





A novel mistranslating tRNA model in *Drosophila melanogaster* has diverse, sexually dimorphic effects

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Abstract

Transfer RNAs (tRNAs) are the adaptor molecules required for reading the genetic code and producing proteins. Transfer RNA variants can lead to genome-wide mistranslation, the misincorporation of amino acids not specified by the standard genetic code into nascent proteins. While genome sequencing has identified putative mistranslating transfer RNA variants in human populations, little is known regarding how mistranslation affects multicellular organisms. Here, we create a multicellular model of mistranslation by integrating a serine transfer RNA variant that mistranslates serine for proline (tRNA^{Ser}_{UGG,G26A}) into the *Drosophila melanogaster* genome. We confirm mistranslation via mass spectrometry and find that tRNA^{Ser}_{UGG,G26A} misincorporates serine for proline at a frequency of ~0.6% per codon. tRNA^{Ser}_{UGG,G26A} extends development time and decreases the number of flies that reach adulthood. While both sexes of adult flies containing tRNA^{Ser}_{UGG,G26A} present with morphological deformities and poor climbing performance, these effects are more pronounced in female flies and the impact on climbing performance is exacerbated by age. This model will enable studies into the synergistic effects of mistranslating transfer RNA variants and disease-causing alleles.

Keywords: tRNA; mistranslation; *Drosophila melanogaster*; development; locomotion; proteostasis; deformity; sex-specific

Introduction

Mistranslation occurs when an amino acid that differs from what is specified by the standard genetic code is incorporated into nascent proteins. Mistranslation is implicated in various disease phenotypes. Editing-defective transfer RNA (tRNA) synthetases that induce mistranslation cause cardiac abnormalities and neurodegeneration in mice (Lee et al. 2006; Liu et al. 2014), and impaired locomotion, reduced lifespan, and neurodegeneration in flies (Lu et al. 2014). Ectopically expressed mistranslating tRNAs cause developmental deformities in zebrafish (Reverendo et al. 2014) and promote tumor growth in mouse cell lines (Santos et al. 2018). tRNA variants can cause mistranslation and are also directly linked to human disease, as mitochondrial tRNA variants cause MELAS and MERRF in humans (Goto et al. 1990; Shoffner et al. 1990). Despite the profound impact of mistranslation and the prevalence of cytoplasmic tRNA variants with the potential to mistranslate in humans (Berg et al. 2019a), the impact of these variants on the biology of multicellular organisms is not well described.

Mutations in tRNAs that cause mistranslation arise spontaneously and were identified initially in *Escherichia coli* as suppressors of nonsense and missense mutations (see e.g. Stadler and Yanofsky 1959; Gorini and Beckwith 1966; Goodman et al. 1968). Subsequently, mistranslating tRNAs have been identified as

suppressors of deleterious phenotypes in other organisms (e.g. Goodman et al. 1977; Wills et al. 1983; Chiu and Morris 1997; El Meziane et al. 1998; Murakami et al. 2005). While no spontaneous tRNA variants have been detected through suppression screens in *Drosophila*, researchers have engineered amber suppressing tRNA^{Tyr} and tRNA^{Leu} variants, respectively, with a low level of amber stop codon suppression activity when integrated into the *Drosophila melanogaster* genome (Laski et al. 1989; Garza et al. 1990). In both cases sterility was noted.

Translation requires base pairing of the anticodon, the three nucleotides of the tRNA at positions 34, 35, and 36, with complementary codons in mRNA in the A site of the ribosome. Because the anticodon provides a direct link between the tRNA and its amino acid assignment, it is the main identity element for the aminoacylation (the attachment of an amino acid to the 3' end of a tRNA) for most tRNAs (Giegé et al. 1998), with the exception of tRNA^{Ser}, tRNA^{Ala}, and tRNA^{Leu} (Hou and Schimmel 1988; McClain and Foss 1988; Normanly et al. 1992; Achsel and Gross 1993; Asahara et al. 1993; Breitschopf et al. 1995; Himeno et al. 1997). Changing the anticodon of the gene expressing a serine tRNA (tRNA^{Ser}) does not affect aminoacylation but changes codon recognition (Garza et al. 1990; Geslain et al. 2010; Reverendo et al. 2014; Zimmerman et al. 2018; Berg et al. 2019b), resulting in mistranslation. In this study, we stably integrate the gene expressing a tRNA^{Ser} variant that mistranslates serine at proline codons into

Received: December 16, 2021. Accepted: February 03, 2022

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the *D. melanogaster* genome. Development time of flies containing the mistranslating tRNA was extended and fewer flies reached adulthood compared with wild-type flies. The tRNA variant increased the prevalence of morphological deformities in adult flies, with females being more severely affected than males. Mistranslation also impaired climbing performance. Cytosolic mistranslating tRNA variants thus impact multiple aspects of the biology of a multicellular organism and in a sex-specific manner.

Materials and methods

Fly husbandry and stocks

All fly stocks were obtained from the Bloomington *Drosophila* Stock Centre and maintained on standard Bloomington recipe food medium (Bloomington, Indiana) under a 14:10 light:dark cycle at 24°C and 70% relative humidity.

Creating transgenic stocks

The gene encoding wild-type tRNA^{Ser}_{UGA} (FlyBase ID: FBgn0050201) was amplified from genomic DNA using primers VK3400/VK3401 (primers are listed in [Supplementary Table 1](#)) and cloned into pCDF4, a kind gift from Dr Simon Bullock ([Port et al. 2014](#)), as a *Bgl*II/*Xba*I fragment to create pCB4222. The gene encoding tRNA^{Ser} with a proline UGG anticodon and G26A secondary mutation (tRNA^{Ser}_{UGG,G26A}) were made by 2-step mutagenic PCR with primers VK3400/VK3889 and VK3401/VK3890 and pCB4222 as a template. Products from the first round were amplified with primers VK3400/VK3401 and cloned as a *Bgl*II/*Xba*I fragment into pCDF4 to give pCB4250. Sequences of tRNA^{Ser}_{UGA} and tRNA^{Ser}_{UGG,G26A} are found in [Supplementary Fig. 1](#).

To create flies containing mistranslating tRNAs, a stock expressing phiC31 (ΦC31) integrase in the germ line and containing an attP site in the left arm of the second chromosome was used (stock no. 25709: $y^1 v^1 P\{nos\text{-}phiC31\int int.NLS\}X; P\{CaryP\}attP40$). Plasmids were injected into *D. melanogaster* embryos ([Isaacson 2018](#)). Transgenic flies were identified by their wild-type eye color and balanced using stock no. 3703 ($w^{1118}/Dp(1; Y)y^+; CyO/nub^1 b^1 sna Sco lt^1 stw^3; MKRS/TM6B, Tb^1$) and no. 76359 ($w^{1118}; wg^{Sp-1}/CyO, P\{w^+mC=2xTb^1\text{-}RFP\}CyO; MKRS/TM6B, Tb^1$) to create final stocks of the following genotype: $w^{1118}; P\{CaryP\}attP40[v^+=tRNA_{UGA}^{Ser}]/CyO, P\{w^+mC=2xTb^1\text{-}RFP\}CyO; MKRS/TM6B, Tb^1$. This was the genotype of the flies used in all experiments. After producing offspring, DNA was extracted from both parents of the final cross for PCR amplification using the primer set M13R and VK3400 for sequence confirmation.

Complementation in *Saccharomyces cerevisiae*

The *Bgl*II/*Xba*I fragment of pCB422 encoding *Drosophila* tRNA^{Ser}_{UGG,G26A} was cloned into the *Bam*HI/*Xba*I sites of the yeast-*E. coli* shuttle plasmid YEPlac181 (pCB4877, [Gietz and Sugino 1988](#)). pCB4877 and YEPlac181 were transformed into the yeast strain CY9013 [MAT α his3 Δ 1 leu2 Δ 0 lys2 Δ 0 met15 Δ 0 ura3 Δ 0 tti2 Δ -met5 Δ -mTn10luk containing pRS313 ([Sikorski and Hieter 1989](#)) expressing tti2-L187P ([Berg et al. 2017](#))] selecting for growth on minimal plates lacking leucine and histidine. Transformants were streaked onto yeast-peptone (YP) plates containing 2% glucose and 5% ethanol and grown at 30°C for 4 days.

Mass spectrometry

Detailed mass spectrometry protocols are described in the [Supplementary Appendix](#). Briefly, protein was extracted from 20 pupae per sample, reduced, alkylated, and digested into peptides following the R2-P1 method as described in [Leutert et al. \(2019\)](#).

Peptides were analyzed on a hybrid quadrupole orbitrap mass spectrometer (Orbitrap Exploris 480; Thermo Fisher Scientific). MS/MS spectra were searched against the *D. melanogaster* protein sequence database (downloaded from Uniprot in 2016) using Comet (release 2015.01; [Eng et al. 2013](#)). Mistranslation frequency was calculated using the unique mistranslated peptides for which the nonmistranslated sibling peptide was also observed and defined as the counts of mistranslated peptides, where serine was inserted for proline, divided by the counts of all peptides containing proline, respectively, expressed as a percentage.

Scoring deformities

Virgin, heterozygous flies were collected within ~8 h of eclosion and scored for deformities in adult legs (limbs gnarled or missing segments), wings (blistered, absent, fluid-filled, or abnormal size), or abdomen (fused or incomplete tergites). Flies collected before wing expansion were excluded. Sex and type of deformity was recorded. Flies with multiple deformities had each recorded. Four hundred and thirty-three tRNA^{Ser}_{UGA} flies (227 males and 216 females) and 656 tRNA^{Ser}_{UGG,G26A} flies (345 male and 311 female) were scored. All deformities were photographed through the lens of a stereomicroscope using a Samsung Galaxy S8 camera.

Developmental assays

Approximately 250 flies of each genotype ($w^{1118}; P\{CaryP\}attP40[v^+=tRNA_{UGA}^{Ser}]/CyO, P\{w^+mC=2xTb^1\text{-}RFP\}CyO; MKRS/TM6B, Tb^1$ or $w^{1118}; P\{CaryP\}attP40[v^+=tRNA_{UGG,G26A}^{Ser}]/CyO, P\{w^+mC=2xTb^1\text{-}RFP\}CyO; MKRS/TM6B, Tb^1$) were placed into fly cages and allowed to lay eggs for 1 h. Seven replicates of 30 eggs from each plate were checked every 12 h to record progress through development. Sex, zygosity, and deformities of adults were recorded.

Climbing assays

Virgin adult flies were sorted by sex and scored for deformities. Deformed flies or flies homozygous for the transgenic tRNA were discarded. Equal numbers were collected from each genotype during each collection period. Sixty flies in 11 vials from each genotype were transferred to new food the day before testing. The number of flies that climbed to a 5-cm line in 10 s was recorded. Flies were retested every 3 days until the flies were 51 days old. Each vial was tested 3 times.

Statistical analyses

Statistical analyses were performed using R Studio 1.2.5001. Analyses used for comparisons were: t-test (frequency of proline-to-serine misincorporation between tRNA^{Ser}_{UGA} and tRNA^{Ser}_{UGG,G26A}); Wilcoxon rank-sum tests (developmental time data, corrected using Holm–Bonferroni’s method); Fisher’s exact tests (survival between developmental stages and proportion of deformities, corrected using Holm–Bonferroni’s method). A generalized linear model was constructed from the climbing assay data and performance was compared using F-tests corrected using Bonferroni’s method.

Results

A tRNA^{Ser} variant induces mistranslation in *D. melanogaster*

To characterize mistranslation in a multicellular organism, we integrated genes encoding wild-type tRNA^{Ser}_{UGA} as a control and a tRNA^{Ser} variant that mistranslates serine for proline ([Fig. 1a](#)) into

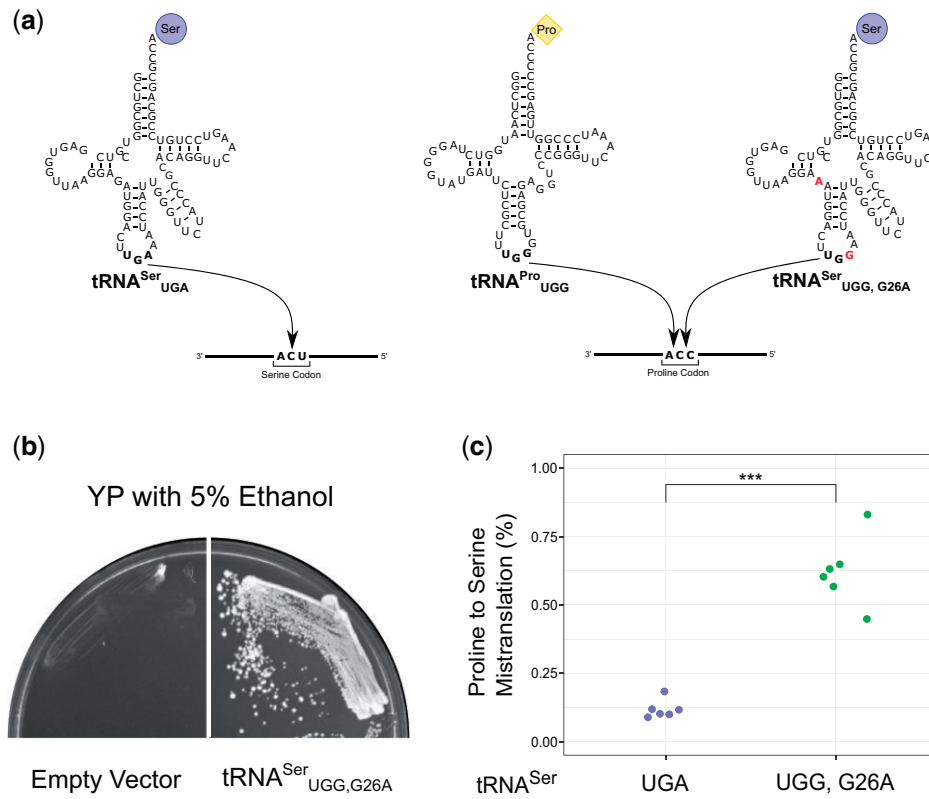


Fig. 1. tRNA^{Ser}_{UGG,G26A} induces mistranslation in *D. melanogaster*. a) Wild-type tRNA^{Ser} base pairs with serine codons, incorporating serine into the growing polypeptide. tRNA^{Ser}_{UGG,G26A} competes with tRNA^{Pro}_{UGG} for CCA codons and inserts serine at proline codons. Red bases indicate mutation compared with the wild-type tRNA^{Ser}_{UGA}. b) *Drosophila melanogaster* tRNA^{Ser}_{UGG,G26A} suppresses the ethanol sensitivity caused by *tti2-L187P* in *S. cerevisiae*. Plasmid encoding the vector alone (left) or the gene expressing tRNA^{Ser}_{UGG,G26A} (right) were transformed into CY9013 (*tti2-L187P*), streaked onto YP medium containing 5% ethanol and grown at 30°C for 4 days. c) Frequency of proline-to-serine mistranslation in tRNA^{Ser}_{UGA} and tRNA^{Ser}_{UGG,G26A} pupae (n = 6 replicates of 20 pupae each). Genotypes were compared using a t-test. **** P < 0.001.

the left arm of the second chromosome of the *D. melanogaster* genome. The tRNA^{Ser} variant has a proline UGG anticodon and G26A secondary mutation (tRNA^{Ser}_{UGG,G26A}). The alleles were balanced over a homolog that has serial inversions, preventing recombinant offspring, and transgene loss. tRNA insertions were validated with PCR using primers specific to the inserted plasmid and confirmed by sequencing. The secondary G26A mutation was included in the mistranslating tRNA to dampen tRNA function as we have previously found a tRNA^{Ser} variant with a proline anticodon causes lethal levels of mistranslation when expressed in yeast (Berg et al. 2017).

Adults homozygous for tRNA^{Ser}_{UGA} or tRNA^{Ser}_{UGG,G26A} can be produced. However, we were unable to propagate the strain homozygous for tRNA^{Ser}_{UGG,G26A} because crosses between male and female tRNA^{Ser}_{UGG,G26A} homozygotes produce no viable offspring. As such, we used heterozygous flies for our experiments with adults. Studying heterozygous flies may be more biologically relevant as mistranslating tRNAs present in populations are likely to arise as single alleles. We determined zygosity by balancing the tRNAs over a CyO homolog containing Tubby-linked RFP and *miniwhite* (Pina and Pignoni 2012). Heterozygous larvae and pupae are identified by the presence of RFP and heterozygous adults by their curly wings and nonwhite eyes.

As an initial test of mistranslation by *Drosophila* tRNA^{Ser}_{UGG,G26A}, we determined if the tRNA rescues growth of a *Saccharomyces cerevisiae* strain containing *tti2-L187P* (CY9013). The *tti2-L187P* allele contains a missense mutation converting a CUA codon for

leucine to CCA for proline and results in the slow growth of yeast in medium containing 5% ethanol (Hoffman et al. 2017). Mistranslation of proline to serine rescues the growth of yeast cells in ethanol medium (Berg et al. 2017). The gene encoding *Drosophila* tRNA^{Ser}_{UGG,G26A} was transformed into a yeast strain that contains *tti2-L187P* as the sole copy of *TTI2*. Cells were transformed with plasmid expressing *Drosophila* tRNA^{Ser}_{UGG,G26A} or vector alone. As shown in Fig. 1b, *Drosophila* tRNA^{Ser}_{UGG,G26A} enabled growth of the strain on medium containing 5% ethanol indicative of mistranslation by *Drosophila* tRNA^{Ser}_{UGG,G26A}.

We then analyzed the proteome of *D. melanogaster* pupae by mass spectrometry to determine the mistranslation frequency (Fig. 1c; Supplementary File 2). Pupae were used because of the extensive cellular remodeling and corresponding rapid changes in protein synthesis that occur during this stage (Mitchell et al. 1977; Mitchell and Petersen 1981), and the potential of mistranslation during this stage to influence adult traits such as anatomy or neuronal function. The frequency of proline to serine mistranslation, calculated as the ratio of peptides containing the mistranslated serine residue to peptides containing the cognate proline residue, was ~0.6% in flies expressing tRNA^{Ser}_{UGG,G26A}. In the control strain, the frequency of proline to serine substitutions was 0.1%.

tRNA^{Ser}_{UGG,G26A} adversely affects *D. melanogaster* development

To determine if tRNA^{Ser}_{UGG,G26A} affects fly development, we collected 210 wild-type tRNA^{Ser}_{UGA} and tRNA^{Ser}_{UGG,G26A} 1-h old embryos,

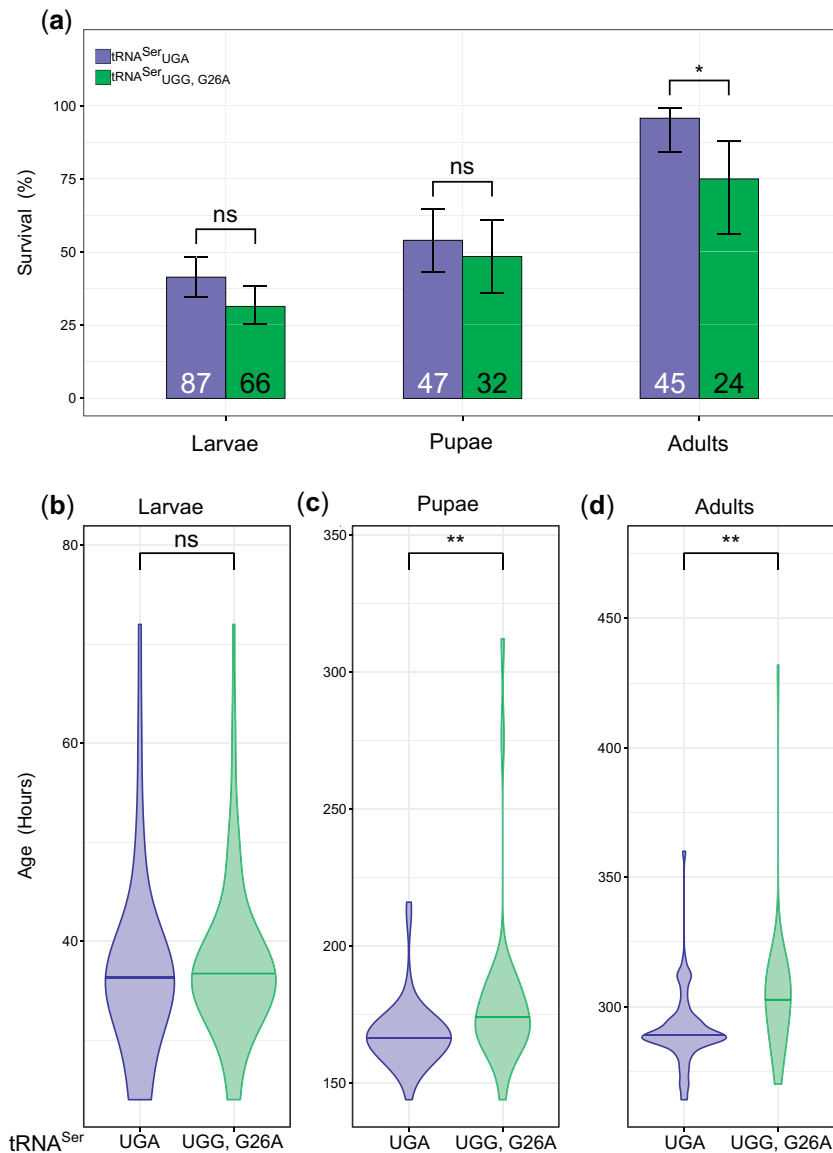


Fig. 2. A mistranslating tRNA variant impacts development of *D. melanogaster*. a) Percentage of the 210 embryos containing tRNA^{Ser}_{UGA} or tRNA^{Ser}_{UGG,G26A} that reached larval, pupal, and adult stages out of survivors from the previous stage. Survival was compared using Fisher's exact test corrected using Holm-Bonferroni's method. Error bars represent the 95% confidence interval of the proportion. Values within bars represent the number of flies that reached that developmental stage. b) Violin plot depicting the distribution of times for tRNA^{Ser}_{UGA} and tRNA^{Ser}_{UGG,G26A} embryos to hatch into larva. The horizontal line within the plot represents the median of the distribution. Genotypes were compared using Wilcoxon's rank-sum tests corrected using Holm-Bonferroni's method. c) Total time until pupation. d) Total time until eclosion. "ns" $P \geq 0.05$; "*" $P < 0.05$; "**" $P < 0.01$; "****" $P < 0.001$.

comparing survival at 12-h intervals through each developmental stage (Fig. 2a) and time to reach each stage (Fig. 2, b-d): embryos to larvae, larvae to pupae, and pupae to adults. Since the RFP marker used to determine tRNA zygosity is not expressed during early embryonic stages, both homozygotes and heterozygotes were pooled in this assay. While there were fewer female and homozygous tRNA^{Ser}_{UGG,G26A} flies compared with tRNA^{Ser}_{UGA} flies, neither the male bias nor heterozygote bias reached statistical significance (Supplementary File 2). Figure 2a shows the percentage of individuals who reached a developmental stage relative to those who reached the previous stage (e.g. how many larvae managed to pupate). Of the 210 tRNA^{Ser}_{UGA} embryos collected, 87 hatched into larvae, 47 larvae pupated, and 45 of those pupae reached adulthood. Survival of tRNA^{Ser}_{UGA} was low due to the presence of three balancers (CyO, MKRS, and TM6B) in heterozygous flies and two (MKRS and TM6B) in homozygous flies. tRNA^{Ser}_{UGG,G26A}

resulted in reduced viability at each stage as only 66 out of 210 embryos containing tRNA^{Ser}_{UGG,G26A} hatched, 32 larvae pupated, and 24 pupae reached adulthood. However, the difference between the proportion of tRNA^{Ser}_{UGA} and tRNA^{Ser}_{UGG,G26A} embryos that hatched (41.4% vs 31.4%, $P = 0.08$, Fig. 2a) and the proportion of larvae that pupated (54.0% vs 48.5%, $P = 0.51$) was not statistically significant (Fisher's exact test corrected using Bonferroni-Holm's method). In contrast, the proportion of tRNA^{Ser}_{UGA} pupae that reached adulthood was significantly higher than tRNA^{Ser}_{UGG,G26A} (95.7% vs 75.0%, $P = 0.012$). This indicates that flies are particularly susceptible to lethal effects of mistranslating tRNA variants during pupation.

Eggs expressing tRNA^{Ser}_{UGG,G26A} had similar hatching times as eggs expressing wild-type tRNA^{Ser}_{UGA} ($P = 0.24$, Wilcoxon rank-sum test corrected using Holm-Bonferroni's method, Fig. 2b). However, larvae expressing tRNA^{Ser}_{UGG,G26A} pupated significantly

slower than the wild-type ($P = 0.004$, Fig. 2c). This trend continued into adulthood, as $\text{tRNA}_{\text{UGG},\text{G26A}}^{\text{Ser}}$ flies eclosed significantly later than control $\text{tRNA}_{\text{UGA}}^{\text{Ser}}$ flies ($P = 0.002$, Fig. 2d). Median development time of $\text{tRNA}_{\text{UGA}}^{\text{Ser}}$ flies was 288 h whereas median development time of $\text{tRNA}_{\text{UGG},\text{G26A}}^{\text{Ser}}$ flies was 303 h. Some extremely late pupation and eclosion events were observed in the mistranslating $\text{tRNA}_{\text{UGG},\text{G26A}}^{\text{Ser}}$ line and were a potential concern as they could have biased the statistical comparisons (Fig. 2, c and d; Supplementary File 2). However, $\text{tRNA}_{\text{UGG},\text{G26A}}^{\text{Ser}}$ flies still pupated and eclosed significantly later than $\text{tRNA}_{\text{UGA}}^{\text{Ser}}$ flies even after removing these values ($P = 0.007$ and $P = 0.002$, respectively, Supplementary Fig. 2). These results show that flies containing this mistranslating tRNA variant show extended development time and increased developmental lethality.

Mutations in genes vital to proteostasis or translation fidelity cause morphological defects (Rutherford and Lindquist 1998; Cui and DiMario 2007; Reverendo et al. 2014). We observed that flies containing one copy of the exogenous $\text{tRNA}_{\text{UGG},\text{G26A}}^{\text{Ser}}$ had deformities including gnarled or blistered legs, notched wings, and misfused tergites (Fig. 3, a–d). Other abnormalities (e.g. haltere aberrations or rough eyes) were rarely observed, so only the more common leg, wing, and tergite deformities were scored. To determine if the frequency of deformities was greater than the control, we calculated the proportion of flies that eclosed with at least one deformity. These flies were collected separately from the development assay described above. From a total of 433 $\text{tRNA}_{\text{UGA}}^{\text{Ser}}$ flies (227 males and 216 females) and 656

$\text{tRNA}_{\text{UGG},\text{G26A}}^{\text{Ser}}$ flies (345 males and 311 females) we identified proportionally more deformities in flies containing $\text{tRNA}_{\text{UGG},\text{G26A}}^{\text{Ser}}$ than $\text{tRNA}_{\text{UGA}}^{\text{Ser}}$ (Fisher's exact test corrected using Holm-Bonferroni's method, $P < 0.001$, Fig. 3e). In addition, female flies containing $\text{tRNA}_{\text{UGG},\text{G26A}}^{\text{Ser}}$ had more deformities than males ($P < 0.001$, Fig. 3f). Interestingly, flies containing $\text{tRNA}_{\text{UGG},\text{G26A}}^{\text{Ser}}$ presented with disproportionately more tergite deformities than flies with the wild-type $\text{tRNA}_{\text{UGA}}^{\text{Ser}}$ [chi-square test with post hoc analysis using the method outlined in (Shan and Gerstenberger 2017), $P = 0.03$, Supplementary File 3], indicating that this mistranslating tRNA variant is particularly deleterious to fly abdominal development. These results suggest that mistranslating tRNA variants can disrupt fly development and that female flies are more sensitive to their effects.

$\text{tRNA}_{\text{UGG},\text{G26A}}^{\text{Ser}}$ impacts fly motility

Negative geotaxis assays are often used as an initial test of neurodegeneration in flies (e.g. 38–40); therefore, we determined if $\text{tRNA}_{\text{UGG},\text{G26A}}^{\text{Ser}}$ impaired climbing performance. Sixty virgin, heterozygous flies of the four genotypes ($\text{tRNA}_{\text{UGA}}^{\text{Ser}}$ males and females, and $\text{tRNA}_{\text{UGG},\text{G26A}}^{\text{Ser}}$ males and females) were collected and tested using a climbing assay every three days; flies with deformities were not used in this experiment. Climbing performance of all genotypes decreased with age (F -tests performed on generalized linear models corrected using Bonferroni's method, Supplementary File 2). For both males and females, climbing performance of $\text{tRNA}_{\text{UGG},\text{G26A}}^{\text{Ser}}$ flies was significantly worse than wild-

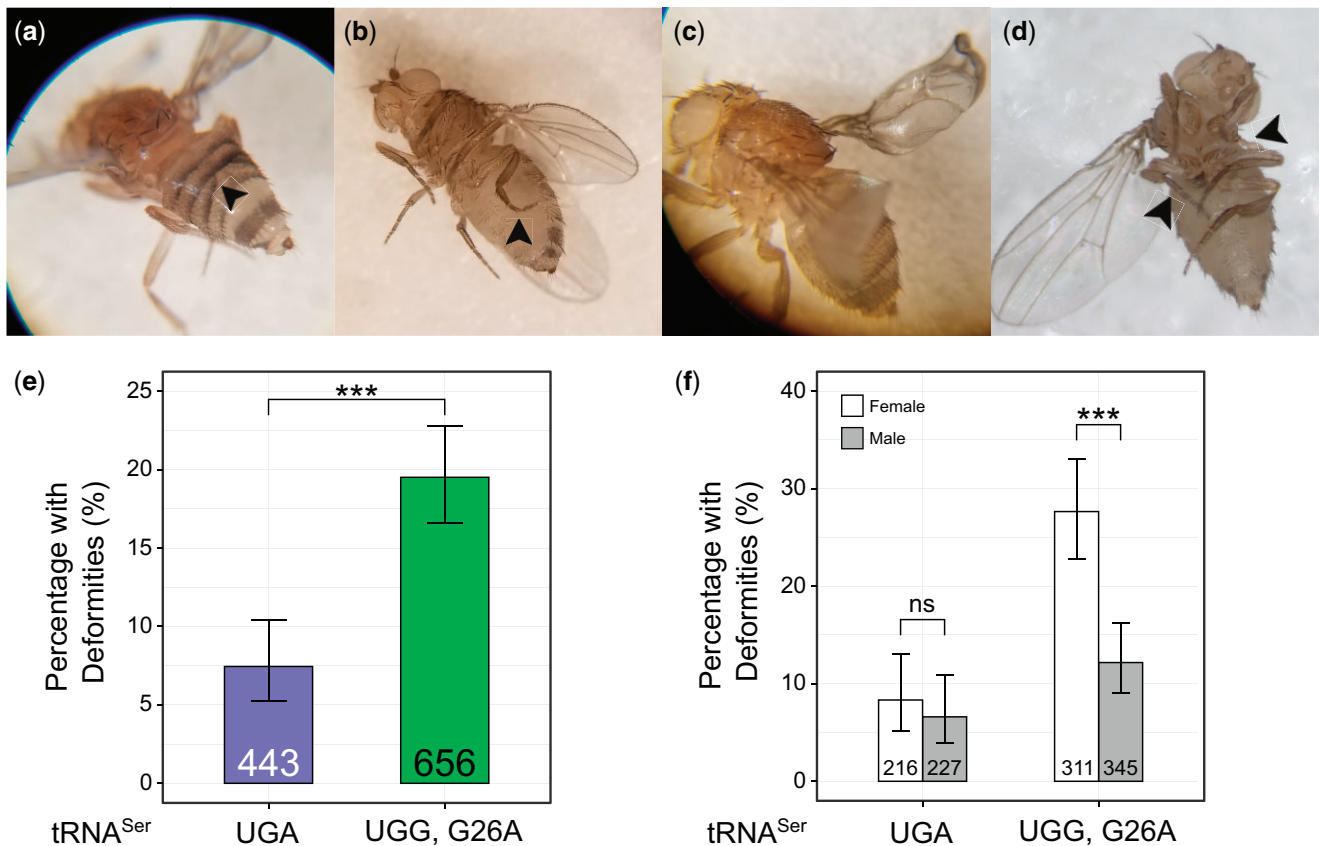


Fig. 3. The $\text{tRNA}_{\text{UGG},\text{G26A}}^{\text{Ser}}$ variant causes morphological deformities in adults in a sex-specific manner. a) Examples of flies with misfused tergites; b) gnarled hindlegs; c) wing blisters; and d) missing wings/legs, as indicated by arrowheads. e) Percentage of $\text{tRNA}_{\text{UGA}}^{\text{Ser}}$ or $\text{tRNA}_{\text{UGG},\text{G26A}}^{\text{Ser}}$ flies that eclosed with at least 1 deformity. Groups were compared using Fisher's exact test and corrected using Holm-Bonferroni's method. Bar height represents the percentage of flies of a genotype that had at least 1 deformity. Error bars represent the 95% confidence interval. Values within bars describe the number of flies examined for deformities. f) Same data as (e) but separated by sex. "ns" $P \geq 0.05$; "***" $P < 0.05$; "****" $P < 0.01$; "*****" $P < 0.001$.

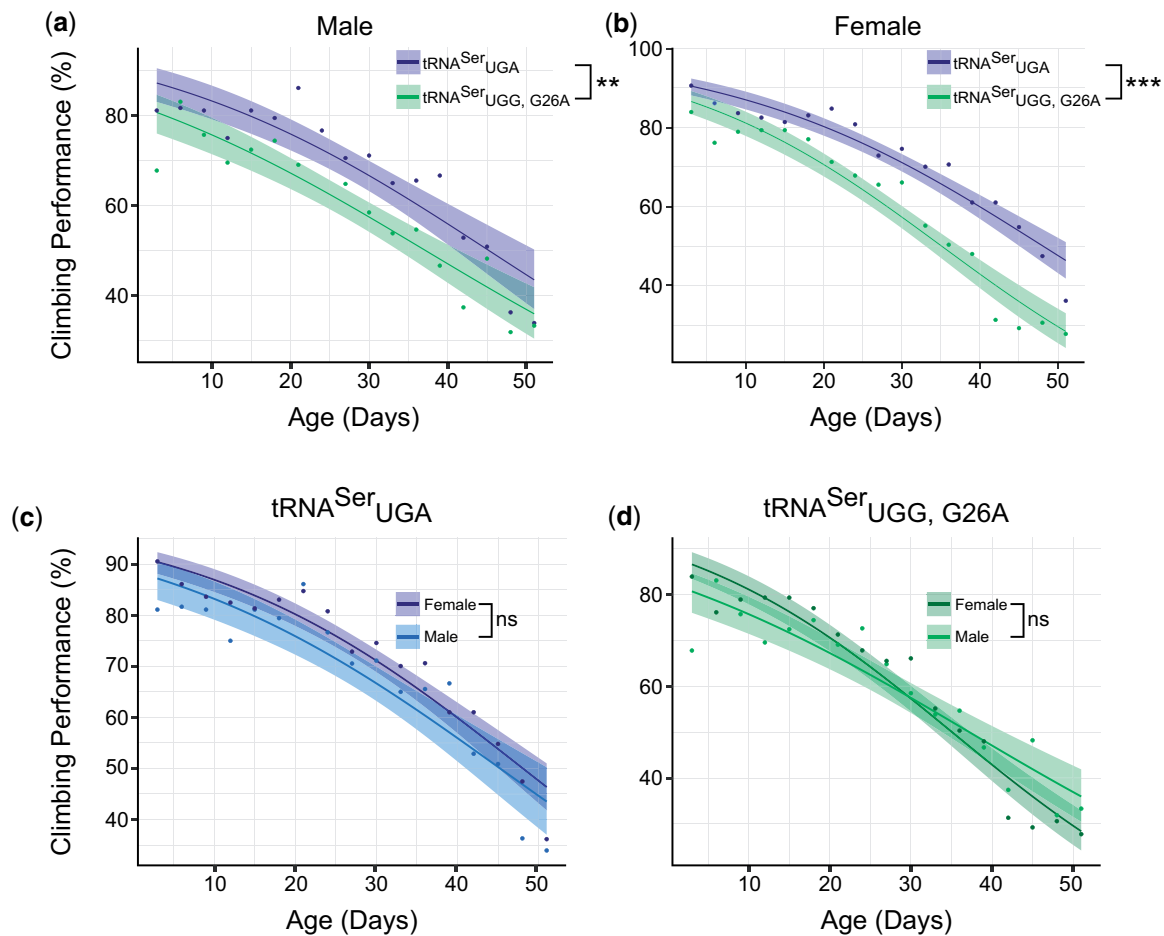


Fig. 4. Fly locomotion is impacted by a mistranslating $tRNA^{Ser}$ variant. Each point represents the percentage of flies (out of 60 individuals from 11 vials) that managed to climb 5 cm in 10 s averaged over 3 trials. Generalized linear models were constructed from the performance data and F-tests were performed on the models. P-values were corrected using Bonferroni's method. Shaded region represents the 95% confidence intervals for the fitted performance curves. a) Climbing performance of male flies containing $tRNA^{Ser}_{UGA}$ or $tRNA^{Ser}_{UGG, G26A}$. b) Climbing performance of female flies containing $tRNA^{Ser}_{UGA}$ or $tRNA^{Ser}_{UGG, G26A}$. c) Climbing performance of male and female flies containing $tRNA^{Ser}_{UGA}$ or d) $tRNA^{Ser}_{UGG, G26A}$. "ns" $P \geq 0.05$; "*" $P < 0.05$; "***" $P < 0.01$; "****" $P < 0.001$.

type $tRNA^{Ser}_{UGA}$ flies (male: $P = 0.001$, female: $P < 0.001$, Fig. 4, a and b). Climbing performance was not significantly different when comparing males to females in either the control $tRNA^{Ser}_{UGA}$ ($P = 0.08$) or mistranslating $tRNA^{Ser}_{UGG, G26A}$ flies ($p \rightarrow 1$, Fig. 4, c and d). The climbing ability of male and female flies containing the wild-type $tRNA^{Ser}_{UGA}$ declined at similar rates, as evidenced by the parallel performance curves ($P = 0.97$, Fig. 4c). However, the climbing performance curve of female flies containing $tRNA^{Ser}_{UGG, G26A}$ intersected the male curve, indicating a significant interaction effect between age and sex ($P = 0.038$, Fig. 4d; Supplementary File 3). Therefore, while overall climbing performance did not differ between $tRNA^{Ser}_{UGG, G26A}$ males and females, rate of performance decline was faster for $tRNA^{Ser}_{UGG, G26A}$ females. These data indicate that the mistranslating $tRNA^{Ser}$ variant negatively affects locomotion and has an accelerated impact on female ability to climb as they age.

Discussion

A fly model of mistranslation

We have created a *D. melanogaster* model containing a genomically integrated cytosolic tRNA that mistranslates serine for proline. The mistranslating fly model allows for studies into sex-

specific or tissue-specific effects of mistranslating tRNA variants and the effect of tRNA variants on development and disease. Our method of transgene integration controlled for positional effects by inserting either wild-type or mistranslating $tRNA^{Ser}_{UGG, G26A}$ into the same locus on chromosome 2L. The fly lines containing $tRNA^{Ser}_{UGG, G26A}$ have not lost the transgene for over two years, indicating that mistranslating tRNA variants can be stably maintained in the genome. We observed a proline-to-serine misincorporation rate of $\sim 0.6\%$ in the pupae for the genomically integrated $tRNA^{Ser}_{UGG, G26A}$ gene. This level of mistranslation was sufficient to cause deleterious phenotypes affecting diverse aspects of fly physiology.

A mistranslating $tRNA^{Ser}$ variant has diverse and sex-specific effects on flies

The mistranslating $tRNA^{Ser}_{UGG, G26A}$ affects fly physiology consistent with organism-wide loss of proteostasis. Our findings resemble other studies of proteostasis loss in flies. Impaired heat shock response exacerbates neurodegeneration and increases development time (Warrick et al. 1999; Gong and Golic 2006), and many of the wing, leg, and tergite deformities observed for heterozygous *Heat shock protein 83* (*Hsp83*) mutants look similar to those observed in this study (Rutherford and Lindquist 1998).

Developmental and neurodegenerative phenotypes including locomotive defects as measured in a climbing assay were likewise observed in flies containing a misacylation-prone PheRS (Lu et al. 2014). It is interesting to note that reduced levels of translation lead to similar deformities as found in mistranslating flies. RNAi knockdown of *Nopp140*, a gene involved in ribosome assembly, causes flies to present with gnarled legs, missing wings, and misfused tergites (Cui and DiMario 2007). *Minute* genes describe a collection of >50 genes required for protein synthesis. Their mutation results in shorter, thinner bristles, delayed development, smaller body size, and anatomical deformities when mutated (Schultz 1929; Marygold et al. 2007), again similar to the developmental and anatomical aberrations seen in flies containing the mistranslating tRNA^{Ser} variant. Though reduced translation and mistranslation are different processes, the similar phenotypes produced demonstrate that development is highly dependent on accurate and efficient translation.

The increased impact of the mistranslating tRNA on female flies was striking. *Drosophila melanogaster* males and females have highly different physiology and experience different developmental challenges. Adult females are larger than males, develop faster, invest more resources into reproduction, and tend to live longer than males (Bonnier 1926; Bakker 1959; Sørensen et al. 2007; Ziehm et al. 2013). Males and females also display dimorphic responses to proteotoxic stress. Fredriksson et al. (2012) examined protein carbonylation in female somatic and germ line cells at different ages to determine how aging affects protein quality control of somatic and reproductive tissues (Fredriksson et al. 2012). They found that as females age, there are fewer carbonylated proteins and reduced protein aggregation (both indicators of proteostasis loss) in eggs compared with the soma. Their work shows that females prioritize maintaining proteostasis of their eggs over their somatic cells, even while unmated. This trade-off could exacerbate the stress of mistranslating tRNAs in females, particularly as they experience aging-induced loss of proteostasis, and could contribute to the faster decline of climbing performance observed in female tRNA^{Ser}_{UGG,G26A} flies compared with males. Many stress-response pathways affect males and females differently. For example, induction of the heat shock response increases male lifespan whereas female lifespan is unaffected (Sørensen et al. 2007, reviewed in Tower 2011). Dietary restriction shows the opposite trend, as it increases female lifespan more than male (Nakagawa et al. 2012; Regan et al. 2016; Garratt 2020). Experiments testing the effects of mistranslating tRNAs on male and female fly longevity are ongoing. It is also possible that expression of the mistranslating tRNA differs between males and females or that the mistranslating tRNA has alternative functions (e.g. tRNA-derived fragments) that differ between males and females.

Implications for human disease

Our work suggests that mistranslating tRNA variants have the potential to influence multiple aspects of human physiology. From a development perspective, the alteration in progression through life stages and increased number of deformities suggest that the proteotoxic stress resulting from mistranslating tRNA variants may contribute to congenital or developmental anomalies. Flies expressing tRNA^{Ser}_{UGG,G26A} have a pattern of locomotion defects similar to those seen for flies expressing alleles associated with neurodegeneration (Feany and Bender 2000; Song et al. 2017; Aggarwal et al. 2019). Interestingly, the mistranslating fly model further resembles human neuropathies in that climbing performance declined faster in female compared with male flies, just

as some neurodegenerative disorders, such as Alzheimer's and Huntington's disease, are more common or severe in women compared with men (Viña and Lloret 2010; Zielonka et al. 2013).

Given the prevalence of putative mistranslating tRNAs in the human population (Berg et al. 2019a) and the potential for mistranslation to disrupt proteostasis, we hypothesize that mistranslating tRNAs can exacerbate diseases characterized by a loss of proteostasis (see also Reverendo et al. 2014), and our results here indicate that these effects may differ in magnitude between sexes. Our previous studies in yeast have shown negative genetic interactions between mistranslation and mutations in genes involved in protein quality control and other pathways that could contribute to disease (Hoffman et al. 2017; Berg et al. 2020, 2021). Our *D. melanogaster* model of mistranslation allows for the expansion of these studies into the investigation of mutant tRNA contribution to disease and development.

Data availability

Fly lines and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and supplementary material. Supplementary File 1 contains an extended methods section and supplementary figures and tables. Supplementary File 2 contains all raw data. Supplementary File 3 contains R code used to analyze mass spectrometry, developmental, deformity, and climbing assay data. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Perez-Riverol et al. 2019) with the dataset identifier PXD028498.

Supplementary material is available at G3 online.

Acknowledgments

We would like to thank the Biotron Integrated Microscopy facility for their aid with this research. In particular, JRI would like to thank Karen Nygard, Reza Khazaei, and Marc Courchesne for their instruction and help. We would also like to thank Dr Yolanda Morbey for guidance on analyzing the climbing assay data, Dr Patrick O'Donoghue and Dr Robert Cumming for their feedback on the work, Ricard Rodriguez-Mias for assisting with the mass spectrometry and maintaining the instruments, and Julie Genereaux for technical assistance.

Funding

This work was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) grants to CJB [RGPIN-2015-04394] and AJM [RGPIN-2020-06464], as well as a University of Western Ontario Medical & Health Science Research Board seed grant to AJM. Mass spectrometry work was supported by a research grant from the Keck Foundation, National Institutes of Health grant R35 GM119536 and associated instrumentation supplement to JV. JRI and MDB were supported by an NSERC Postgraduate Scholarship (Doctoral) and NSERC Canada Graduate Scholarship (Doctoral), respectively.

Conflicts of interest

None declared.

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Communicating editor: B. Reed