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

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Abstract

Biases in synonymous codon use occur in many unicellular and 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, but the dynamics of their use remain understudied. Here we examine non-optimal codon use, using sex-biased genes expressed in the gonads of *Drosophila melanogaster* as a case study. We show that genes with sex-biased expression exhibit a preference for non-optimal codon use, especially testis-biased genes. Further we show that the use of non-optimal codons is not random. Instead, specific non-optimal codons are favored, again especially in testis-biased genes. Non-optimal codon use is positively linked to elevated disorder of the encoded proteins. Remarkably, all 18 degenerate amino acids were associated with higher disorder when encoded by the identified primary non-optimal codon, than when encoded by its sister optimal codon. We hypothesize that selection may have promoted non-optimal codon choice for a subset of favored non-optimal codons 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.

Keywords: Non-optimal codons, sex-biased expression, protein folding, tRNAs, *Drosophila*

Significance Statement

Optimal codons, those codons most commonly used in highly expressed protein-coding genes, are thought to improve translational efficiency in a range of organisms. However, relatively minimal attention has been given to non-optimal codons, those least often used in highly transcribed genes, and their potential roles in translation. Here, using sex-biased gonadal genes of *Drosophila melanogaster* as a model system, we demonstrate that non-optimal codons are preferentially used in sex-biased genes, particularly within highly expressed testis-biased genes, and are associated with disordered proteins. Moreover, the preference for a specific non-optimal codon (per amino acid) is ubiquitous across all degenerate amino acids. We propose that non-optimal codon use is non-random and may have evolved under selection for roles in translational regulation and protein folding in sex-biased genes, and in a manner associated with their tRNA abundances. The findings have significant implications for the understanding of pacing of translation, protein conformation and protein functionality.

Introduction

The genetic code contains 18 amino acids encoded by synonymous codons (Ikemura 1981; Sharp et al. 1986; Shields et al. 1988; Liu et al. 2021; Plotkin & Kudla 2011). Optimal codons are defined as those codons most frequently used for amino acids in abundant transcripts, and are thought to promote cost efficient or more accurate translation, a phenomenon often described as optimal codon choice (Ikemura 1981; Hershberg & Petrov 2009). To date, optimal codons have been identified in unicellular (Ikemura 1985, 1981; Akashi 2003) and some multicellular organisms, including insects such as flies, beetles, crickets and mosquitoes (Galtier et al. 2018; Ikemura 1985; Moriyama & Powell 1997; Whittle et al. 2021, 2019; Hambuch & Parsch 2005; Williford & Demuth 2012; Whittle & Extavour 2017; Shields et al. 1988; Behura & Severson 2011). The optimization of codons in highly expressed genes is thought to have resulted from adaptation of the coding sequence (CDS) to favor use of the most common tRNAs in the cells, often measured as the number of matching tRNA genes in the genome, which gives rise to a high ratio of tRNA supply to codon demand (Gingold et al. 2012; Du et al. 2017; Duret 2000; Akashi 2003; Percudani et al. 1997; Zhou et al. 2013; Reis et al. 2004). However, a number of studies have found that some optimal codons have no tRNAs (wobble codons) in certain taxa, a scenario that may slow translation (Whittle et al. 2021, 2019; Stadler & Fire 2011). While studies in multicellular taxa have largely focused on optimal codons (Akashi 2001; Ikemura 1981; Cutter et al. 2006; Ingvarsson 2008; Whittle et al. 2007; Behura & Severson 2011; Duret 2000; Duret & Mouchiroud 1999; Hershberg & Petrov 2009), non-optimal codons, defined as those least commonly used in highly expressed genes, may also have translational roles (Whittle et al. 2021, 2019; Zhou et al. 2015; Fu et al. 2016; Liu et al. 2021; Brule & Grayhack 2017; Tuller et al. 2010; Wang et al. 2011). When non-optimal codons have few or no matching tRNA genes, some data suggest that their roles could include targeted slowing of translation for co-translational protein folding or to prevent ribosome jamming (Yu et al. 2015; Liu et al. 2021; Tuller et al. 2010; Stein & Frydman 2019; Letzring et al. 2010; Pechmann & Frydman 2013; Zhou et al. 2015). In addition, under a somewhat paradoxical scenario, some non-optimal codons have been found to have abundant tRNAs, and yet are not used as optimal codons. In these cases, this high tRNA abundance to codon use ratio could lead to rapid and preferential translation of specific mRNAs, namely, those mRNAs with unusually high use of the non-optimal codons (Whittle et al. 2021, 2019; Guimaraes et al. 2020; Torrent et al. 2018). At present however, studies on non-optimal codon use and its potential molecular consequences remain sparse. Key outstanding questions include whether some non-optimal codons may be favored over others, that is, whether non-optimal codons may exhibit non-random use, and if so,

92 whether favored non-optimal codons may be linked to specific properties of the proteins whose transcripts
93 deploy them.

94 An attractive model system to study codon use, particularly non-optimal codon use, is sex-biased
95 genes expressed in the gonads of multicellular organisms. Because these genes are thought to play an
96 important role in reproductive success, factors influencing their expression and protein functionality may
97 be especially relevant to fitness. Indeed, pioneering studies in the insect *D. melanogaster* and in certain
98 plants have shown that optimal codon use differs between sexes, and is lower in male- than in female-
99 biased reproductive genes (Hambuch & Parsch 2005; Zhang et al. 2004; Whittle et al. 2007). Thus,
100 gonadal genes exhibit sufficient selection differences in codon use to be detectable on a gene-wide level
101 (e.g., Fop, or frequency of optimal codons (Bahiri-Elitzur & Tuller 2021) (Hambuch & Parsch 2005;
102 Zhang et al. 2004; Whittle et al. 2007)), and thus provide an informative system to fill gaps in our
103 knowledge on the dynamics of non-optimal codon use. *D. melanogaster* as a study system is especially
104 valuable given its vast genomic and gene expression resources available for study (Gramates et al. 2022;
105 Li et al. 2014; Graveley et al. 2011).

106 Some previous observations suggest that the dynamics of non-optimal codon use in genes may be
107 dependent on protein-folding properties (Liu et al. 2021; Zalucki & Jennings 2007; Yu et al. 2015). For
108 example, studies of the circadian clock proteins FQR (in *Neurospor*a) and PER in *D. melanogaster* have
109 found that experimentally changing non-optimal codons to optimal codons in disordered regions impaired
110 protein form and function, a pattern not found upon optimization of codons in structurally ordered regions
111 (α-helixes) (Zhou et al. 2015; Yu et al. 2015; Fu et al. 2016). Thus, non-optional codons in those two
112 genes may contribute to proper protein structure, particularly for disordered regions (Zhou et al. 2015; Yu
113 et al. 2015). Further, changes in a single synonymous codon, including non-optimal codons, have been
114 linked to altered protein folding and disease phenotypes (Fu et al. 2016; Kimchi-Sarfaty et al. 2007;
115 Letzring et al. 2010; Kirchner et al. 2017; Goodarzi et al. 2016). Thus, the extent and accuracy of protein
116 folding may be a factor linked to a gene's overall non-optimal codon use (Zhou et al. 2015), or potentially
117 to the use of specific non-optimal codons. One approach to quantify the contribution to protein folding of
118 individual amino acid sites (and thus, on a codon-by-codon level) and also on a gene-wide basis, is to
119 assess the relative solvent accessibility (RSA). The RSA is an index that quantifies the exposed surface
120 area of an amino acid within the protein structure (that reflects secondary and tertiary structures), relative
121 to its maximum possible surface area in Angstroms (Braun 1998; Tien et al. 2013). Low RSA values
122 typically indicate deeply buried hydrophobic amino acids or protein regions, while exposed residues at

the protein surface are typically hydrophilic with relatively high RSA values. RSA analyses have proven effective in revealing links between protein folding and the rate of protein sequence evolution, including slow protein evolution in highly ordered (low RSA) regions (Scherrer et al. 2012; Conant & Stadler 2009; Franzosa & Xia 2009; Moutinho et al. 2019; Echave et al. 2016; Bricout et al. 2023; Ramsey et al. 2011). In this regard, it is reasonable to infer that RSA may also provide an effective means to study protein folding properties with respect to optimal and non-optimal codon use.

Here, from the study of highly expressed sex-biased genes in *D. melanogaster*, we provide evidence of non-random non-optimal codon use, that is, selection for the use of non-optimal codons. Specifically, we report a stepwise increase in non-optimal codon use from highly transcribed sexually unbiased, to ovary-biased, to testis-biased genes. We show that certain non-optimal codons are markedly preferred over other sister non-optimal codons, especially in the testis-biased genes. In addition, genes with the highest frequency of non-optimal codon use (>60% of amino acids) show significantly elevated gene wide RSA, and this pattern is most prevalent in testis-biased genes. We further reveal that for a single amino acid, the most strongly favored non-optimal codon per amino acid is consistently linked to elevated RSA as compared to its sister optimal codon, a pattern observed for 18 of 18 degenerate amino acids in the genetic code. The favored use of certain 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 specific non-optimal codons in testis-biased genes could enhance at least two potential functions, namely slowed translation and targeted up-translation (Stein & Frydman 2019; Liu et al. 2021; Whittle et al. 2021)). We hypothesize that the use of non-optimal codons in sex-biased genes, and especially in male-biased genes, may be mediated by very low cross-tissue gene pleiotropy (Otto 2004; Mank & Ellegren 2009; Yanai et al. 2005), and by weak or absent potential for sexual conflict (Ellegren & Parsch 2007; Wong & Holman 2023).

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) (Sharp & Li 1987) of a set of highly expressed (ribosomal protein genes) versus all genes in the genome, and

154 defined this value as the $\Delta RSCU_{Ribosome\text{-}All}$ (see Materials and Methods (Subramanian et al. 2022)). This
155 approach allows a means to identify the optimal (most common), and the non-optimal (least common)
156 codons used in highly expressed genes in the genome (as compared to low or moderately expressed genes),
157 which is the foundation for our subsequent analysis of codon use in highly transcribed sex-biased genes
158 (Table S1). All codon classifications obtained with this method, and their tRNA abundance statuses, are
159 provided in Table S1. We identified optimal codons as universally G3 and C3 codons, as previously
160 suggested for *D. melanogaster* (Duret & Mouchiroud 1999; Hambuch & Parsch 2005; Heger & Ponting
161 2007; Shields et al. 1988). This pattern of codon use is not explainable by mutational biases, but rather
162 likely arose from selection pressures (Shields et al. 1988; Duret & Mouchiroud 1999; Heger & Ponting
163 2007). Herein, for our study, for each of the 18 degenerate amino acids we explicitly defined the (primary)
164 optimal codon (Whittle et al. 2019, 2021) as that with the largest positive $\Delta RSCU_{Ribosome\text{-}All}$ value
165 compared with its sister codons (Table S1). All other codons for a given amino acid (a total of 41) were
166 classed as non-optimal codons. These typically had a negative or near-zero ΔRSCU value (and an RSCU
167 value 1.5-fold to six-fold smaller than that of the optimal codon). We then focused on further analysis of
168 these non-optimal codons, which have been minimally considered or excluded in codon use studies to date
169 (Duret & Mouchiroud 1999; Hambuch & Parsch 2005; Shields et al. 1988; Heger & Ponting 2007)(see
170 Supplementary Text File S1 for details).

171 We classified all optimal and non-optimal codons based on the number of exact matching tRNA
172 genes in the *D. melanogaster* genome (Table S1, GtRNA database (Chan & Lowe 2016)), because tRNA
173 abundances, often well reflected by gene copy numbers, underlie purported translational functions
174 (Gingold et al. 2012; Du et al. 2017; Duret 2000; Akashi 2003; Percudani et al. 1997; Stein & Frydman
175 2019; Stadler & Fire 2011; Whittle & Extavour 2019)). 12 of the 18 optimal codons had the most abundant
176 perfectly matching tRNA genes among its sister codons (e.g., GCC for Gly with 14 tRNAs; denoted as
177 $Opt_{high\text{-}tRNAs}$ status; high/low tRNAs were demarcated by the median of 5 tRNA genes). The other six
178 optimal codons had no matching tRNAs, and were classed as wobble codons (Opt_{wobble}; e.g. Pro-CCC). In
179 turn, 26 of the 41 non-optimal codons had low tRNA numbers ($Non\text{-}opt_{low\text{-}tRNAs}$, status; e.g., ATA for Ile)
180 and the remaining 15 had abundant tRNA genes ($Non\text{-}opt_{high\text{-}tRNAs}$ status; e.g., ACT for Thr) (Table S1).
181 Hereafter, we focus on the study of sex-biased genes and their patterns of optimal and non-optimal and
182 optimal codon use (Table S1). When pertinent, we also consider statuses of their tRNA gene abundances.
183

184 **Identification and Characterization of the Sex-Biased Gene Sets**

185 We identified highly expressed testis-biased (N=916), ovary-biased (N=258) and unbiased
186 (N=605) genes in *D. melanogaster* (Fig. S1A) using the strict criteria that a gene have ≥100 RPKM and
187 at least five-fold-bias in one sex versus the other to be classified as sex-biased (testis versus ovaries
188 (mated)), where genes classified as unbiased had <5 fold bias (and ≥100 RPKM in at least one sex), based
189 on transcriptome data from modEncode (Table S2, (Graveley et al. 2011; Gramates et al. 2022) see
190 Materials and Methods). For stringency, we also compared gene expression in the male accessory glands
191 and virgin ovaries (Table S2). We found that 86.9% of the identified testis-biased genes were also male-
192 biased in accessory glands (using a cutoff of ≥ 5-fold sex-bias), and that 77.1% of the identified ovary-
193 biased genes were also female-biased in virgin ovaries, indicating that strong male-female transcriptional
194 differences were also present in those alternate reproductive tissue types. The predicted functions of these
195 genes based on GO analysis showed testis-biased genes were strongly linked to testicular roles such as
196 spermatogenesis, and ovary-biased genes were associated with female reproductive process roles such as
197 the chorion and oogenesis. In contrast, unbiased genes were preferentially involved in the ribosome and
198 other housekeeping functions (Table S3; chromosomal locations of genes are discussed in Supplementary
199 Text File S1). In terms of characteristics of these genes with sex-biased expression, the CDS lengths were
200 shorter for testis-biased (median=273 codons) and unbiased genes (median=269 codons) than for the ovary
201 biased genes (median=498 codons; MWU tests P<0.05) (Fig. S1B). We then determined the *tau* value, an
202 index of gene expression specificity with values from 0 to 1 (Yanai et al. 2005), across 59 tissue stages in
203 *D. melanogaster* (Table S2). *tau* exhibited a stepwise decline from testis-biased genes (median=0.95) to
204 ovary-biased genes (median=0.83) to unbiased genes (median=0.74) (ranked ANOVA and Dunn's P<0.05
205 for each paired contrast; median genome-wide *tau*=0.85, Fig. S1C). Thus, the testis-biased genes
206 exhibited higher specificity, and lower cross-tissue pleiotropy (Meisel 2011; Assis et al. 2012; Mank &
207 Ellegren 2009), than ovary-biased and unbiased genes.
208 We assessed the relationships between tRNA gene numbers, amino acid use, and amino acid size
209 complexity (S/C) scores, which are thought to reflect biosynthetic costs of amino acids (provided in Table
210 S4 (Dufton 1997). The tRNA gene copy number per amino acid (pooled across all codons) was strongly
211 positively correlated to the amino acid use (percentage of each amino acid relative to all concatenated
212 amino acids per gene set) for all genes (testis-biased genes: Spearman's R=0.91, $P<10^{-7}$; ovary-biased
213 genes: R=0.85, $P<10^{-7}$; unbiased genes: R=0.83, $P<10^{-7}$; Fig. S2A-C). Moreover, the S/C score per amino
214 acid (Dufton 1997) was inversely correlated to the organismal tRNA gene numbers (R=-0.59, $P<10^{-7}$, Fig.
215 S2D). In sum, the patterns suggest that all three gene sets have a history of selection on codon use (Du et

al. 2017; Akashi 2003; Whittle et al. 2021), further affirming their utility as a model to study dynamics of codon use in this taxon.

By examining the codon adaptation index (CAI), an index that reflects the gene-wide codon frequencies relative to a highly expressed reference gene set (here, ribosomal genes) (Subramanian et al. 2022; Sharp & Li 1987), we found a stepwise decrease in CAI from unbiased, to ovary-biased, to testis-biased genes (ranked ANOVA and Dunn's $P<0.05$, Fig. S3A; note that CAI was highly correlated to Fop across the genome, Spearman's $R=0.967$, $P<10^{-7}$). In other words, as genes exhibited increased gonad-biased expression, they also presented increased non-optimal codon use, with the highest non-optimal codon use in testis-biased genes (see Supplementary Text File S1). Further, within our highly expressed sex-biased genes sets (the targeted gene sets herein), we subdivided expression into two classes, ≥200 RPKM (extremely high expression), and ≥100-200 RPKM (high expression) and subdivided genes by CDS length (short and long CDS lengths, with long defined as ≥272 codons; cutoff at the 33rd percentile of all gene lengths), which were linked to CAI within each of the sex-biased gene sets (Fig. S3B). The differences in CAI between gene sets (Fig. S3A) were robust to both transcription level and length cutoffs (Fig. S3A, B; see also Supplementary Text File S1). The high non-optimal codon use in testis-biased genes is not due to enhanced genetic interference, as those processes would be expected to ameliorate non-optimal codon use in longer rather than shorter genes, a pattern that was absent from our data (Fig. S3) (Loewe & Charlesworth 2007; Betancourt & Presgraves 2002; Comeron et al. 1999; Hambuch & Parsch 2005). The testis-biased genes herein were short (Fig. S1B), and even within the testis-biased gene set (Fig. S3B), shorter genes (<272 codons) had greater non-optimal codon use (lower CAI) than longer genes for both expression classes ((MWU-tests $P<0.05$, Fig. S3B; see Supplementary Text File S1). Thus, other factors must explain the stepwise increase in non-optimal codon use from unbiased, to ovary-biased to testis-biased genes. Moreover, it is notable that the extremely highly expressed testis-biased genes tended to have an elevated degree of sex-bias relative to the highly expressed testis-biased genes (the median was 149.5 and 210 fold bias for long and short genes in the former, and 71.0 and 69.5 fold bias in the latter, consistent with high extent of specialization in the testis in each group), suggesting the more biased the expression in the testis (among genes with ≥100RPKM), the greater the use of non-optimal codons.

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

To further characterize codon use in unbiased and sex-biased genes, we assessed codon use on a codon-by-codon basis for each gene set described above. We determined the ΔRSCU between testis-

247 biased genes versus ovary-biased genes ($\Delta RSCU_{Testis\text{-}Ovary}$), and between testis-biased genes versus
248 unbiased genes ($\Delta RSCU_{Testis\text{-}Unbiased}$), for each of the 59 codons with synonymous codons (*cf.* (Whittle et
249 al. 2007)). In these contrasts, a positive ΔRSCU value indicates higher use of a codon in the testis-biased
250 genes, and a negative value denotes a reduction in its use within testis-biased genes (and thus elevated use
251 in the respective ovary or unbiased genes, see Materials and Methods). The $\Delta RSCU_{Testis\text{-}Ovary}$ and
252 $\Delta RSCU_{Testis\text{-}Unbiased}$ values per codon are shown in Table S5. Remarkably, we found that testis-biased
253 genes reduced their use of optimal codons, and this pattern was universal to all of the degenerate amino
254 acids. Specifically, 16 of the 18 optimal codons in *D. melanogaster* had a statistically significant negative
255 $\Delta RSCU_{Testis\text{-}Ovary}$ ($P<0.05$, only exceptions were for Ser and Thr $P>0.05$), and 18 of 18 optimal codons
256 had a negative value for $\Delta RSCU_{Testis\text{-}Unbiased}$. The magnitude of negative ΔRSCU values was consistently
257 larger for the $\Delta RSCU_{Testis\text{-}Unbiased}$ contrast than for the $\Delta RSCU_{Testis\text{-}Ovary}$ contrast, consistent with a stepwise
258 decline in the use of optimal codons from unbiased genes to ovary-biased genes to testis-biased genes
259 (Table S5, Fig. S3). This suggests the possibility that there are weaker translational selection pressures for
260 optimal codons in both types of sex-biased genes, and especially in the testis-biased genes. However,
261 another significant hypothesis worthy of consideration here is that the patterns may reflect a non-random
262 displacement of optimal codons by the favored use of non-optimal codons in testis-biased genes. In other
263 words, it could be the case that testis-biased genes reduce their use of optimal codons not arbitrarily, but
264 rather in favor of preferential use of non-optimal codons.

265 To test this hypothesis, we asked whether there was any pattern to the non-optimal codons that
266 were used in testis-biased genes. We found that for the nine amino acids encoded by two synonymous
267 codons (Asn, Asp, Cys, Gln, Glu, His. Lys, Phe, Tyr), the universal reduction in use of the optimal codon
268 in testis-biased genes was compensated by increased use of the sister non-optimal codon (as indicated by
269 positive $\Delta RSCU_{Testis\text{-}Unbiased}$ and $\Delta RSCU_{Testis\text{-}Ovary}$ values, Table S5), consistent with a hypothesis of
270 preferential use of the non-optimal codons in testis-biased genes. Nonetheless, the two-fold degenerate
271 amino acids cannot be used to fully differentiate whether there may also be codon choice among the non-
272 optimal codons. Thus, we examined the nine amino acids that were encoded by three, four or six
273 synonymous codons (Ala, Arg, Gly, Ile, Leu, Pro, Ser, Thr, Val). If the low use of optimal codons in testis-
274 biased genes were solely due to weaker selection for optimal codons, then one would expect a similar
275 degree of enhanced use of all the synonymous non-optimal codons per amino acid in testis-biased genes
276 (under a neutral model). In contrast, however, we found that the elevated use of non-optimal codons in
277 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 (Non-opt$_{high\text{-}tRNAs}$) had elevated use in testis-biased genes versus ovary-biased genes ($\Delta RSCU_{Testis\text{-}Ovary}$ =+0.166; t-test P<0.05. Fig. 1B), while the other two non-optimal codons showed no elevation in use. This is not consistent with a neutral model of non-optimal codon use, and instead suggests a preference for one specific non-optimal codon over the others (Fig. 1B). The same preference for ACT was also observed using the $\Delta RSCU_{Testis\text{-}Unbiased}$ comparison (ΔRSCU=+0.201, Table S5), a codon that was detected even though the ovary-biased and unbiased genes were from fully non-overlapping gene sets, indicating that the preference for this non-optimal codon in the testis-biased genes is robust to the two contrasts. Another example is Ile (three-fold degenerate) where the reduced frequency of the optimal codon ATC in testis-biased versus unbiased genes contrast was accompanied by a strong preferential use of just one of the two non-optimal codons (ATA; $\Delta RSCU_{Testis\text{-}Ovary}$ =+0.114; 9.5 fold higher than that of its synonymous non-optimal codon ATT; Table S5), and the same non-optimal codon preference was also identified using the $\Delta RSCU_{Testis\text{-}Unbiased}$ contrast (value was +0.266. t-test P<0.05, Fig. 1C, Table S5). While the same primary non-optimal codons (largest positive ΔRSCU) tended to occur in both the $\Delta RSCU_{Testis\text{-}Ovary}$ and the $\Delta RSCU_{Testis\text{-}Unbiased}$ contrasts, the latter were more often statistically significant (Fig. 1, Table S5). This likely reflects not only the larger ΔRSCU values in the latter contrasts, but also the larger N values and thus power for the unbiased gene set (N=605 for unbiased, and 258 for ovary-biased). Overall, the pattern of the highly favored use of a specific non-optimal codon over others per synonymous codon family in testis-biased genes was also observed for Gly (GGA, Fig. 1D), Ala (GCA), Leu (TTG), Ser (AGT) and Val (GTA as primary non-optimal codon, GTT as secondary), Pro (CCT, particularly $\Delta RSCU_{Testis\text{-}Ovary}$ contrast) and Arg (AGG as primary, AGA as a close secondary) (Table S5, Fig. 1A). All primary non-optimal codons ended in A3, T3 or G3 (Table S5), thus excluding a role of mutational biases for a particular nucleotide at the 3rd "silent" position, and suggesting instead that selective processes have influenced the use of those codons. Notably, the identified primary non-optimal codon per amino acid always had a positive value for both $\Delta RSCU_{Testis\text{-}Ovary}$ and for $\Delta RSCU_{Testis\text{-}Unbiased}$, (P<0.001 for all latter contrasts) but was typically smaller in value for the former contrast, as expected if non-optimal codons are preferred in sex-biased over unbiased genes (and also more favored in testis-biased than ovary-biased genes). Together, these patterns show that testis-biased genes show lowered use of use optimal codons, and preferentially use specific non-optimal codons, rather than randomly choosing among the available non-optimal codons.

309 Notably, the primary non-optimal codon per amino acid (Table S5, Fig. 1A) in testis-biased genes
310 was not predicted by tRNA status. Specifically, for Arg, Leu and Ser, the primary non-optimal codon had
311 low tRNA abundance (Non-opt$_{low\text{-}tRNAs}$ status), while others such as Gly, Pro and Thr favored the use of
312 codons with highly abundant tRNAs (Non-opt$_{high\text{-}tRNAs}$ status (Table S5)). We speculate that this pattern
313 suggests that selection of non-optimal codons with few tRNAs (Arg, Leu and Ser) may typically be
314 involved in slowing translation for protein folding or to limit ribosome jamming, while those with
315 abundant tRNAs (Gly, Pro and Thr) may often be involved in ensuring up-translation of mRNAs carrying
316 those codons. These predictions are based on presumed supply-demand ratios of tRNAs:codons (Brackley
317 et al. 2011; Zhou et al. 2015; Stein & Frydman 2019; Brule & Grayhack 2017; Whittle et al. 2021). Thus,
318 we speculate that the primary non-optimal codons identified here could regulate gene function by
319 influencing translation. It should be noted that the tRNA populations for *D. melanogaster* closely match
320 those of its sister species *D. simulans, D. sechellia*, *D. yakuba* and *D. erecta* (Spearman's R≥0.998 ($P<10^{-}$
321 ^{7}), and thus these tRNA populations have existed for a substantial period of time (at least 13 Mya since
322 divergence (Tamura et al. 2004) (see Supplementary Text File 1 for details).
323 We determined the percentage of genes in each gene set under study that exhibited extreme use of
324 the primary non-optimal codons (defined as RSCU values ≥1.5). As shown in Fig. S4A, a larger
325 percentage of testis-biased genes exhibited extreme use of the primary non-optimal codons than did the
326 comparable ovary-biased and/or unbiased genes (Chi-square P<0.05; shown as examples are Thr-ACT,
327 Gly-GGA, Ile-ATA and Val-GTA; see Supplementary Text File S1 for parallel data for optimal codons,
328 Fig. S4B). It is worth noting that the percent of genes with extreme use of optimal codons with plentiful
329 tRNAs differed from those optimal codons with wobble status (Fig. S4B; in all gene sets, P<0.05). This
330 observation is important as it allows us to infer that these two classes of optimal codons may not be
331 equivalent in functions (see Supplementary Text File S1), and thus in future studies should not be treated
332 as equal optimal codons, nor studied without including tRNA gene data.
333
334 **Amino Acids Using Non-optimal Codons Have High Surface Exposure and Disorder**
335 To determine whether these patterns of non-optimal codon use were linked to specific biophysical
336 properties of the encoded proteins, we first assessed the solvent accessibility RSA value for every amino
337 acid (and its associated codon) in the testis-biased genes (N=299,817 amino acids), ovary-biased genes
338 (155,282 amino acids) and unbiased genes (218,580 amino acids). We found that RSA value was strongly
339 correlated to the disorder probability scores (from NetsurfP (Hoie et al. 2022)) in all three datasets

340 (Spearman's R ≥0.68 for each of three datasets, $P<10^{-7}$). Thus, higher RSA reflects both surface exposure
341 and intrinsic disorder (Piovesan et al. 2022; Akdel et al. 2022; Ilzhofer et al. 2022). In terms of major
342 secondary structures of proteins (α-helices, β-strands and random coils (Hoie et al. 2022)), a majority of
343 amino acids in the proteins of all three gene sets were located within random coils (60.6% for testis-biased,
344 57.2% of unbiased, and 70.8% for ovary-biased genes, Chi-square tests P<0.05). α-helices were the next
345 most common structures in all three gene sets (31.1, 22.4 and 31.5%, respectively), and β-strands the least
346 common (11.2, 8.3 and 6.8%, Fig. S5A). The lowest RSA values occurred for amino acids located in β-
347 strands, while these values were intermediate for α-helices and highest for random coils for all three gene
348 sets (Fig. S5B, median RSA values across all residues for testis-biased genes were 0.13, 0.34 and 0.53,
349 for ovary-biased genes were 0.11, 0.33 and 0.60, and for unbiased genes were 0.11, 0.30 and 0.57 for β-
350 strands, α-helices and random coils respectively; MWU-tests P<0.05 for all contrasts per gene set). These
351 patterns are consistent with disordered regions having the greatest surface exposure.

352 For each gene under study, we then determined the percent of amino acids encoded by non-optimal
353 codons, as a gene-wide direct measure of non-optimal codon use (denoted as Percent-Non-opt). The genes
354 were placed into bins of low (≤50%), moderate (>50 to ≤60%) and high Percent-Non-opt codon use
355 (>60%; note that we excluded Met and Typ from this analysis, discussed in Supplementary Text File S1).
356 We found the average RSA per gene increased from the lowest Percent-Non-opt bin (median RSA=0.40),
357 to the moderate (median=0.43) to high (median=0.47) bins (MWU-tests each P<0.05) in testis-biased
358 genes (Fig. 2A). In other words, the elevated non-optimal codon use in these testis-biased genes was linked
359 to greater disorder in their encoded proteins, whereas genes using more optimal codons tended to be more
360 well-folded (Fig. 2A). For ovary-biased genes and unbiased genes, no statistically significant differences
361 were observed in RSA between the highest Percent-Non-opt bin and the moderate and low bins (P>0.05,
362 Fig. 2B, C), likely because the highest non-optimal codon class was so uncommon in those gene sets for
363 testing (N≤23 genes in each case). Nonetheless, the moderate Percent-Non-Opt bins had elevated average
364 RSA per gene as compared to the low bins for both the ovary-biased and the unbiased genes (MWU-tests
365 P<0.05). Overall, elevated non-optimal codon use is associated with greater protein surface exposure in
366 testis-biased, ovary-biased and unbiased genes, with the most striking stepwise correlation observed for
367 the testis-biased genes (Fig. 2A-C).

368 Importantly, of the 255 genes (out of 1,779 highly expressed genes under study; Fig. S1A) within
369 the highest Percent-Non-opt class (>60% non-optimal codons, Fig. 2D), 83% were testis-biased (N=212),
370 while only 9.0% were ovary-biased (N=23) and 7.8% were unbiased (N=20; Fig. 2D). Thus, the highest

371 degree of non-optimal codon use is most commonly observed in the testis-biased genes, and is much less
372 common among ovary-biased and unbiased genes. Given that non-optimal codons, when they occur, tend
373 to be found in higher RSA regions (Fig, 2A-C), this implies a concentration of non-optimal codons in the
374 high RSA regions of testis-biased genes (i.e. non-optimal codons less common overall in ovary-biased or
375 unbiased genes). Even when taking into account that there were more testis-biased genes than ovary-
376 biased or unbiased genes, we find that 23.1% (212 of 916) of the testis-biased gene set belongs to the
377 highest class bin of non-optimal codon use, versus only 8.9% (23 of 258) and 3.3% (20 of 605) of the
378 ovary-biased and unbiased gene sets respectively. In terms of genes in the moderate Percent-Non-opt bin
379 (Fig. 2E), testis-biased genes were most common in absolute numbers (N=325; that 35.4% of all testis-
380 biased genes), with less than a third as many ovary-biased genes (N=99 of 258, 38.3% of the ovary genes),
381 and even fewer unbiased genes (N=78; 12.9% of unbiased genes). In the lowest Percent-Non-opt bin (with
382 the highest optimal codon use), unbiased genes were most strongly represented with 509 genes (84.1% of
383 the unbiased gene set), while 389 were testis-biased (42.5% of testis-biased gene set), and 136 were ovary-
384 biased genes (52.7% of the ovary-biased gene set; Fig. 2F). Overall, it is evident that a major portion of
385 testis-biased genes were linked to the highest non-optimal codon use bin (Percent-Non-opt>60%), a group
386 that also had very high protein-wide RSA (Fig. 2A-C). These RSA patterns add another layer of support
387 (in addition to the results in Fig. 1) to the conclusion that the high non-optimal codon use in *D.*
388 *melanogaster* genes is non-random, given that it is also negatively associated with the degree of protein
389 folding in all gene sets under study (Fig. 2A-C), and that that the highest degree of non-optimal codon use
390 (and thus high RSA) is especially common in testis-biased genes (Fig. 2A,D).

391 In Figs. 2G-I, we provide examples of the predicted structures of a testis-biased gene from the
392 highest Percent-Non-opt bin, an ovary-biased gene from the moderate Percent-Non-opt gene bin, and an
393 unbiased gene from the low Percent-Non-opt bin. The testis-biased sperm-related gene protein Mst57Da
394 (FBgn0259139) encodes 73.0% of its amino acids with non-optimal codons, and is predicted to be highly
395 unstructured (Fig.2G) with an average CDS-wide RSA of 0.72±0.09. The ovary-biased gene protein Jabba
396 (FBgn0259682), that is involved in early embryogenesis (Gramates et al. 2022), has 55.1% of its amino
397 acids encoded by non-optimal codons, and shows more helical structures and a lower RSA value (0.50
398 ±0.01; Fig. 2H) than the sperm protein (Fig. 2G). The unbiased gene encoding ribosomal protein RpL3
399 has 73.2% optimal codon use an average RSA of 0.36 ±0.01 (Fig. 2I), and high levels of helical and beta-
400 sheets illustrating its extensive degree of folding (NetsurfP predictions for all three proteins are in Fig.

401 S6). In sum, these patterns demonstrate the marked positive associations in non-optimal codon use and
402 RSA.
403 Functional clustering shows that the 212 testis-biased genes in the highest Percent-Non-opt bin
404 (>60% non-optimal codons, Fig. 2D) are enriched in genes involved in sperm-related functions (Table
405 S6). Examples include genes encoding accessory gland proteins Acp54A1, Acp26Aa and Acp36DE, the
406 testis-enriched gene *RpL10Aa,* the seminal fluid protein Sfp87B, and spermatogenesis genes *soti*, *Mst89B*,
407 M*st57Da*, and other Mst genes (Table S7; gene functions from DAVID and FlyBase (Sherman et al. 2022;
408 Gramates et al. 2022)). Thus, these testis-biased genes with the highest percentage of non-optimal codons
409 were highly specialized to testicular functions, including spermatogenesis.
410
411 ***Tissue specificity suggests a mechanism to accumulate non-optimal codons***
412 Analyses of gene expression specificity across 59 tissues/stages in *D. melanogaster* (Table S2),
413 revealed that testis-biased genes had exceptionally elevated *tau* values, with a median near the maximum
414 possible value of 1 for genes in the high, moderate and low Percent-Non-opt bins (median values≥0.96,
415 Fig. 2D-F; see Materials and Methods). Thus, the expression of these genes is highly specific to the testis.
416 In turn, ovary-biased genes, had intermediate *tau* values (median between 0.83 to 0.87) among low,
417 moderate and high Percent-Non-opt bins, while unbiased genes had the lowest *tau* (0.71 to 0.77) (Fig. 2D-
418 F). Assuming expression breadth reflects a gene's cross-tissue pleiotropy (Meisel 2011; Mank & Ellegren
419 2009; Assis et al. 2012), then testis-biased genes comprise the least pleiotropic gene set under study. The
420 low pleiotropy of these testis-biased genes may allow them to be under less severe functional constraint
421 than those involved in a wide range of tissues or pathways (Otto 2004; Meisel 2011; Dean & Mank 2016;
422 Mank et al. 2008), possibly allowing them the flexibility to evolve the observed high non-optimal codon
423 use (Figs. 1, 2). Moreover, sex-biased gene expression, including sex-specific expression (79.0% of testis-
424 biased genes were sex-specific herein, Fig. S1 legend), may act as a means to limit sexual conflict
425 (Ellegren & Parsch 2007; Wong & Holman 2023; Ingleby et al. 2014). Thus, we predict that non-optimal
426 codon use in the testis-biased genes may typically be of little or no fitness consequence to the ovaries or
427 other tissues, facilitating accumulation of non-optimal codons in testis genes to regulate gene expression
428 for specialized testicular functions.
429
430 **All 18 Degenerate Amino Acids have Higher RSA Values When Encoded by the Primary Non-**
431 **optimal Codon than by the Optimal Codon**

Finally, we assessed the RSA value for each individual type of amino acid when it was encoded by the primary non-optimal codon (Table S5) versus when it was encoded by the optimal codon (Table S1) in the testis-biased genes, Our analyses of all amino acids encoded by testis-biased genes showed that for all 18 degenerate amino acids, the primary non-optimal codon was linked to a higher average RSA than the primary optimal codon (Fig. 3; sign test $P<0.001$; averages and standard errors are in Table S8). Thus, the elevated RSA of non-optimal codons included every degenerate amino acid, regardless of its maximum solvent accessibility and its polarity (which vary among amino acids (Tien et al. 2013)). When encoded by a non-optimal codon, residues were consistently located in less deeply folded protein regions, than when that same amino acid was encoded by its optimal codon. This result includes the primary non-optimal codons Thr-ACT, Ile-ATA, and Gly-GGA (Fig. 2B-D) and spans all two-fold, three-fold, four-fold and six-fold degenerate amino acids (Table S5). Thus, the favored use of the primary non-optimal codons versus the optimal codon in more disordered regions was universal across all 18 degenerate amino acids.

In addition, the primary non-optimal codons, which all exhibited elevated RSA compared to optimal codons, included those with few and those with plentiful matching tRNA genes (i.e. with Non-$opt_{low\text{-}tRNAs}$ and Non-$opt_{high\text{-}tRNAs}$ statuses (Fig. 3, Table S1, Table S5; optimal codons included both $Opt_{high\text{-}tRNAs}$ and Opt_{wobble} statuses). The rarity of exact matching tRNAs for Non-$opt_{low\text{-}tRNAs}$ codons (Table S1, Table S5, Fig. 3) may make these types of codons poised to slow translation, and allow proper co-translational protein folding (Brule & Grayhack 2017; Stein & Frydman 2019), including in disordered regions (Zhou et al. 2015). However, the high ratio of tRNA genes to codon use for Non-$opt_{high\text{-}tRNAs}$ codons suggests that this class of codons likely has different functions, possibly in the rapid translation of mRNA segments containing the codons, or the up-translation of any mRNAs with high use of these (otherwise) rare codons within a cellular mRNA pool (Whittle et al. 2021; Torrent et al. 2018; Whittle et al. 2019). Thus, despite potentially having distinct functions in slowing translation (Non-$opt_{low\text{-}tRNAs}$) or in targeted enhanced translation of mRNAs (Non-$opt_{high\text{-}tRNAs)}$) (Brackley et al. 2011; Zhou et al. 2015; Stein & Frydman 2019; Brule & Grayhack 2017; Whittle et al. 2021; Liu et al. 2021; Fu et al. 2016), the two types of non-optimal codons (Table S1), are each consistently favored over optimal codons in less-structured protein segments (Fig. 3). Based on these findings, we speculate that the combined use of primary Non-$opt_{low\text{-}tRNAs}$ and Non-$opt_{high\text{-}tRNAs}$ codons (Fig. 3) may respectively act to slow down and speed up local rates of translation within specific types of mRNAs (for instance, in highly transcribed mRNAs that have ameliorated use these otherwise rare codons, such as testis and sperm genes (Table S5,

463 Fig. 3, and/or in some regions of ovary-biased and unbiased genes (Fig. 2B, C), thereby providing a means
464 to control translational pacing and potentially allowing proper protein conformation and functionality.
465
466 **The Potential Role of Selection in Shaping Non-optimal Codon Use**
467 Formally, we cannot exclude the possibility that the non-optimal codon use patterns we have
468 described here are the result of relaxation of constraint. However, several lines of evidence herein support
469 the hypothesis that natural selection has contributed to the use of a subset of preferred non-optimal codons.
470 First, we *a priori* identified optimal and non-optimal codons from analysis of the most highly expressed
471 genes in the *D. melanogaster* genome (Table S1), which are most apt to experience translation-related
472 selection pressures (including tRNA abundance pressures), using an approach parallel to that historically
473 used to identify optimal codons (Duret & Mouchiroud 1999; Whittle & Extavour 2016; Whittle et al.
474 2021, 2007; Duret 2000; Akashi 2001; Wang et al. 2011; Whittle et al. 2019). This *a priori* analysis
475 automatically (in Table S1) controls for codon use in lowly and moderately expressed genes, wherein
476 selection is weak or absent and neutral processes tend to be prevalent (Wang et al. 2011; Whittle et al.
477 2019, 2021). Second, a specific subset of non-optimal codons, typically one specific codon per amino
478 acid, exhibited much higher use than the other sister non-optimal codons in highly transcribed testis-biased
479 genes (Fig. 1, Table S5). We posit that this pattern, remarkably observed for 18 of 18 degenerate amino
480 acids (Table S5), is unlikely to be explained by a neutral process, whereby all non-optimal codons would
481 be expected to have similar, arbitrary use patterns. Third, the same primary non-optimal codon was
482 identified when using the ovary-biased genes and when using the unbiased genes as the reference dataset
483 to compare with testis-biased genes (Fig. 1, Table S5). the ovary-biased and unbiased groups are mutually
484 exclusive gene sets, but the same primary non-optimal codon per amino acid was nevertheless identified
485 in in the testis-biased genes. Fourth, the primary (preferred) non-optimal codon for amino acids ended in
486 different nucleotides (A, T, G; Fig. 1, Table S5) and thus a mutation-bias or gene conversion process (e.g.,
487 favoring G) associated with high gene expression in the testis is a poor explanation for this pattern. Fifth,
488 our findings that 18 of 18 degenerate amino acids in *D. melanogaster* showed preferential use of the
489 primary non-optimal codon in disordered regions (higher RSA, Fig. 3), infers a remarkably consistent
490 pattern of non-arbitrary codon use among sister non-optimal codons, across every degenerate amino acid,
491 again unlikely to be the result of a neutral process. Further, we showed this pattern was observed for non-
492 optimal codons that we *a priori* identified that had enhanced use in highly expressed genes (Table S1),
493 which are most apt to experience translation-related selection pressures (as compared to low or moderately

494 expressed genes (similar to optimal codons in highly expressed genes (Duret & Mouchiroud 1999; Whittle
495 & Extavour 2016; Whittle et al. 2021, 2007; Duret 2000; Akashi 2001; Wang et al. 2011; Whittle et al.
496 2019)). While neutral processes are apt also to play a role in shaping the use of the non-primary non-
497 optimal codons per amino acid for highly expressed testis-biased and ovary-biased genes (for those amino
498 acids with three or more codons and thus, more than one non-optimal codon, Fig. 1), we propose the
499 collective evidence best supports a model whereby one non-optimal codon is heavily favored over others,
500 and that use of these codons tends to be preferentially located in genes and gene regions that encode
501 disordered proteins (Fig. 2, Fig. 3).
502

503 **Conclusions and Future Directions**

504 The collective evidence herein supports the hypothesis that selection has contributed to shaping
505 the use of a specific subset of (preferred) non-optimal codons. Non-optimal codons are non-arbitrarily
506 used in sex-related genes in *D. melanogaster*, and certain non-optimal codons are favored over others.
507 This pattern is particularly prevalent in testis-biased genes, and suggests that certain (primary, or
508 preferred) non-optimal codons could have functions in regulating translation.

509 Future studies should assess the prevalence and evidence for selection for non-optimal codon use
510 in other multicellular organisms, including a system whereby non-optimal codons are more common in
511 the ovaries (Whittle & Extavour 2017) or in other specific organ systems. In addition, investigation of the
512 placement of all four categories of codons studied herein, namely $\text{Opt}_{\text{high-tRNAs}}$, $\text{Opt}_{\text{wobble,}}$ $\text{Non-opt}_{\text{low-tRNAs}}$
513 and $\text{Non-opt}_{\text{high-tRNAs}}$, with respect to the starting ramp of translation, versus middle and terminal CDS
514 regions, and especially with respect to the RSA in disordered regions, will help reveal whether these
515 codons may cause localized pacing (fast/slow) of translation (Quax et al. 2015; Stein & Frydman 2019;
516 Miller et al. 2019) in *D. melanogaster* and other multicellular models. Experimental studies to quantify
517 translation rates and protein folding of testis genes with extreme use of $\text{Non-opt}_{\text{high-tRNAs}}$ codons (Fig. S4,
518 Table S5; cf. (Zhou et al. 2015; Allen et al. 2022)) may further reveal their putative roles in the rapid
519 translation of specific mRNAs in a cellular mRNA pool or of mRNA regions (Torrent et al. 2018; Whittle
520 et al. 2021), while investigations of those genes with extreme use of $\text{Non-opt}_{\text{low-tRNAs}}$ codons (Fig. S4)
521 may inform on their function in the slowed translation and precise protein conformation in testis-involved
522 genes, as inferred for circadian genes (Fu et al. 2016; Zhou et al. 2015; Liu et al. 2021). Additional data
523 on the tRNA populations in the testes and ovaries in this species as the technology improves (Guimaraes
524 et al. 2020) (discussed in Supplementary Text File S1), will also help determine whether or how there is

525 variation in tRNA populations among the gonads, beyond that predicted by tRNA gene frequency (Fig.
526 2). Future research should also evaluate whether younger testis-biased genes have greater flexibility to
527 evolve a set of preferred non-optimal codons with functional roles (*cf.*(Allen et al. 2022)), and whether
528 non-optimal codon use may have particular roles in shorter genes (as testis-biased genes tended to be short
529 herein, Fig. S3, Fig. 2). Finally, *in vitro* or *in vivo* studies that replace non-optimal codons with optimal
530 codons (Zhou et al. 2015; Fu et al. 2016; Allen et al. 2022), and vice-versa, to evaluate their effects on
531 protein structure and functions for testis-biased and ovary-biased genes studied here, may provide further
532 insights into the potential specific protein biophysical roles of the non-optimal codons in this species. As
533 part of such analyses, an evaluation of altered Non-opt$_{low\text{-}tRNAs}$ and Non-opt$_{high\text{-}tRNAs}$ statuses within
534 disordered protein regions may reveal their individual roles on the pacing of translation, protein
535 conformation and protein functionality.
536

537 ## Materials and Methods

538

539 **Genomic Data and Expression Analyses**

540 We downloaded the genome of *D. melanogaster* (13,986 protein coding genes) version 6.50
541 (Gramates et al. 2022). For our analysis, we extracted the longest CDS per gene (Whittle et al. 2021, 2019;
542 Sandmann et al. 2011). To identify sex-biased genes, we used the modEncode database that contains
543 expression data (Graveley et al. 2011; Gramates et al. 2022) for a wide range of *D. melanogaster* tissue
544 types and developmental stages (N=59 tissues/stages studied herein, Table S2 (Li et al. 2014; Gramates
545 et al. 2022; Graveley et al. 2011)), and that we also used to measure *tau* (Yanai et al. 2005) across all
546 tissues. We identified highly expressed sex-biased genes as those with ≥100RPKM and ≥5-fold sex-bias
547 in one sex versus the other in the contrast of 4-day mated testes versus 4-day mated ovaries, and unbiased
548 genes as those with <5-fold bias (and ≥100RPKM in at least one gonad). For additional rigor, we also
549 identified sex-biased genes using male accessory glands versus virgin ovaries (Table S2). Further details
550 on identification of sex-biased genes and calculations of expression specificity using *tau* ((Yanai et al.
551 2005)(described previously in (Whittle & Extavour 2023)) using the data in Table S2, are provided in
552 Supplementary Text File S1.
553

554 **Codon Use and ΔRSCU**

555 The organism-wide RSCU values (Table S1) for *D. melanogaster* were obtained from the Codon
556 Statistics Database (CSD) (Subramanian et al. 2022). From these, we calculated the $\Delta RSCU_{Ribosome\text{-}All}$
557 $=RSCU_{Ribosomal\ genes} - RSCU_{All\ genes}$ (using the concatenated genes per dataset), which is a more conservative
558 version of the approach using the highest and least expressed genes (Wang et al. 2011; Whittle et al. 2007;
559 Cutter et al. 2006; Ingvarsson 2008; Duret & Mouchiroud 1999). We identified the largest positive
560 $\Delta RSCU_{Ribosome\text{-}All}$ values as the optimal codon (and each was also identified as optimal at the CSD, with
561 P-values<0.05). All other codons per amino acid were classed as non-optimal, given their large disparity
562 in use from the optimal codon (see Supplementary Text File S1 section "Genome Datasets and Gonadal
563 Expression Analyses"). The tRNA gene counts in the *D. melanogaster* genome were determined using the
564 GtRNA database ((http://gtrnadb.ucsc.edu) (Chan & Lowe 2016) which utilizes tRNAscan-SE to extract
565 tRNAs from the genome (Lowe & Chan 2016; Chan & Lowe 2019). CAI values that reflect similarity of
566 codon use to the highly expressed ribosomal gene set, and the Fop values, were obtained from the CSD
567 (Subramanian et al. 2022).
568 For the study of differences codon use among sex-biased genes, we determined herein the RSCU
569 values and the codon raw counts per codon for every testis-biased, ovary-biased and unbiased gene using
570 Codon W (Peden 1999). For each of the studied 59 codons encoding a degenerate amino acid, the RSCU
571 values were used to calculate $\Delta RSCU_{Testis\text{-}Ovary}$=average $RSCU_{Testis\text{-}biased\ genes}$ – average $RSCU_{Ovary\text{-}biased}$
572 $_{genes}$ and the $\Delta RSCU_{Testis\text{-}Unbiased}$=average $RSCU_{Testis\text{-}biased\ genes}$ – average $RSCU_{Unbiased\ genes}$. The RSCU
573 values per codon were compared across all genes per contrast using t-tests to obtain P-values (Whittle et
574 al. 2007). The codon counts per gene under study (for each testis-biased, ovary-biased and unbiased gene)
575 were used to calculate the Percent-Non-opt codon use per gene (relative to all optimal and non-optimal
576 codons per gene), based on the non-optimal and optimal codon identities that were defined in Table S1
577 (see also Supplementary Text File S1).
578
579 **Protein Folding, Relative Solvent Accessibility and Codon Use in the Sex-biased Genes**
580 To determine the RSA per amino acid we used NetsurfP v3.0 set to default parameters (Hoie et al.
581 2022). This program is a machine-learning based tool proven effective for determining RSA and other
582 structural data of proteins (Hoie et al. 2022; Klausen et al. 2019). NetsurfP v3.0 has been widely used to
583 characterize protein folding and structures (Hoie et al. 2022; Klausen et al. 2019), and its RSA calculations
584 have been shown to be equivalent to those predicted by other top predictor tools such as SPOT-1D-Single
585 (Hoie et al. 2022). We also found strong correlations using protein structures from the Protein Data Base

586 and RSA from GETAREA (Braun 1998) (data not shown). We determined the type of secondary structure
587 that each amino acid was located within, namely a helix, string (β-string/sheet) or a random coil, and the
588 disorder scores per amino acid, using NetsurfP 3.0 (Hoie et al. 2022).

589 Protein folding predictions for the visualization of structures in Fig. 2G-I were determined using
590 Alphafold2 (Juniper et al. 2021), available for the *D. melanogaster* proteins (Uniprot proteome Identity
591 UP000000803, taxon identity= 7227) from the AlphaFold2 database (https://alphafold.ebi.ac.uk) (Varadi
592 et al. 2022). Visualization was conducted using Geneious Prime (Kearse et al. 2012).

593 For Fig. 3, using all concatenated testis-biased genes, the RSA was determined for each type of
594 amino acid (18 degenerate amino acids) when it was encoded by the preferred non-optimal codon and
595 when the same amino acid was encoded by the optimal codon, across all amino acid sites. Then, the
596 average RSA was determined for all cases where the amino acid was encoded by the preferred non-optimal
597 codon and when encoded by the optimal codon (averages and standard errors are provided in Table S8).
598

599 **GO Functions**

600 Gene ontology and functional analysis was performed using DAVID (Sherman et al. 2022) .
601

602 ## Data Availability

603 All data used in this study are publicly available. The locations of the data are provided in the Materials
604 and Methods.
605

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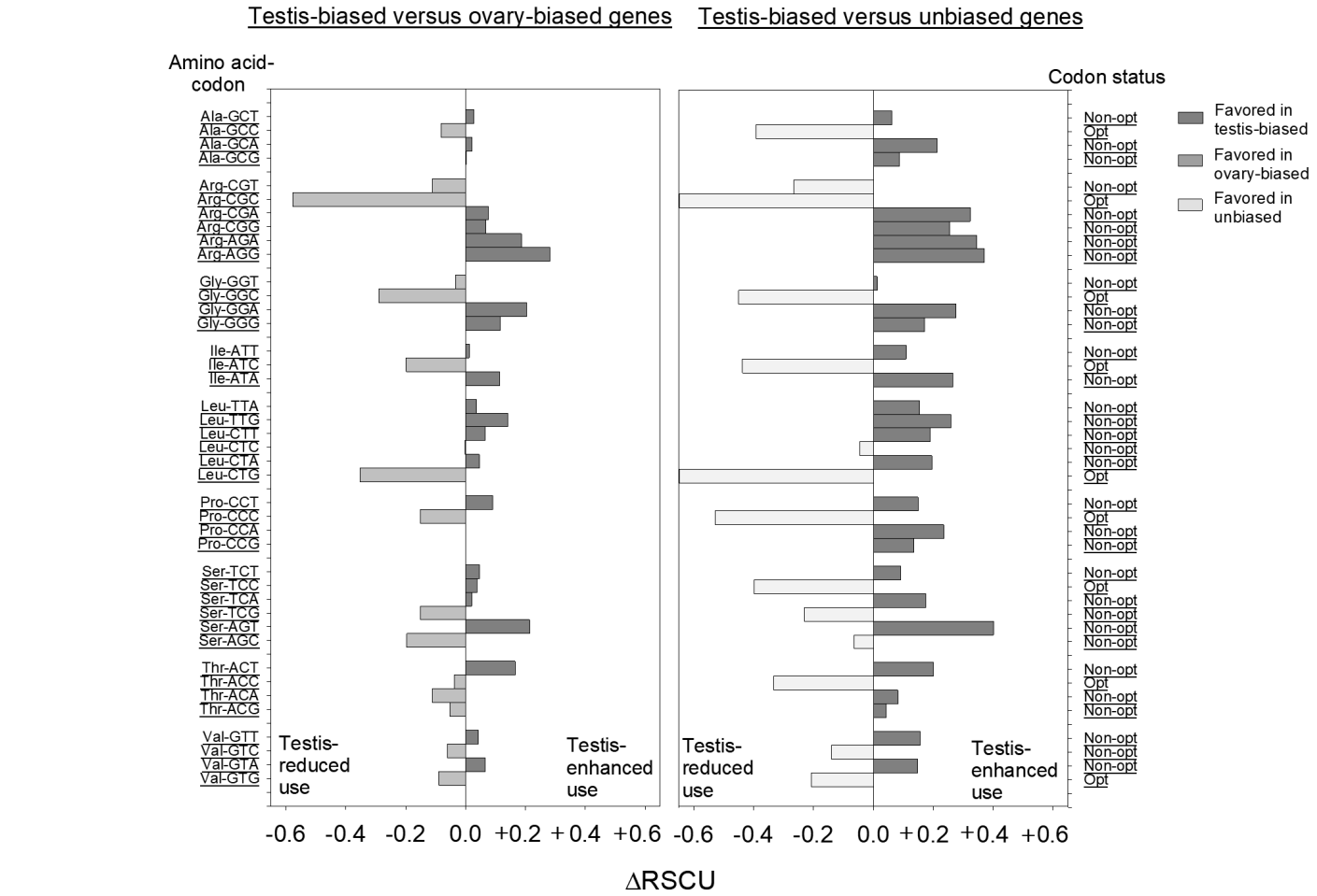

Fig. 1. A) The values for $\Delta RSCU_{Testis\text{-}Ovary}$ (left panel) and $\Delta RSCU_{Testis\text{-}Unbiased}$ (right panel) per codon for amino acids with three or more synonymous codons (P-values in Table S5). The amino acid-codon and the codon status (left) with respect to tRNAs (right) are 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.

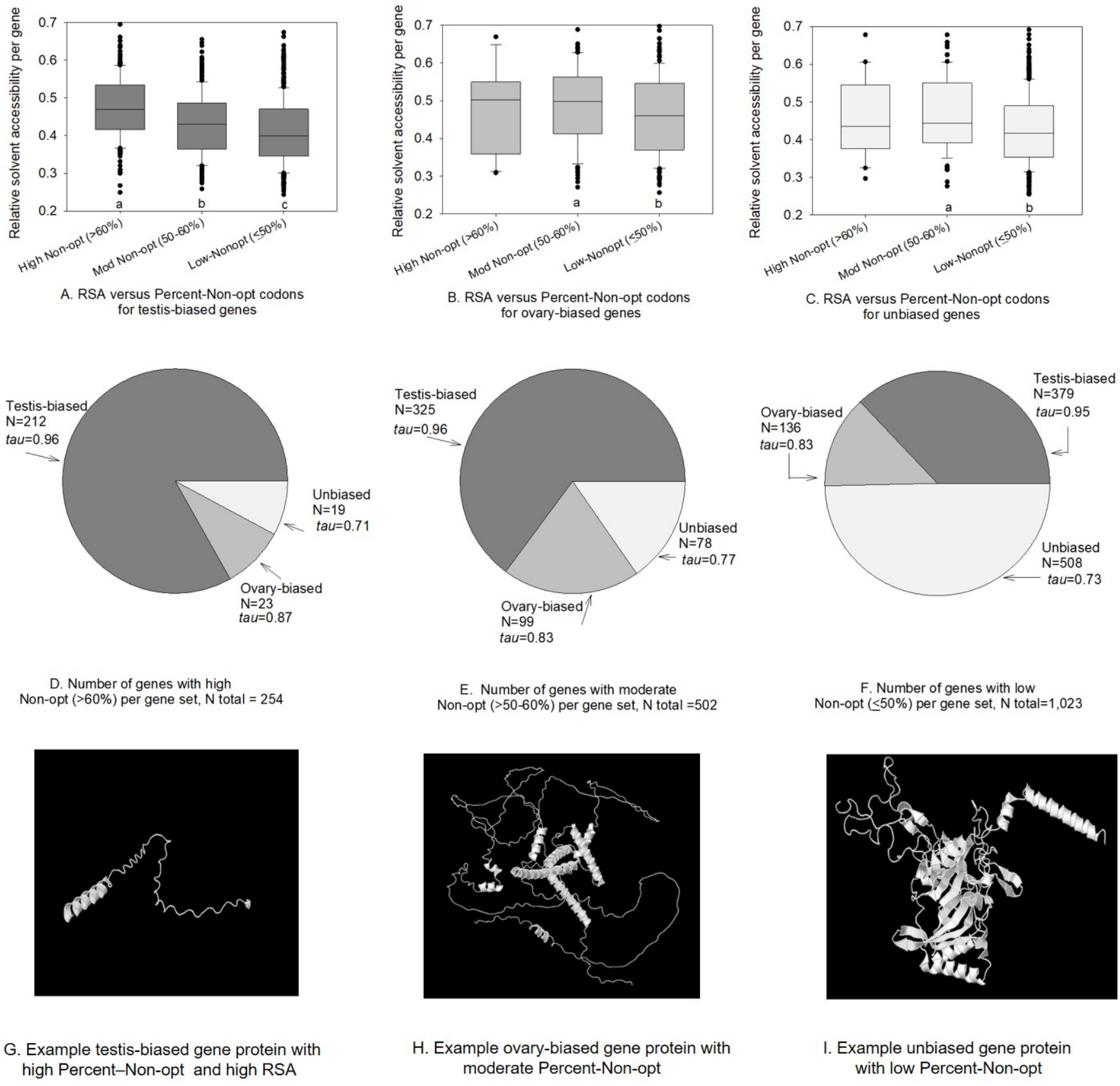

Fig. 2. A-C: The relationships between high non-optimal codon use (>60% of amino acids), moderate non-optimal use (50 to ≤60%) and low optimal codon use (≤50%) and the relative solvent accessibility for: A) testis-biased genes; B) ovary-biased genes; and C) unbiased genes in *D. melanogaster*. Pie charts 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 unbiased 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)

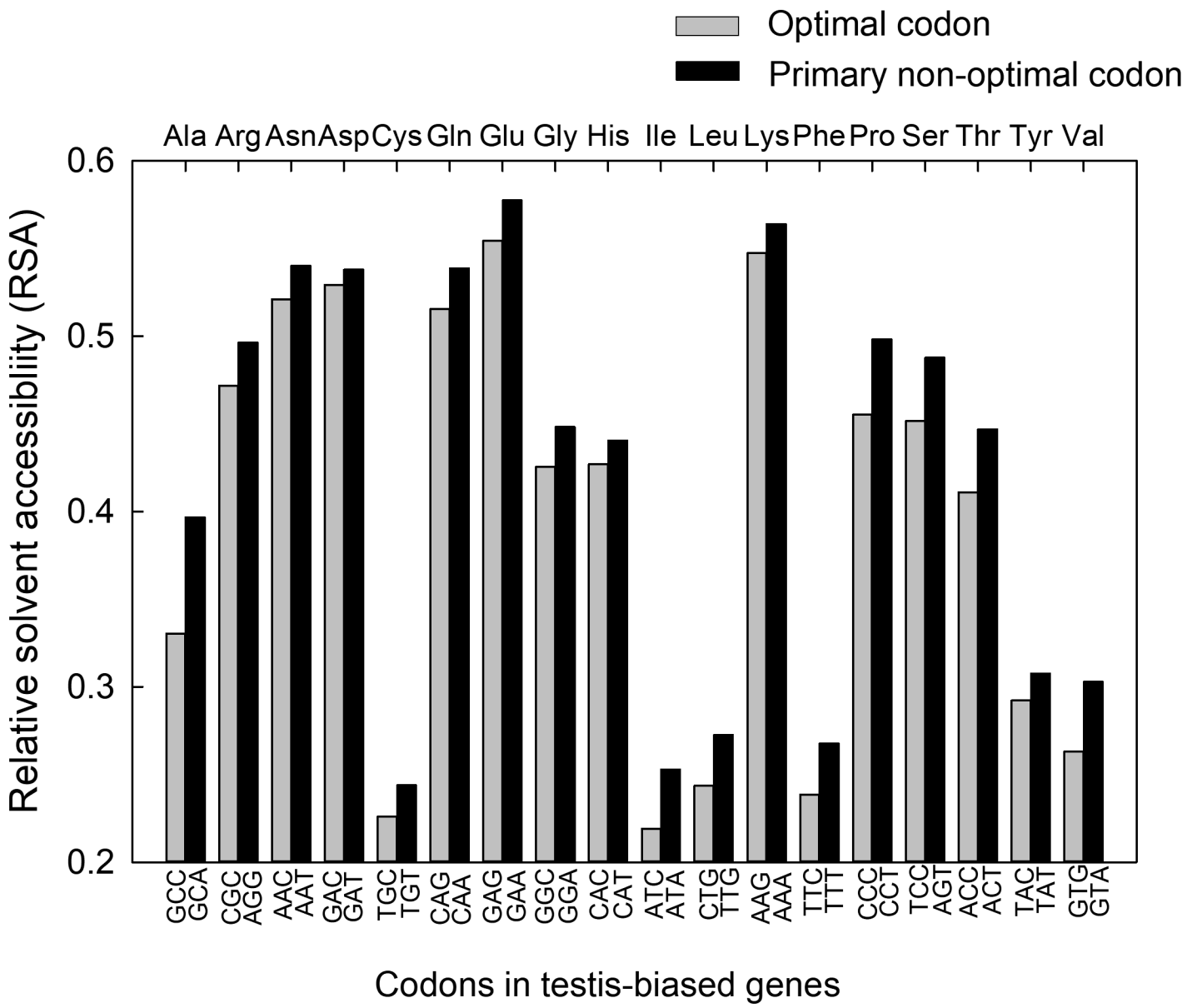

Fig. 3. The average RSA of testis-biased genes when encoded by the primary non-optimal codon (defined in Table S5) versus the optimal codon (Table S1) for all 18 degenerate amino acids. Results are from all amino acids and codons in testis-biased genes (N=299,817 amino acids). In total, 18 of 18 degenerate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