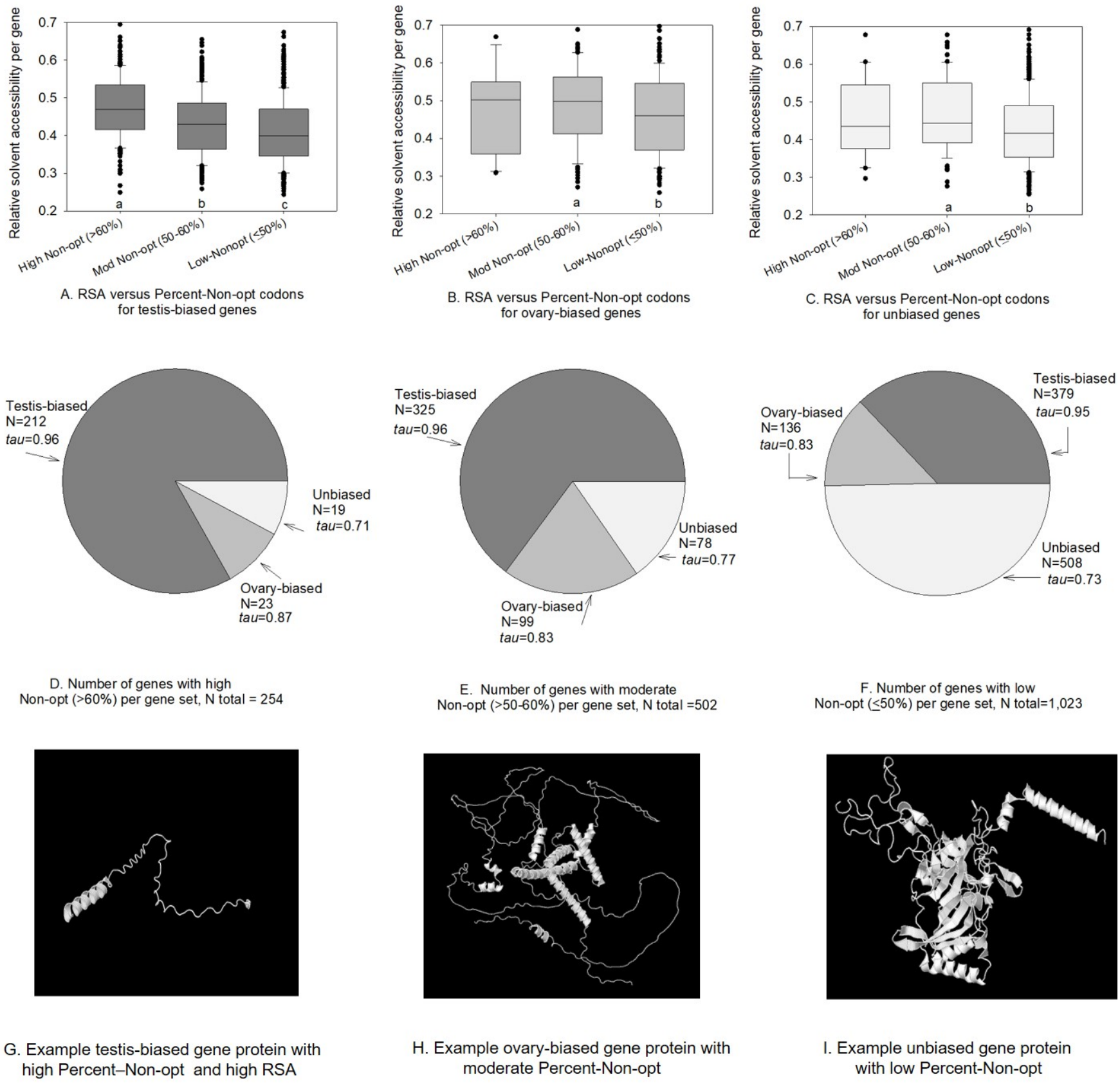


Fig. 2. A-C: The relationships between high non-optimal codon use (>60% of amino acids), moderate non-optimal use (50 to ≤60%) and low optimal codon use (≤50%) and the relative solvent accessibility for: A) testis-biased genes; B) ovary-biased genes; and C) unbiased genes in *D. melanogaster*. Pie charts of the number genes with D) high non-optimal codon use; E) moderate non-optimal; and F) high optimal codon use, also showing the median values of *tau* across genes. Examples of AlphaFold2 predicted protein structures of a typical: G) testis-biased gene with high non-optimal codons and RSA; H) ovary-biased genes wth moderate non-optimal codons and RSA; and I) an unibiased gene with low non-optimal codons and RSA. In A-C, different letters below bars indicate a statistically signficant difference (MWU-test P<0.05)

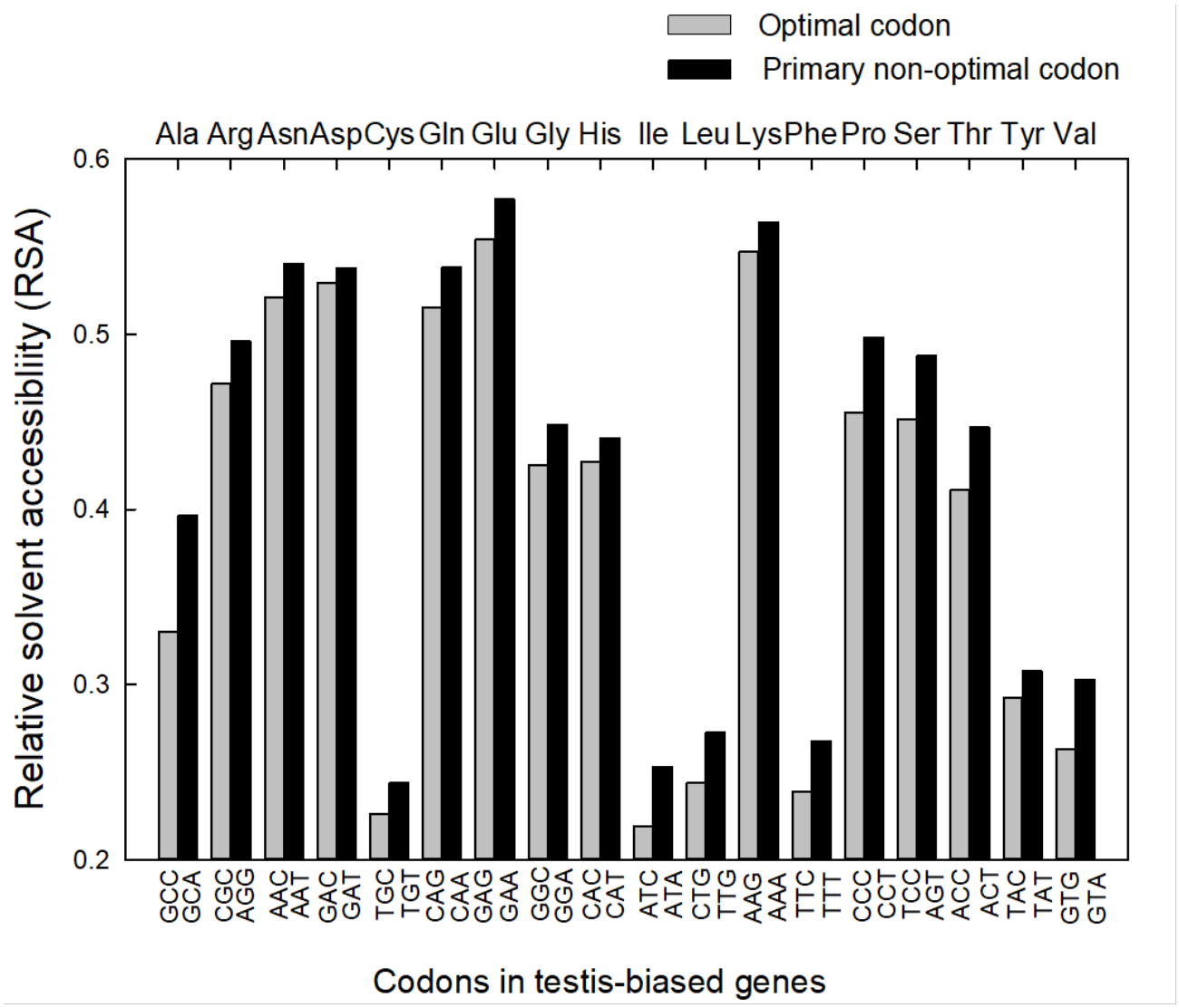


Fig. 3. The average RSA of testis-biased genes when encoded by the primary non-optimal codon (defined in Table S5) versus the optimal codon (Table S1) for all 18 degenerate amino acids. Results are from all amino acids and codons in testis-biased genes (N= N=299,817 amino acids). In total, 18 of 18 degenerate amino acids had higher average RSA when encoded by the non-optimal codon, and sign test across all amino acids had P<0.001. The standard errors were very small (all <0.005) and are provided in Table S8.