Title: Time-Restricted Feeding Extends Healthspan in Female and Lifespan in Male C57BL/6J Mice

Authors: Samantha E. Iiams[1 †], Nathan J. Skinner[1 †], Victoria A. Acosta-Rodríguez[1], Mary Wight-Carter[3], Carla B. Green[1]*, and Joseph S. Takahashi[1,2]*

Affiliations:

[1]Department of Neuroscience, Peter O'Donnell Jr. Brain Institute, University of Texas Southwestern Medical Center, Dallas, TX, USA

[2]Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX, USA

[3]Animal Resource Center, University of Texas Southwestern Medical Center, Dallas, TX, USA

* Corresponding authors: Carla B. Green (CBG); carla.green@utsouthwestern.edu, Joseph S. Takahashi (JST); joseph.takahashi@utsouthwestern.edu

† These two authors contributed equally to this work and are co-first authors

Abstract: Time-restricted feeding (TRF) aligned with an organism's circadian rhythm has been shown to improve health, but its long-term effects on healthspan and lifespan in mammals, especially under normal dietary conditions, remain unclear. In this study, we tested the impact of 12-hour (h) and 8h nightly TRF windows on aging in male and female mice fed regular chow. TRF improved several health measures, including the regularity of behavioral patterns, body weight and composition, frailty, and disease onset, with stronger effects observed in the 8h-TRF group, which self-imposed a caloric restriction. When integrated into a composite Healthspan Index, these improvements revealed that TRF prolonged healthspan in females more effectively than in males. Only 8h-TRF extended lifespan in males by 12%, suggesting that TRF exerts sex-specific effects on aging.

Main Text:

While human life expectancy has nearly doubled since the early 20th century (*1*), healthspan remains limited, with many spending over 10% of their lives in poor health due to age-related functional declines within tissue and the accompanying development of disease (*2*). Caloric restriction (CR), a potent aging intervention involving a 30-50% reduction in food intake without malnutrition, has been extensively studied for its ability to delay the onset of age-associated diseases, including cancer, cardiovascular disorders, and neurodegeneration, while extending lifespan across multiple model organisms (*3-5*). Notably, incorporating daily feeding/fasting cycles with CR enhances these geroprotective effects (*6-9*), and aligning feeding with the active phase of an organism's circadian rhythm further extends lifespan (*10*). This synergy likely arises from both synchronizing food intake with internal metabolic processes and from the ability of feed-fasting rhythms to enhance daily oscillations in the expression of genes regulated by circadian clocks in the liver and other peripheral tissues (*11-17*).

With age, circadian rhythms in behavior and physiology weaken as the amplitude of clock-controlled gene expression damps or shifts in phase (*18*). However, interventions that rescue circadian rhythms have been shown to improve health and extend longevity (*19*). Thus, optimization of meal timing presents a promising strategy to counteract aging-related rhythm disruptions and promote healthy longevity.

Given that CR can be challenging to maintain in people (*20*), meal timing alone via time-restricted feeding (TRF), or time-restricted eating (TRE) in humans, has emerged as an alternative strategy to delay aging (*21, 22*). TRF, which involves daily windows of feeding and fasting without imposing CR, confers similar benefits as timed CR, including improved rhythmic gene expression, cognitive function, and cardiometabolic health, even in models of obesity fed a high-fat, high-

52 sucrose diet (*23-29*). Clinical trials testing TRE have noted high adherence rates and improvements
53 in cardiometabolic health, altogether demonstrating its potential as a feasible and potentially
54 effective intervention to enhance human healthspan (*30-32*). However, few studies have examined
55 the benefits of TRF/TRE under non-obese conditions (*33-35*), and even fewer have compared
56 impacts between the sexes (*36*). The long-term impact of TRF on healthspan and lifespan in
57 mammals also remains unclear (*37, 38*). In this study, we sought to address these key knowledge
58 gaps and determine whether TRF could serve as an effective aging intervention for healthy males
59 and females.

60

61 **Results:**

62 **Early onset circadian aligned TRF has sex-specific impacts on longitudinal feeding and**
63 **wheel-running activity levels**

64 To determine how time-restricted feeding (TRF) impacts healthspan and lifespan in mammals, we
65 individually housed 264 female and 264 male C57BL/6J mice in wheel cages with automated
66 feeders at 2 months of age. To measure impacts under normal nutritional conditions, mice were
67 fed purified precision pellets (Bioserv F0075). For the first 8-weeks, all mice were fed *ad libitum*
68 (free-feeding; AL) with access to a maximum of 22 pellets or 6.6g (23.8 Kcal) per day. At 4 months
69 of age, N=78/sex were switched to a 12-hour (h) TRF window (Zeitgeber time (ZT) 12-24) and
70 another N=78/sex to an 8h-TRF window (ZT14-22) both circadian aligned to the night, the active
71 phase of nocturnal rodents. The remaining N=108/sex were maintained AL to serve as the control.
72 Across all feeding groups mice had access to the same amount of food, so as not to impose caloric
73 restriction (CR), and were maintained on these regimens for life (Fig. 1A and Fig. S1) (*39*).

Measurements of daily food intake throughout each mouse's lifespan showed that feeding increased over time in both sexes, then declined near the end of life in males, similar to a previous study (Fig. 1, A and B, and Fig. S2-3) (*39*). In line with other longitudinal recordings (*40*), females in our study consumed more food relative to their body weight at older ages than male. We also observed sex-specific responses to TRF where 12h-TRF females consumed the same amount as AL, while 12h-TRF males significantly reduced intake compared to AL by 8-14% from 140-539 days (~5-18 months) (two-way ANOVA: Tukey's post-hoc, $P \leq 0.05$). In contrast, 8h-TRF self-imposed CR for the majority of life in both sexes, with intake in females reduced by 10-22% between 140-728 days (~5-24 months) and males reduced by 9-23% between 140-980 days (~5-33 months) relative to sex- and age-matched AL controls. This 8h-TRF schedule, which narrows the daily eating window without explicitly limiting calorie intake, as seen in clinical trials (*41*), presents an alternative strategy for achieving long-term CR in both sexes.

Analysis of daily wheel-running recordings for each mouse revealed that individuals in all feeding groups, of both sexes, consistently displayed nocturnal activity, which subsequently declined with age, as anticipated (Fig. 1, A and C and Figs. S4-5) (*42, 43*). Only 8h-TRF males had increased levels of wheel activity from 224-833 days of age (~7-28 months) with the enhanced activity ranging +36-82% from 329-833 days (~11-28 months) relative to AL (two-way ANOVA: Tukey's post-hoc, $P \leq 0.05$). This male-specific difference further underscores the sex-specific effects of TRF on behavior. Additionally, in line with previous work (*10, 44*), the sustained increases in activity under 8h-TRF suggest a healthspan-enhancing effect in males.

95 **TRF improves the amplitude of daily rhythmic behaviors**

96 We next examined age-related changes in the diurnal profiles of feeding and wheel-running, two
97 well-established rhythmic behaviors, as high-amplitude behavioral rhythms are closely associated
98 with improved health outcomes (Fig. 2, A and B) (*18, 19, 45, 46*). Across all groups, food intake
99 became consolidated to the night, from 6 to 24 months of age. Both mean and median fasting
100 durations, which account for daytime fasting and the short intervals between pellets during the
101 feeding phase, declined with the most significant consolidation observed in the 8h-TRF group (Fig.
102 2, A, C, and D). A Fast Fourier Transform (FFT) analysis of diurnal amplitude, reflecting the
103 strength of daily rhythms, unsurprisingly confirmed that TRF increased feeding amplitude and
104 lead to a preservation of enhanced rhythmicity with age, particularly in the 8h-TRF group. (Fig. 2,
105 A and E). While the diurnal amplitude of wheel-running declined with age across all groups, 8h-
106 TRF males maintained stronger rhythms throughout life compared to controls (Fig. 2, B and F).
107 Females in both TRF groups also showed modest increases in wheel-running amplitude, with 8h-
108 TRF sustaining enhanced rhythms relative to controls from 350 days of age (~12 months) through
109 the end of life. Together, these findings highlight that distinct behavioral rhythms, such as feeding
110 and activity, are differentially affected by aging.

111

112 **TRF reduces age-related gains in body weight and fat**

113 Body weights were recorded every 21 days (Fig. 3, A and B, and Fig. S6), and body composition
114 was analyzed every 6 months using nuclear magnetic resonance imaging (EchoMRI, Houston, TX)
115 (Fig. 3, C and D). In females, 12h-TRF without CR still weakened age-related gains in body weight
116 by 5-8% from 350-686 days of age (~12-23 months) (two-way ANOVA: Tukey's post-hoc, $P \leq$

117 0.05). This feeding window also improved fat vs lean body composition, reducing fat mass while
118 increasing lean mass by 3–4% at 12 and 18 months of age. Unexpectedly, 8h-TRF females with
119 self-imposed CR did not exhibit any further improvements beyond 12h-TRF, in weight or fat/lean
120 composition. In males, 12h-TRF reduced body weight gain by 5-7% from 224-476 days (~7-16
121 months) and improved fat vs lean composition by 3% at 12 months. 8h-TRF in males enhanced
122 these benefits, lowering body weight gain by 5-16% from 161-749 days (~5-25 months) and
123 improving fat vs lean composition by 3-7% from 6-12 months. Overall, this shows that 12h-TRF
124 is sufficient to attenuate body weight gain and improve body composition in both sexes, with
125 longer-term benefits in 12h-TRF females, but that 8h-TRF with long-term CR provides enhanced
126 benefits for males.

127

128 **TRF slows age-related increases in frailty index values**

129 Frailty, a widely used measure of healthspan in aging mice, integrates 31 physiological and
130 behavioral parameters into a composite health index (*47*). Frailty assessments were conducted
131 every 6 months and showed that TRF significantly reduced frailty index scores, with varying
132 impacts between the two feeding windows (Fig. 4, A-C, and Figs. S7-8). In females, 12h-TRF
133 significantly reduced frailty index scores at 18 months, with hearing loss and body condition
134 assessments defining the key differences between the two groups. 8h-TRF further extended
135 reductions in frailty from 12 to 24 months, with improvements observed in grimace, piloerection,
136 forelimb grip strength, coat condition, ulcerative dermatitis, and body condition scores. Males
137 showed similar trends: 12h-TRF reduced frailty indices between 12 and 18 months, primarily
138 improving grimace and body condition, while 8h-TRF sustained these benefits through 24 months
139 and improved additional parameters, including grimace, hearing, piloerection, menace reflex, fur

140 color loss, coat condition, and body condition. Although both TRF regimens reduced frailty,
141 supporting the intervention's effectiveness in extending healthspan, the responses were dose-
142 dependent to the degree of TRF, with 8h-TRF providing the most prolonged benefits and
143 improving the greatest number of health measures.

144

145 **The age-related onset of health reports reveals sex-specific differences and a delayed onset**
146 **in 8h-TRF males**

147 As mice developed health issues they were reported to veterinary staff for more frequent
148 monitoring. These reports revealed that TRF did not reduce the relative frequency of commonly
149 reported ailments compared to AL (Fig. 4D). In fact, 8h-TRF males has significantly more reports
150 of body condition scores declining to 2 (Scored 1-5 with: 1 being emaciated, 3 ideal condition, and
151 5 obese) (Fisher's exact test 8h-TRF vs AL: Body Condition Score=2, $P < 0.05$). However, we
152 observed a clear sex-dependent pattern in the types and frequencies of reported conditions.
153 Females, regardless of feeding group, were significantly more likely to be reported for ulcerative
154 dermatitis (Fisher's exact test females vs males: $P < 0.0001$), rectal prolapse ($P < 0.0001$), and
155 hind limb paralysis (often associated with spinal tumors, $P < 0.001$). In contrast, males were more
156 frequently reported for external tumors and abdominal masses (Fisher's exact test females vs
157 males: $P < 0.0001$), as well as Body Condition Score=2 ($P < 0.0001$). The onset of age-related
158 health issues also differed by sex, with 8h-TRF delaying the median health report onset by 52 days
159 in males compared to AL controls (Log-rank Mantel-Cox, $P = 0.0018$) (Fig. 4E). Additionally,
160 while there were no significant differences under TRF, overall, females spent significantly fewer
161 days on health report until death than males (two-way ANOVA: feeding, $P > 0.05$; sex, $P < 0.01$;

interaction, P > 0.05) (Fig. 4F). Altogether this demonstrates there is sex-specificity in the development of and resistance to disease.

8h-TRF extends median and maximal lifespan in males

For the first time, we were able to investigate the impact circadian aligned TRF with regular chow diet, on lifespan in mice (Fig. 5A, and Tables S1-3). In females, 12h-TRF alone did not extend median lifespan, with females surviving to a median of 715 days compared to 694 days in AL. However, 8h-TRF with self-imposed CR resulted in a ~5% increase in median lifespan to 729 days (Fisher's exact test, P = 0.0442), though overall survival curves showed no significant difference. In males, 12h-TRF also failed to extend lifespan, with a median survival of 839 days compared to 818 days in AL, a finding now reproduced across two independent experiments (Fig. S9). Interestingly, 8h-TRF with lifelong self-imposed CR, significantly improved overall survival for males (Log-rank Mantel-Cox, P = 0.001). Median lifespan increased by approximately 12%, reaching 916 days (Fisher's exact test, P = 0.0019), and maximal lifespan was prolonged by about 3% (Fisher's exact test, P = 0.0038). Though TRF is beneficial for improving health in both sexes, its capacity to extend lifespan appears to be feeding window- and sex-dependent.

Sex-dependent differences in disease types and incidence at death

After death each mouse underwent gross necropsy and histopathological analysis by a veterinary pathologist to determine the types of diseases that contributed to its debilitation (Fig. 5B, and Tables S4-6). As shown previously (*10*), the types and relative frequency of diseases were similar for males across all feeding conditions with the most common cause of death or debilitation being

184 neoplasia. Along with the delayed onset of health reports, this supports that rather than preventing
185 disease, 8h-TRF delays the development of age-related diseases in males. TRF did not reduce the
186 overall occurrence of disease in females. However, females developed neoplasms far less
187 frequently than males (Fisher's exact tests females vs males: Adenoma in liver, $P < 0.05$;
188 Carcinoma in liver and lung, each $P < 0.0001$; Histiocytic sarcoma in liver and lung, each $P <$
189 0.0001; Histiocytic sarcoma in spleen, $P < 0.001$; Acidophilic macrophage pneumonia in lung, P
190 < 0.0001; Adenoma in lung, $P < 0.0001$). Instead, most females were debilitated by
191 glomerulonephritis in the kidneys and had a higher incidence of lymphoma than males (Fisher's
192 exact tests females vs males: Lymphoma in liver, kidney, spleen, each $P < 0.05$;
193 Glomerulonephritis, $P < 0.0001$). These results have revealed pronounced differences in neoplasia
194 and development of other age-related morbidities between the sexes.

195

196 **Sex-dependent strategies for achieving a healthy lifespan**

197 Identifying biomarkers of health that correlate with lifespan is crucial both for understanding the
198 aging process and for evaluating the effectiveness of interventions. Here, we correlated 13 health
199 measures recorded throughout each mouse's life, including measures of behavior, body
200 physiology, frailty, and disease (Fig. 5C). In both sexes, higher activity levels and amplitudes
201 consistently correlated with longer lifespan, especially at older ages. Although voluntary wheel
202 running does not extend lifespan (*44*), these findings support activity as a valuable healthspan
203 biomarker (*10*).

204 In females, lower food consumption and amplitude during midlife, but higher values in early life
205 and late life correlated with longevity. Shorter fasting durations and lower maximum fasting times

206 (i.e., longer feeding windows) also showed modest associations. While not formally quantified,
207 we frequently observed a marked consolidation of feeding shortly before death, potentially
208 explaining this trend. Conversely, males tended to correlate longer lifespan with higher feeding
209 amplitude, longer fasting durations, and greater maximum fasting times during early to midlife,
210 with this trend reversing in late life.

211 Body physiology measures also showed sex-specific correlations. In females, lower lean mass and
212 body weight in early-life correlated with longer lifespans, but higher body weight, fat composition,
213 and body condition scores correlated with longevity in late life. Males showed a modest trend for
214 higher fat in late-life as well but reversed patterns for body weight, condition score, and lean mass.
215 The late-life fat retention trend aligns with studies suggesting fat provides protective health
216 benefits in old age (*48*). However, the opposing body mass patterns indicate sex-specific strategies
217 for maintaining healthy physiology where males appear to benefit from limiting weight gain, while
218 females need to maintain higher body weights in late life, to support longevity.

219 Frailty index showed inconsistent correlations with lifespan in females, whereas in males, lower
220 frailty scores consistently associated with longer lifespan (*49*). For both sexes, delayed onset and
221 shorter duration of veterinary health reports correlated with increased longevity, indicating that
222 delayed and reduced disease burden supports extended lifespans.

223

224 **Healthspan is more prolonged in females and can be extended by 12h-TRF**

225 While TRF improved and attenuated age-related changes in several markers of health, we next
226 aimed to quantify both the magnitude and duration of its overall impact by measuring healthspan.
227 Frailty index is commonly used to determine healthspan (*47, 49*), but it cannot incorporate the

228 impact of behavioral changes in wheel-activity and food consumption and can miss important
229 physiological changes in the time between assessments. To generate a continuous healthspan
230 index, we integrated frailty with 12 additional health measures (the same 13 in Fig. 5C) by first
231 normalizing each data set (mean = 0, standard deviation = 1), then scaling the parameter
232 directionally by providing context based on whether higher or lower values reflected better health
233 (e.g., high wheel-running activity was scaled positively, while low frailty scores were inverted and
234 also scaled positively) (Fig. 6A), and finally summing the values across age points (Fig. 6B). With
235 higher values reflecting better health, females not only begin in a healthier state than males during
236 the AL baseline but also cross the subjective thresholds of '0' (red crosshairs) and '–5' (blue
237 crosshairs) 100–200 days later than their male counterparts in the same feeding group. Importantly,
238 we find that in females at '0', 12h-TRF is sufficient to extend healthspan by more than 100 days
239 relative to AL, while in males both AL and 12h-TRF cross this threshold at similar ages. In both
240 sexes 8h-TRF extends healthspan across this threshold by more than 200 days compared to AL.
241 Together, these results suggest that although females do not live longer under TRF, they remain
242 healthier for significantly longer.

243

244 **Discussion:**

245 Previous studies have established that TRF improves circadian rhythms, ameliorates metabolic
246 syndrome, and improves overall health in obesity models consuming a 'Western diet' high in fats
247 and sugars (*25-29*). We expand on these findings by revealing that circadian aligned TRF confers
248 significant, long-term health benefits even under normal nutritional conditions. We show that,
249 depending on the feeding window and sex, TRF prolonged higher amplitudes of daily rhythms in
250 feeding and wheel-running, which are known to feed back on the circadian clock to drive robust

molecular and behavioral oscillations (*14, 15, 17, 50-53*). Although the mice were not obese, the intervention still significantly reduced age-related increases in weight and fat mass. TRF similarly slowed the age-related decline in lean composition, which may also contribute to the observed improvements in adiposity and suggests that TRF can delay the onset of functional disabilities (*54-56*). We also present novel findings that both TRF windows reduce frailty index in females and males, as in CR and intermittent fasting studies (*57-61*). Altogether, this demonstrates that TRF does not merely act as a remedial strategy to 'rescue' poor health but can actively improve behavioral and physiological markers of health under normal metabolic aging conditions.

Our findings further suggest that, when the health measures recorded throughout the experiment are integrated into a comprehensive healthspan index, females are overall healthier than males. Moreover, timing alone through 12h-TRF is sufficient to extend healthspan in females, but only weakly so in males. Although 8h-TRF more noticeably improved healthspan in males, the benefits remained substantially greater in females. However, this approach may oversimplify sex-specific differences, as males and females exhibit distinct patterns of health deficits that correlate differently with longevity. Consequently, a single healthspan scale may not adequately reflect these complex, sex-dependent impacts on aging. Additionally, although females generally show better health scores, we found they had shorter lifespans than males, highlighting a potential disconnect between this healthspan index and ultimate survival outcomes. Regardless, this sex-specific response in healthspan likely reflects a combination of biological factors. Estrogen is known to confer broad protective effects on health and aging (*62*), which may explain the overall female advantage even under AL feeding, and is known to feedback onto the circadian clock (*63-65*). With recent literature showing that females possess a more robust circadian system (*66*), this may underlie their enhanced responsiveness to circadian-based interventions. Indeed, studies have

274 found that females express a greater number of rhythmic genes, display higher amplitude rhythms,
275 and are more sensitive to external cues (*67-69*). As such, TRF may more effectively enhance
276 rhythms at the molecular level to promote health in females (*18, 19*).

277 For the first time, we show that aligning feeding to the active phase under 12h-TRF does not extend
278 lifespan. However, a narrower feeding window of 8h-TRF along with self-induced CR conferred
279 marginal or strong lifespan extension in females and males, respectively. These results contrast
280 with previous studies showing that lifelong 20% CR, adjusted relative to age-related changes in
281 AL feeding, extends lifespan by 40.6% in female and 24.4% in male C57BL/6J mice (*70*). While
282 our 8h-TRF mice maintained ~20% CR for nearly half of their lifespan, both males and females
283 gradually lessened the degree of CR relative to AL feeding shortly before reaching median
284 lifespan. Despite this, 8h-TRF males gained a 12% extension in median lifespan, indicating that
285 even a lower degree of CR is still impactful in males. In contrast, females appear to require a
286 sustained degree of CR ≥20% to see lifespan benefits. Alternatively, the duration of fasting may
287 be more crucial for female lifespan, as longer fasting periods with CR have enhanced lifespan
288 effects in females (*8, 70*), but not in males (*10*). The mechanisms underlying these sex-dependent
289 differences in response to CR and TRF are not fully understood, especially between different
290 strains of mice (*5, 70*), but are likely influenced by a complex interplay of factors such as
291 differences in nutrient sensing and metabolic pathways, DNA repair, and the effects of sex
292 hormones (*36, 66, 71*).

293 Survival curves for AL mice unexpectedly showed an 18% increase in median survival for males
294 compared to females, contrary to the common trend in mammalian studies where females typically
295 live longer due to the genetic advantage of a backup X chromosome and the protective effects of
296 estrogen (*72, 73*). However, in C57BL/6J mice, lifespan outcomes are inconsistent possibly due to

297 genetic drift within the isogenic line as well as environmental variations between experiments (*73-*
298 *76*). While genetic factors may play a role, the shorter lifespan in females could stem from
299 enhanced cold sensitivity and higher thermoregulatory energy demands (*77-80*). The smaller
300 bodied females, needing more energy to maintain body temperature as evidenced by the
301 pronounced increase in food intake with age, may have been adversely affected by the harsher
302 individual housing conditions without nesting material. Although lower body temperatures can
303 extend lifespan (*81, 82*), cold stress can also divert energy away from and impair metabolic,
304 immune, and reproductive functions (*83, 84*).

305 Health report monitoring and post-death histopathological analyses revealed significant
306 differences between the sexes in the progression of age-related diseases, particularly in neoplasia.
307 With the exception of lymphoma, female mice had significantly lower occurrences of neoplasms
308 in the liver, lung, kidney, and spleen which supports a growing body of data that females have
309 greater resistance to cancer (*85-87*). This resistance is largely attributed to sex differences in
310 immunity, mutational burden, and DNA repair, as well as the protective role of X-linked tumor
311 suppressor genes and their interaction with the p53 pathway in females. Females in this cohort
312 were instead most susceptible to kidney failure due to glomerulonephritis, similar to a previous
313 study (*88*). This condition has been linked to menopause in women, with its progression often
314 accelerating after the decline in estrogen levels (*89*). In mice, this disease can be further
315 exacerbated in the absence of the *estrogen receptor alpha* gene (*90*), emphasizing the protective
316 role of estrogen signaling and uncovering renal disease as a potential biomarker of reproductive
317 aging.

318 One limitation of this study was the inability of the automated feeders to retract or block a food
319 pellet that goes uneaten beyond the restricted food intake window. This meant that often, a single

320 pellet would be by the mouse, outside of its 12h or 8h time restricted feeding window.
321 Additionally, some mice had the tendency to hoard pellets within their cage, leading again to food
322 availability outside of the TRF window, a trait that has already been shown in aged mouse
323 populations (*39*). However, a single daytime pellet accounted for ≤10% of total caloric intake, and
324 after subtracting hoarded food, mice consumed ≥90% of the pellets taken from the feeder during
325 the correct feeding windows. These findings, along with evidence that TRF on 5 out of 7 days per
326 week regimens still improves metabolic health (*34*), suggest that moderate TRE adherence may
327 still prolong healthspan and be compatible with modern human lifestyles. Additionally, since our
328 TRF regimens were started in early adulthood and maintained throughout life, it is unclear whether
329 late-life implementation would yield similar benefits. Short-term studies indicate that TRF in the
330 elderly can reduce high-fat diet-induced inflammation and metabolic dysfunction, and studies with
331 CR in aged mice still provided health and lifespan benefits (*91-93*). However, these benefits are
332 not as strong as when started in early adulthood.

333 In conclusion, our study provides compelling evidence that early onset of 12h or 8h of circadian-
334 aligned TRF can significantly improve healthspan, more potently so in females, and that 8h-TRF
335 can extend lifespan in males. While this still warrants further exploration in additional mouse
336 strains and at varying ages, altogether this highlights the potential of TRF as a practical, non-
337 pharmacological intervention to prolong the health quality of human lifespans.

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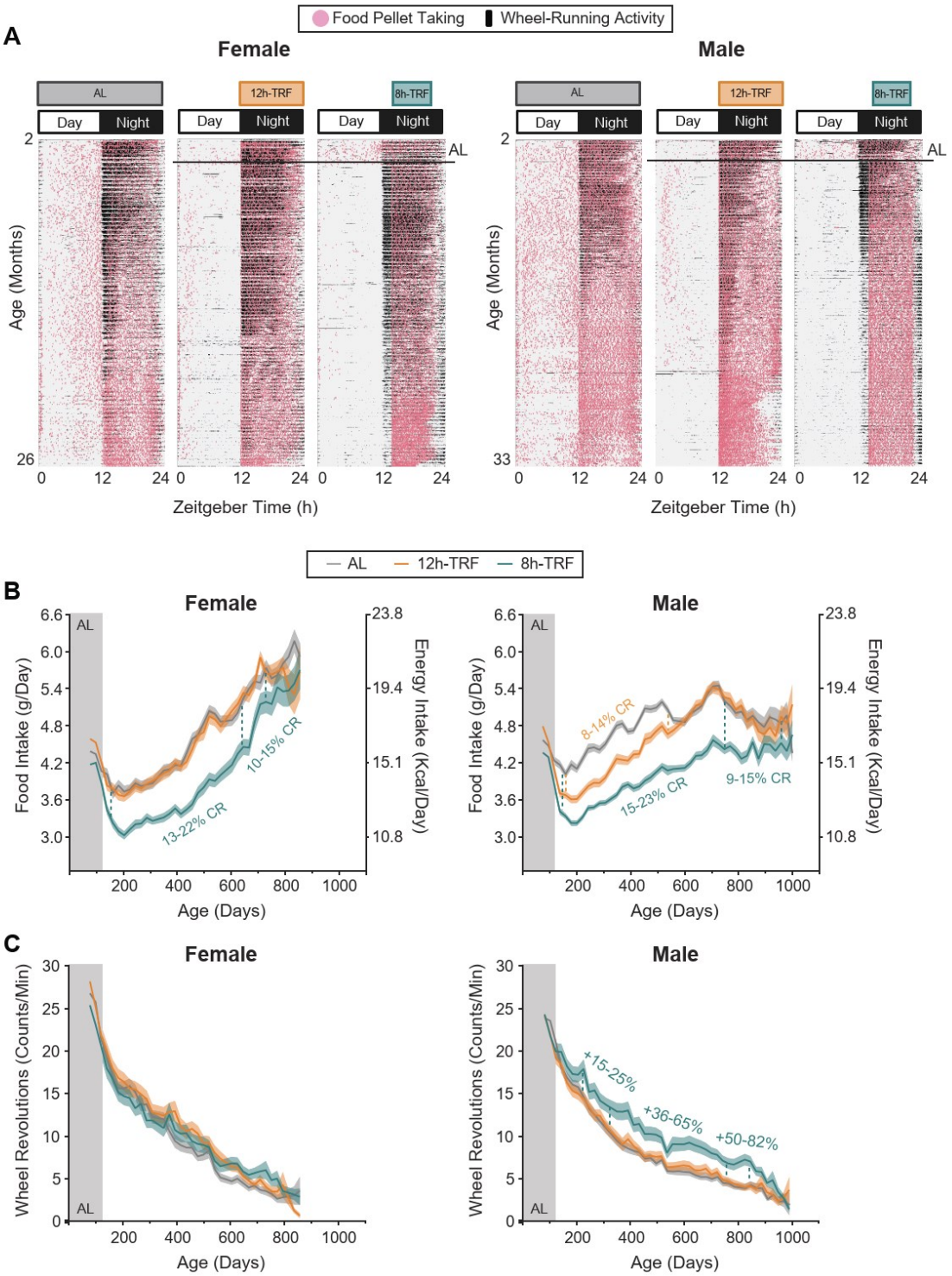
341

342 **Figures and Legends:**

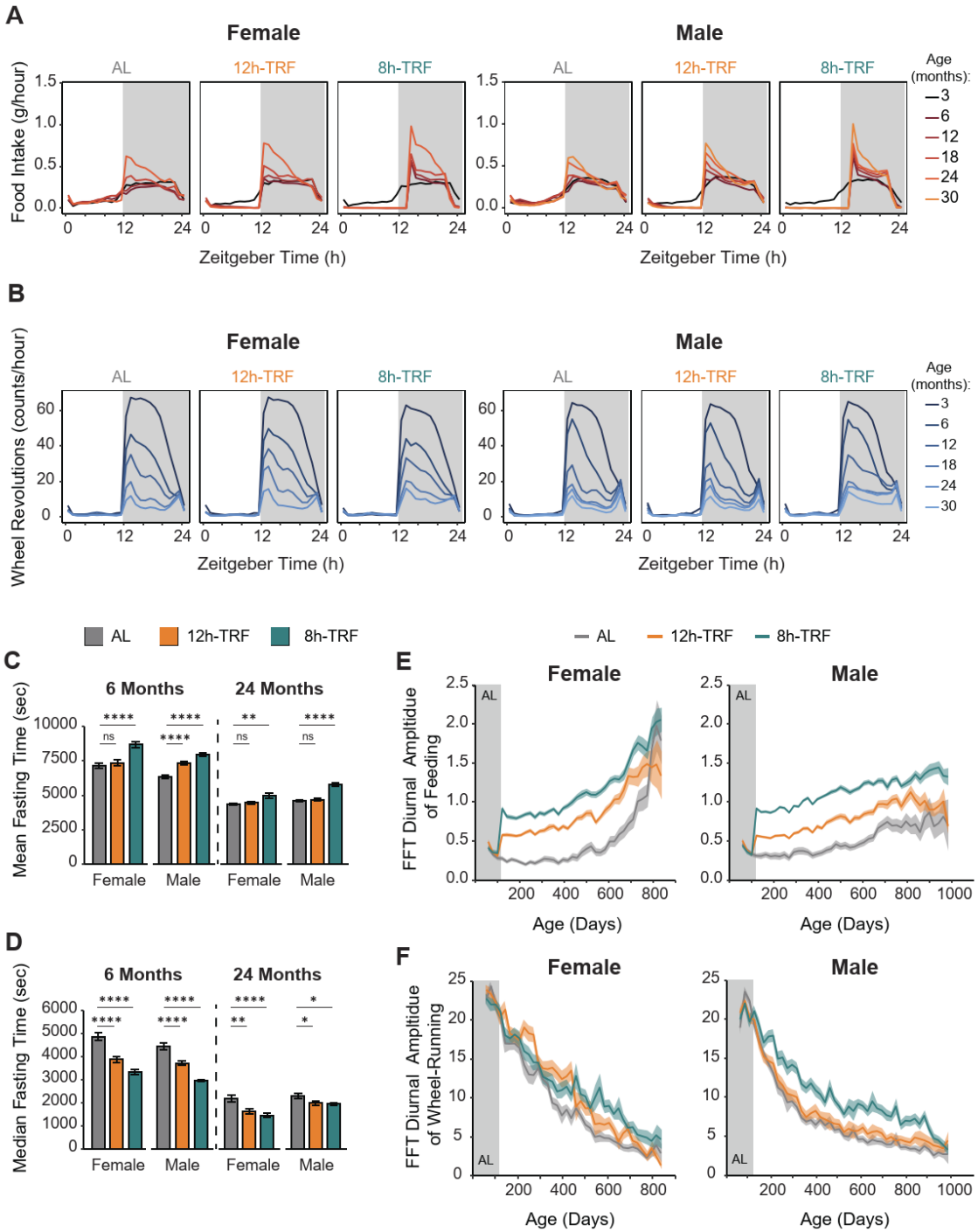


343 **Figure 1: Circadian aligned TRF reduces feeding and improves wheel-running activity in a**

344 **feeding window- and sex-dependent manner.** (**A**) In females 2-26 months of age and males 2-

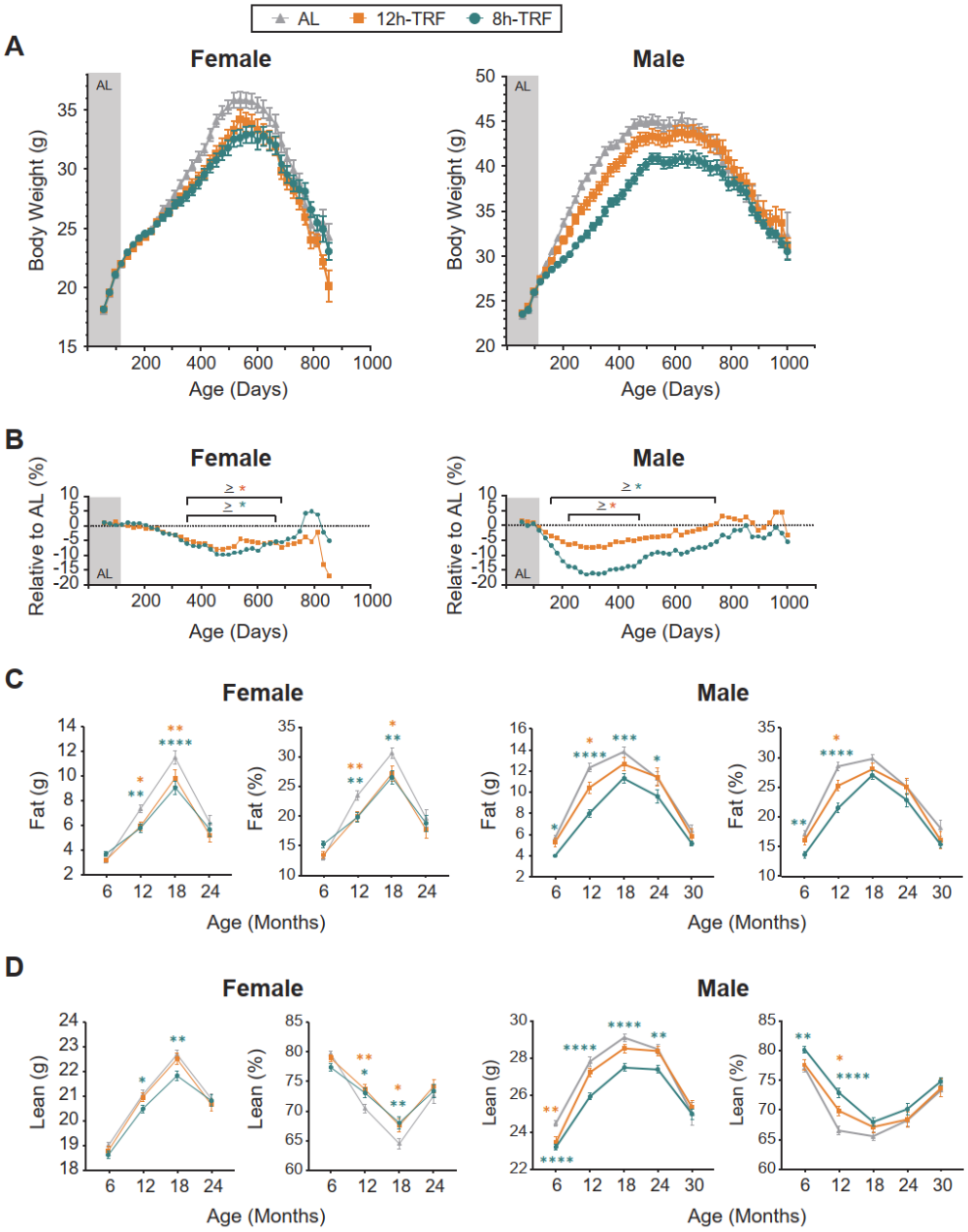
345 33 months of age, representative actograms of individual mice showing wheel-running (black

346 mark) and food pellet taking (pink dot) activity over the course of the 24-hour light:dark cycle.
347 Black line shows ends of *ad libitum* (AL) baseline feeding for the TRF groups at 4 months of age.
348 (**B**) Longitudinal profiles of average daily food intake and energy intake with age. (**C**) Longitudinal
349 profiles of average daily wheel revolutions with age. B and C: AL baseline feeding (Gray area).
350 TRF feeding conditions (White area). Each point represents mean of 21 days ± SEM (shaded
351 regions). Significant differences TRF vs AL shown as % difference ($P < 0.05$) as determined by
352 two-way ANOVA, Tukey's post-hoc. Females N=71-103 per group. Males N=71-97 per group.



353 **Figure 2: TRF improves the amplitude of daily feeding and wheel-running rhythms.** (**A**)
354 Twenty-four-hour profiles of feeding and (**B**) wheel-running starting at 3 and then every 6 months
355 of age. 12h light (white area), 12h dark (gray area). (**C**) Mean and (**D**) median fasting times (sec)
356 throughout the feeding/fasting cycle at 6 and 24 months of age. Data represented as mean ± SEM.

Significant differences TRF vs AL shown with asterisks ($P < 0.05$) as determined by two-way ANOVA and Holm's post-hoc. (**E**) Longitudinal FFT plots of the diurnal amplitude of feeding and (**F**) wheel-running with age. AL baseline feeding (Gray area). TRF feeding conditions (White area). Each point represents mean of 15 days ± SEM (shaded regions). For all panels: Females N=71-103 per group. Males N=71-97 per group.



362 **Figure 3: A 12h TRF window is sufficient to reduce body weight and fat mass in both sexes.**
363 (**A**) Longitudinal profiles of body weight and (**B**) percent differences in body weight relative to
364 AL controls with age. For panels A and B: AL baseline feeding (Gray area). TRF feeding
365 conditions (White area). Group means every 21 days (at cage change) ± SEM (bars). Females
366 N=76-103 per group. Males N=76-102 per group. (**C**) Total fat mass and percentage of body
367 weight with age. (**D**) Total lean mass and percentage of body weight with age. For panels C and

368 D: Group means every 6 months ± SEM (bars). Females 6-24 months: AL, N=43-101. 12h-TRF,
369 N=32-71. 8h-TRF, N=38-72. Males 6-30 months: AL, N=23-102. 12h-TRF, N=19-77. 8h-TRF,
370 N=36-75. For all panels: Significant differences TRF vs AL shown with colored asterisks ($P < 0.05$; Orange=12h-TRF; Teal=8h-TRF) as determined by two-way ANOVA, Tukey's post-hoc.
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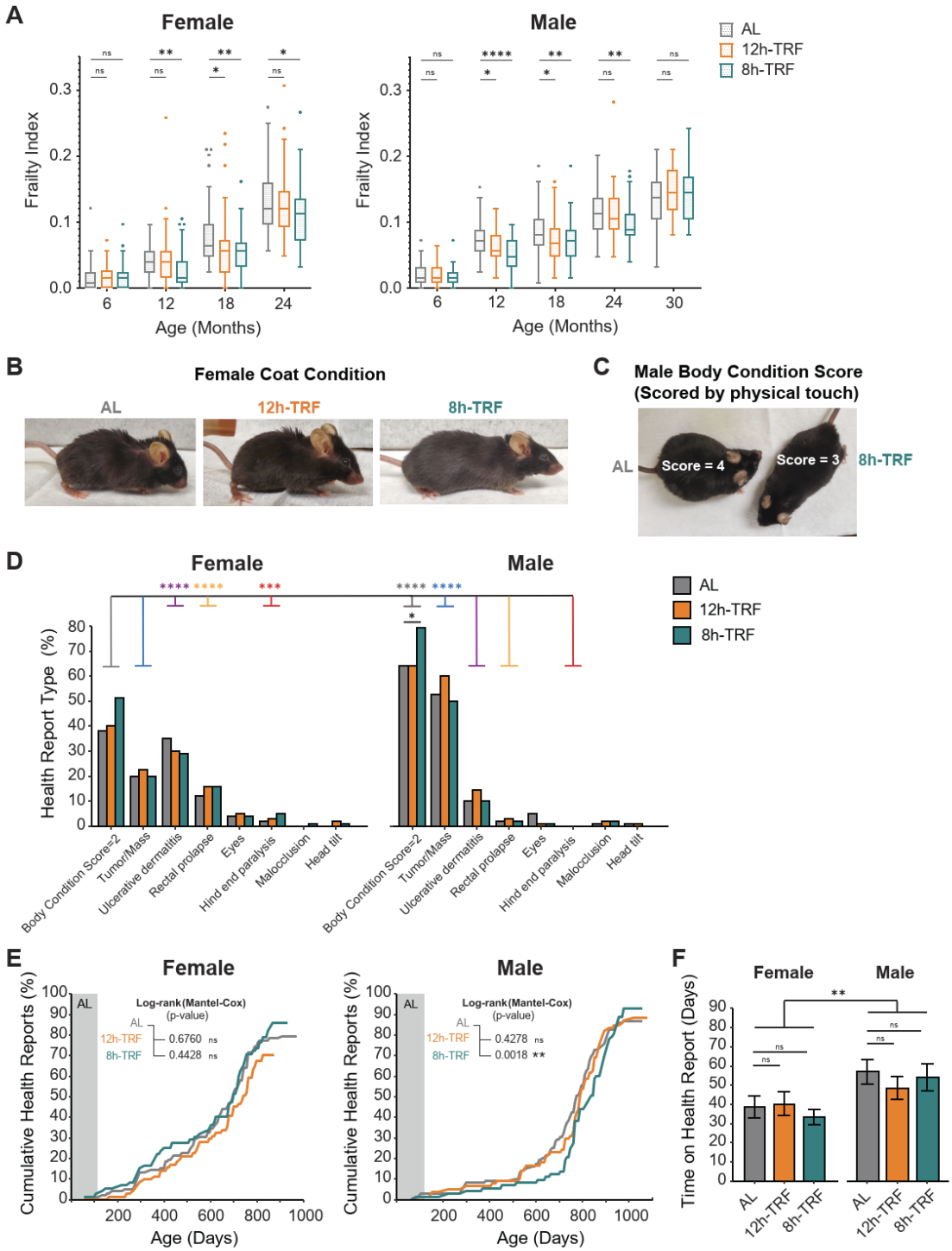

Figure 4: TRF reduces frailty index in both sexes but only delays onset of health reports in males. (**A**) Boxplots of frailty index scores every 6 months. Females: AL, N=44-79. 12h-TRF, N=34-74. 8h-TRF, N=40-74. Males: AL, N=23-79. 12h-TRF, N=22-78. 8h-TRF, N=39-76.

375 Significant differences TRF vs AL shown with asterisks ($P < 0.05$) as determined by two-way
376 ANOVA, Tukey's post-hoc. (**B**) One example parameter contributing to reduced frailty index in
377 8h-TRF females: coat condition, $P < 0.05$ at 24 months compared to AL. (Full list see Fig. S7).
378 (**C**) One example parameter contributing to reduced frailty index in 8h-TRF males: body condition
379 score, $P < 0.05$ 12-18 months compared to AL. (Full list see Fig. S8). (**D**) Percentage of mice in
380 each feeding group and sex reported to veterinary staff for Body Condition Score=2, Tumor/Mass,
381 Ulcerative dermatitis, Rectal prolapses, Eye ulcerations/swelling, Hind end paralysis,
382 Malocclusions, and Head tilts. Significant differences TRF vs AL shown with black asterisk ($P <$
383 0.05; Body Condition Score=2) and females vs males with colored asterisks ($P < 0.05$; Gray= Body
384 Condition Score=2; Blue=Tumor/Mass; Purple=Ulcerative dermatitis; Yellow=Rectal prolapse;
385 and Red=Hind end paralysis) as determined by contingency tables and Fisher's exact tests. (**E**)
386 Cumulative curve showing the percentage of veterinary health reports filed in each group with age.
387 Log-Rank Mantel-Cox Test for significant difference in overall curve TRF vs AL. (**F**) Time spent
388 on health report until death. Data shown as mean ± SEM and significant differences with asterisks
389 ($P<0.05$) as determined by two-way ANOVA, Holm's post-hoc.

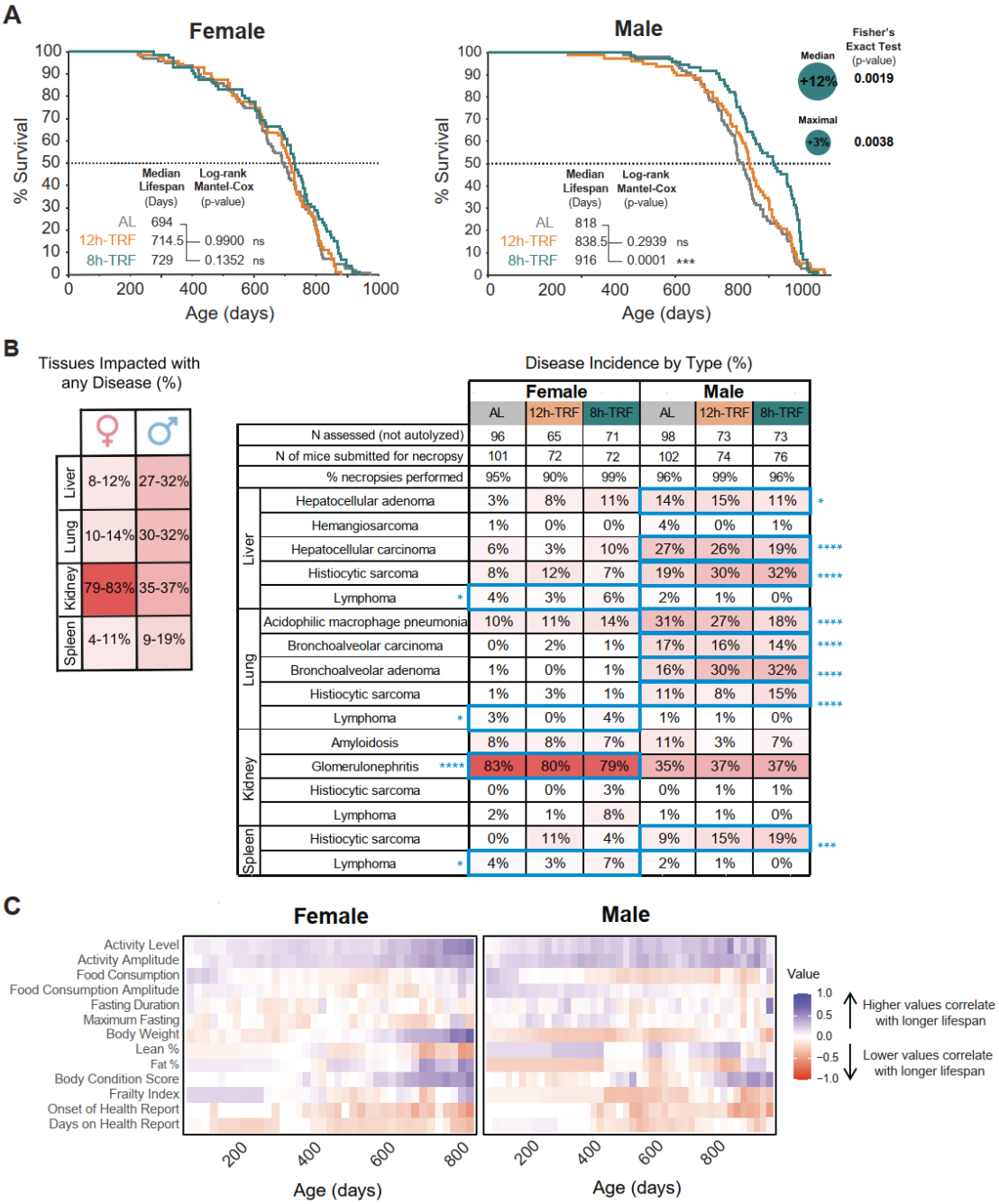

Figure 5: 8h-TRF extends lifespan in males. (**A**) Kaplan-Meier survival curves and day median lifespan reached. Log-Rank Mantel-Cox Test for significant difference in overall survival TRF vs AL. Fisher's exact test for median and maximal survival TRF vs AL. Females N=72-99 per group. Males N=73-99 per group. (**B**) (*Left*) Histopathology results showing the percentage incidence of any disease type in the most impacted tissue types between females and males and (*Right*)

395 incidence by specific disease type within these tissues separated by sex and feeding group (Full
396 table with all tissues and diseases shown in Tables S4-6). Significant differences females vs males
397 highlighted by blue boxes and shown with blue asterisks ($P < 0.05$) as determined by contingency
398 tables and Fisher's exact tests. (**C**) Heatmaps of correlations between lifespan and 13 health
399 measures recorded throughout the experiment. Colors range from blue (parameters where higher
400 values correlate with longer lifespan) to red (parameters where lower values correlate with longer
401 lifespan).

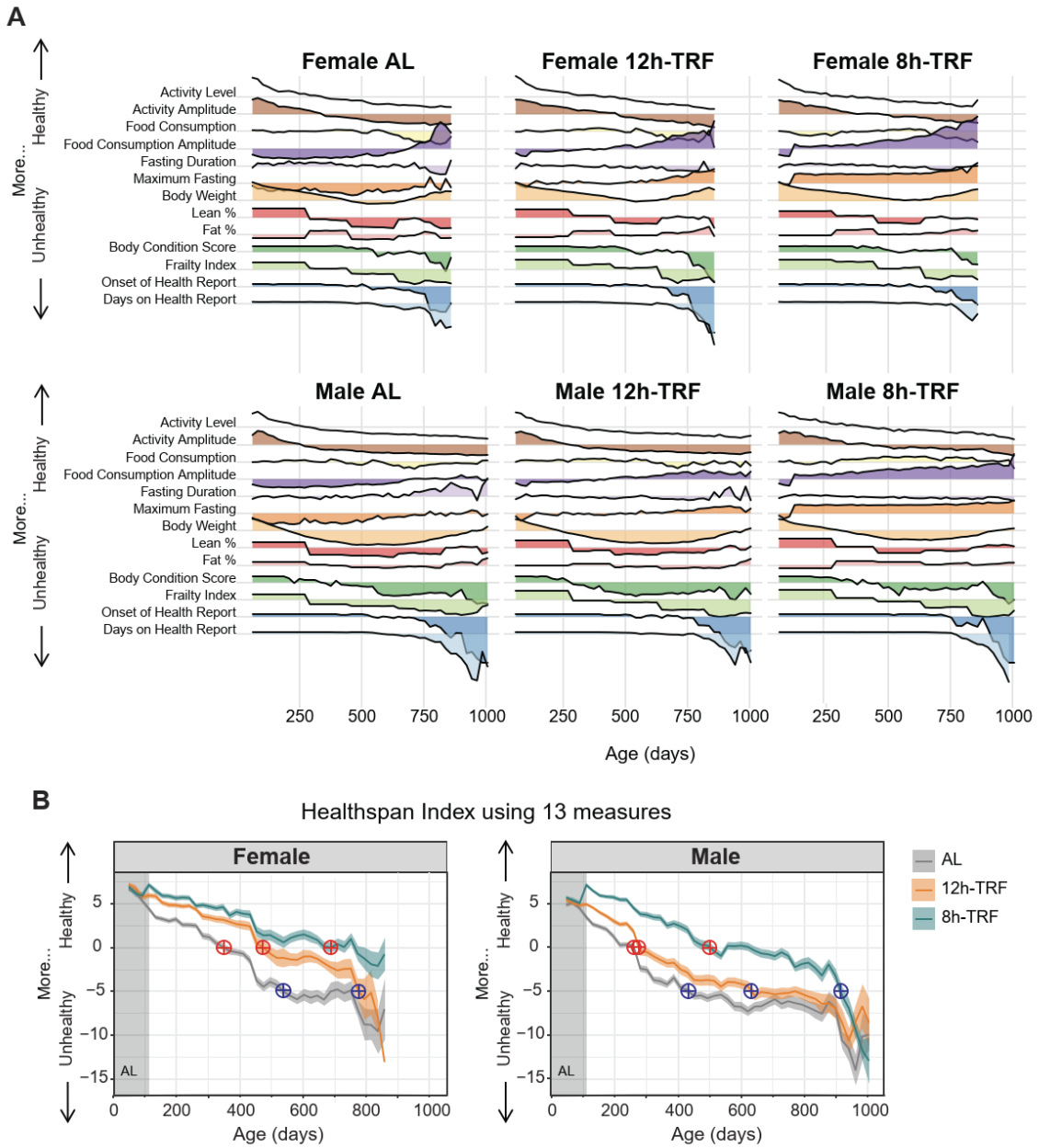

Figure 6: TRF confers greater healthspan benefits to females. (**A**) Ridgeline plots for each of the 13 health measures recorded throughout the experiment. All parameters were standardized (mean = 0, SD = 1) and, where appropriate, oriented in the positive direction to reflect better health. (**B**) Healthspan index summing the 13 health measures for each group from panel A. Higher values indicate better health and lower values poorer health. Red crosshairs mark the age at which each group's index crosses 0; blue crosshairs mark when it crosses –5.

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Author Contributions: SEI, NJS, VAR, CBG, and JST conceptualized the study, developed the experimental design, and oversaw all aspects of project execution and supervision. MW-C performed all histopathological assessments and interpretations. SEI, NJS, and VAR conducted all remaining experimental work, including animal handling, data curation, and statistical analyses. SEI, NJS, VAR, CBG, and JST wrote and revised the manuscript with additional edits from MW-C. Funding acquisition was provided by CBG and JST.

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Data and materials availability: Data are available in the main text or the supplementary materials

Supplementary Materials:

Materials and Methods

Figures S1 to S9

Tables S1 to S6

659 **Materials and Methods:**

660 **Animals and Housing Conditions**

661 264 male and 264 female C57BL/6J mice 6 weeks of age were ordered from Jackson Laboratories,
662 Bar Harbor, ME. As done previously, starting at 2 months of age the mice were: **(i)** individually
663 housed in standard polycarbonate cages with stainless steel running wheels inside isolation
664 cabinets under light:dark (LD) of 12:12 hours, **(ii)** fed 300mg pellets of purified diet (F0075, Bio-
665 Serv) using automated feeders with water provided *ad libitum* (AL), and **(iii)** cage changed every
666 21 days (*9, 10, 94*). The Institutional Animal Care and Use Committee (IACUC) of the University
667 of Texas Southwestern Medical Center approved the animal protocol (APN 2015-100925), which
668 has been renewed every 3 years (in 2018, 2021, and 2024).

669

670 **Time-Restricted Feeding (TRF) Regimens**

671 At 2 months of age, mice were placed in individual cages as described above and fed *ad libitum*
672 (AL) for 8 weeks using the automated feeder system (*9, 10*). At 4 months of age, the mice were
673 divided into three feeding groups for the remainder of the experiment: **(i)** 108 of each sex continued
674 in AL as a control group, **(ii)** 78 of each sex in a 12-hour TRF group where the automated feeder
675 restricted food dispensing to the 12-hour night (12h-TRF, ZT12-24), and **(iii)** 78 of each sex in an
676 8-hour TRF group with food dispensing restricted to the middle 8 hours of the night (8h-TRF,
677 ZT14-22). In addition to the timing of the feeding window, the feeders were also programmed with
678 a 10 min delay between pellet dispensing to reduce hoarding behavior. Regardless of the feeding
679 group, all mice had access to 22 pellets/day so as not to impose caloric restriction.

680

681 For a minimum detection of 10% life extension with 80% power a minimum of 96 animals were
682 needed in the AL group and 72 in each of the TRF groups (*95*). Extra mice were included in each
683 group to account for any that may not adapt to the feeder/diet or for non-aging-related deaths.

684

685 **Daily Monitoring of Feeding and Wheel-Running Activity**

686 As before, the feeders were set and continuously recorded quantity and timing of food intake via
687 ClockLab Chamber Control Software v3.401 (Actimetrics Inc., Wilmette, IL, USA) (*9, 10*).
688 Uneaten food was removed from the bedding every 21 days during cage change, or weekly for
689 chronic hoarders under TRF. The feeding data presented in Figure 1B and Supplemental figures
690 S2 and S3 represent food consumption with hoarding removed. All other feeding figures represent
691 the quantity and pattern of pellet taking from the feeder, not excluding hoarded food. Wheel-
692 running activity was also continuously recorded as before using an updated version of ClockLab
693 Data Acquisition System v3.604 (Actimetrics Inc., Wilmette, IL, USA).

694

695 **Body Weight and Composition Measurements**

696 The body weight of each mouse was measured every 21 days during cage change (morning, ZT4-
697 7) throughout its lifespan. Body composition measurements of lean and fat mass (g) were assessed
698 every 6 months during cage change (ZT4-7) using a 100H-EchoMRI Body Composition Analyzer
699 (EchoMRI, Houston, TX, USA).

700

701 **Frailty Scoring**

702 Every 6 months the mice were assessed for frailty, or physical vulnerabilities, according to 31
703 parameters of aging modified from Whitehead et al. (*47*). These parameters include a range of
704 physiological evaluations, scoring coat condition, ocular and auditory responses, physical and
705 musculoskeletal state, respiration rate, body weight, and temperature. Each assessment was scored
706 either 0=absent, 0.5=mild, or 1=severe. The scores were totaled to calculate an overall index score.

707

708 **Health Monitoring and Survival Study**

709 Mice were physically health checked every 21 days during cage change and visually inspected
710 every 10 days during refilling of the feeders. Using the feeding and wheel monitoring software,
711 mice were also checked virtually every day and only physically health checked if feeding fell <5
712 pellets and/or wheel running fell below set thresholds (1.14 counts/min within 24hr, reduced by
713 10% every 6 months as the mice age) as performed previously (*10*).

714 If any mouse was found to have a mild/moderate health ailment (e.g. dermatitis, external tumor,
715 abdominal mass, head tilt) or a body condition score < 3 (Scored 1-5 with: 1 being emaciated, 3
716 ideal condition, and 5 obese) it was placed on health report. These records were also used to
717 generate Figures 4D-F. Mice on health report were physically health checked weekly by the UT
718 Southwestern Animal Resource Facility (ARC) veterinary staff and twice a week by our own
719 laboratory staff. In order to obtain accurate but humane lifespan data, mice were monitored
720 carefully for euthanasia criteria that would indicate imminent death (moribund) or unacceptable
721 levels of pain (analgesics were avoided to avoid confounding the results). Criteria were determined
722 in conjunction with the ARC veterinarians according to AAALAC guidelines and include: Non-

723 responsive to touch, hypothermic or cold to touch, slow or labored breathing, failure or inability
724 to eat/drink after moist chow is given, a body condition score of 1, body weight loss of >20% from
725 baseline or between cage changes, a broken/fractured/dislocated limb, head tilt that is resulting in
726 an animal being unable to maintain sternal body position which would affect their ability to
727 consume food and water, severe eye protrusion/infection, severe dermatitis, grade 3 rectal or penile
728 prolapse, urinary obstruction, or a subcutaneous tumor >2cm.

729 The number and date of those euthanized or found dead across the feeding groups and sexes were
730 recorded and used to generate the Kaplan-Meier survival curves. A total of 35 animals (35/528)
731 were censored from the survival curve if death occurred earlier than 6 months or due to non-aging
732 related injuries or death: 9 AL females, 6 12h-night TRF females, 6 8h-night TRF females, 9 AL
733 males and 5 8h-night TRF males.

734

735 **Necropsy and Histopathology**

736 Mice euthanized or found dead were submitted for gross necropsy and blinded histopathological
737 analysis was performed, as done previously (*10*). Less than 10% of the animals submitted were
738 too autolyzed to analyze. Disease types were tallied together by tissue type, sex, and feeding group.

739

740 **Health Measures vs Lifespan Correlation**

741 Throughout the experiment we recorded thirteen health measures including: wheel-running
742 activity to determine overall activity level and diurnal amplitude, food consumption to determine
743 overall level, diurnal amplitude, average fasting duration, and maximum fasting times, body

weight, composition of lean and fat mass as a percentage of body weight, body condition score, frailty index, and the onset and duration of veterinary health reports.

Healthspan Index

Healthspan index was calculated by summing the calculated scaled values of each of the thirteen health measures. In the case of body condition score, an absolute difference from BCS=3 was calculated. For body fat percentage, an absolute difference in value from 24.45% was calculated. The value 24.45% was used based on previous literature showing the average body fat percentage across multiple healthy mouse strains at the age of 16 weeks (*96*). All measures were then normalized to a mean of 0 and a standard deviation of 1, within each measure. Certain values were then inverted, given their context, in order to have positive numbers representing a healthier state, and negative values representing an unhealthy state. For example, days_on_hr is inverted, as higher values would be less healthy, and lower values are healthier. All normalized values were then summed together at 21-day intervals.

Statistical Analyses

As performed previously, all feeding and wheel-running activity was collected using Chamber Control and ClockLab, respectively (Actimetrics, Inc) (*10*). Each point on the longitudinal profiles represents a 21-day mean. Twenty-four-hour profiles were binned by hour, with each age point reflecting a 15-day mean (taken from the middle of each 21-day cage cycle to avoid behavioral changes due to cage change stress). For each mouse, each 15-day bin is analyzed by 1-dimensional Discrete Fast Fourier Transform (FFT) using the mixed-radix algorithm for real-valued input

766 (Python function from the scipy library: scipy.fft.rfft) (*97*). The Blackman window function is
767 applied to the data prior to FFT analysis to inhibit spectral leakage (*98*). The power of each
768 frequency component is then normalized, with the total normalized power of all frequency
769 components equaling 100. The normalized power from the 24h frequency component is then taken
770 as the diurnal amplitude of either food intake behavior or running wheel activity. This value
771 represents the percentage of contribution of the 24h frequency component, to the entire 15-day
772 behavioral period. For example, if the diurnal amplitude for food intake is 21, it indicates that 21%
773 of the observed variation in food intake can be directly attributed to the animal's consistent 24-
774 hour daily cycle. The output of this analysis for each mouse was then averaged within group, to
775 provide a group-wise indication of diurnal amplitude.

776 Cumulative health report and survival curves were analyzed using Log-rank Mantel Cox and
777 Fisher's exact test, health report type and necropsy report disease type percentages analyzed by
778 contingency tables and Fisher's exact test and, and all other plots analyzed using two-way ANOVA
779 with Tukey's or Holm's post-hoc analyses (*10, 99*). Data plots were generated and statistically
780 analyzed by Prism 10 (Graphpad Software Inc), Python, and R.

Supplemental Figures and Legends:

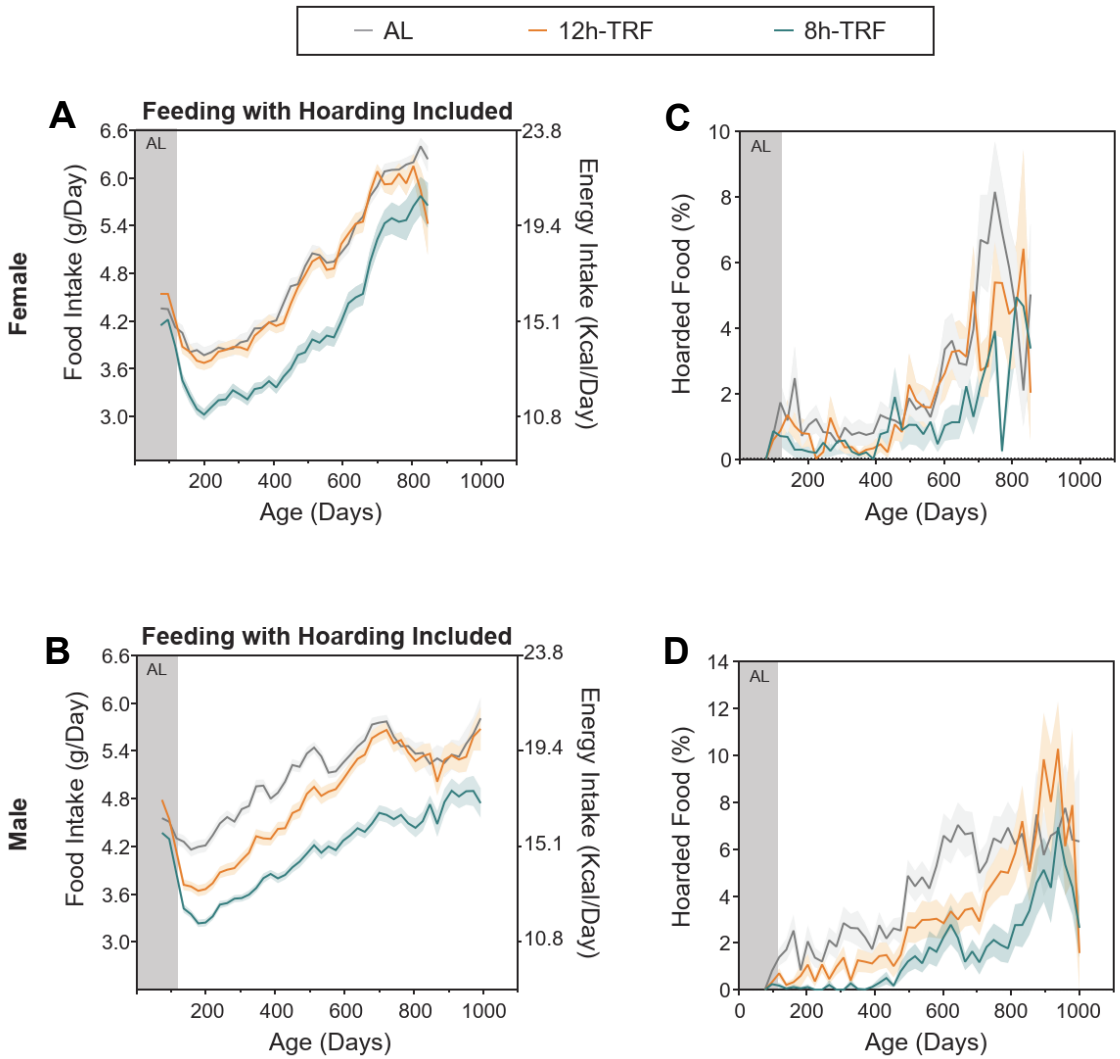


Figure S1: Food taking and hoarding behavior: (A) In females and (B) males, longitudinal profiles of daily food taken from the feeder including uneaten (hoarded) food found in the cage (g/Day) and the equivalent energy intake (Kcal/Day). (C) In females and (D) males, longitudinal profiles of the percent (%) of hoarded food in the cage. Mean of 21 days ± SEM (shaded regions). *Ad libitum* (AL) baseline feeding (Gray area). Time-restricted feeding (TRF) (White area).

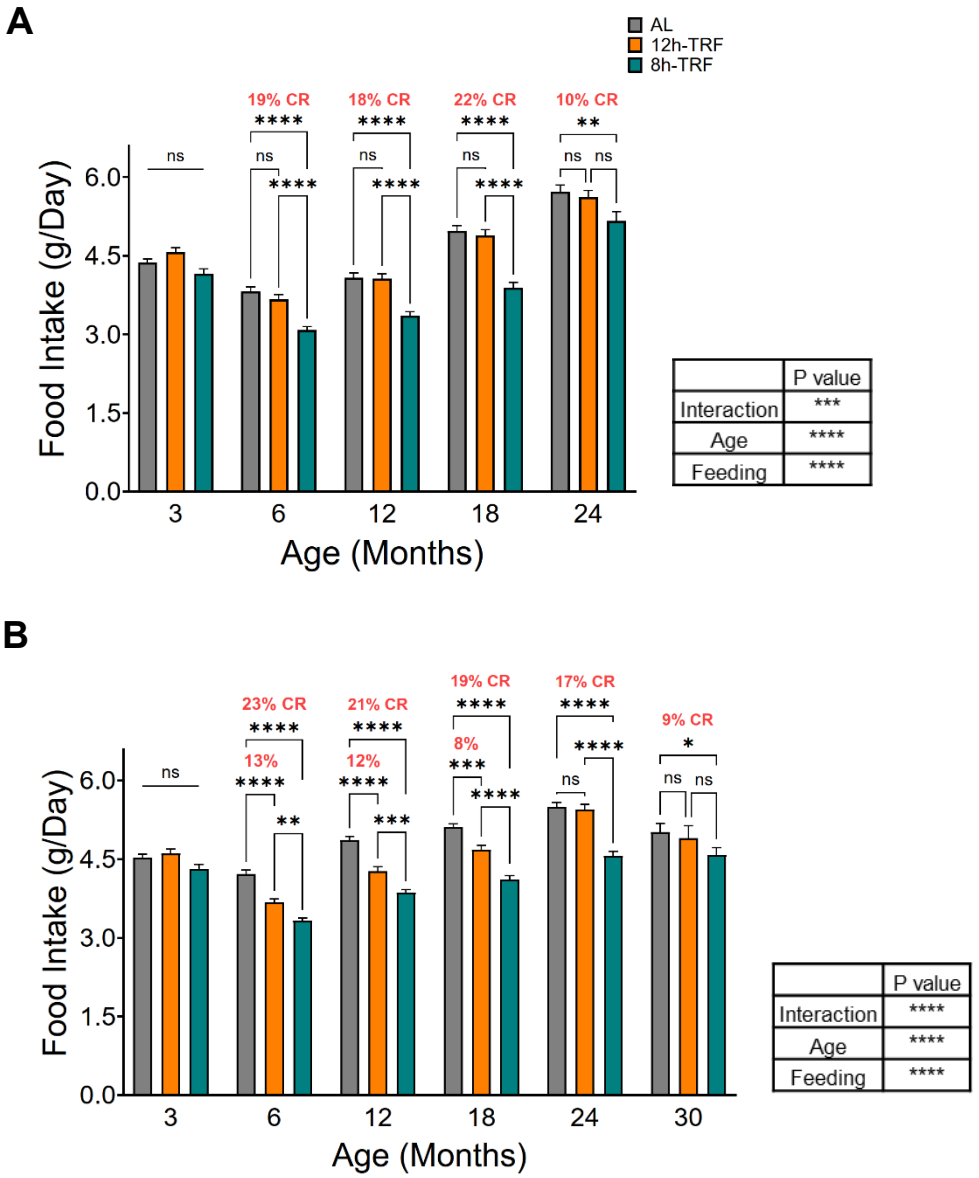


Figure S2: Food intake by monthly age epochs: (A) In females and (B) males, comparison of food intake with hoarding removed (g/Day) at 3 months of age and then every 6 months. Mean of 21 days at each age point ± SEM. Any caloric restriction (CR) relative to the *Ad Lib* control at each age epoch shown as %. Two-way ANOVA, Tukey's post-hoc.

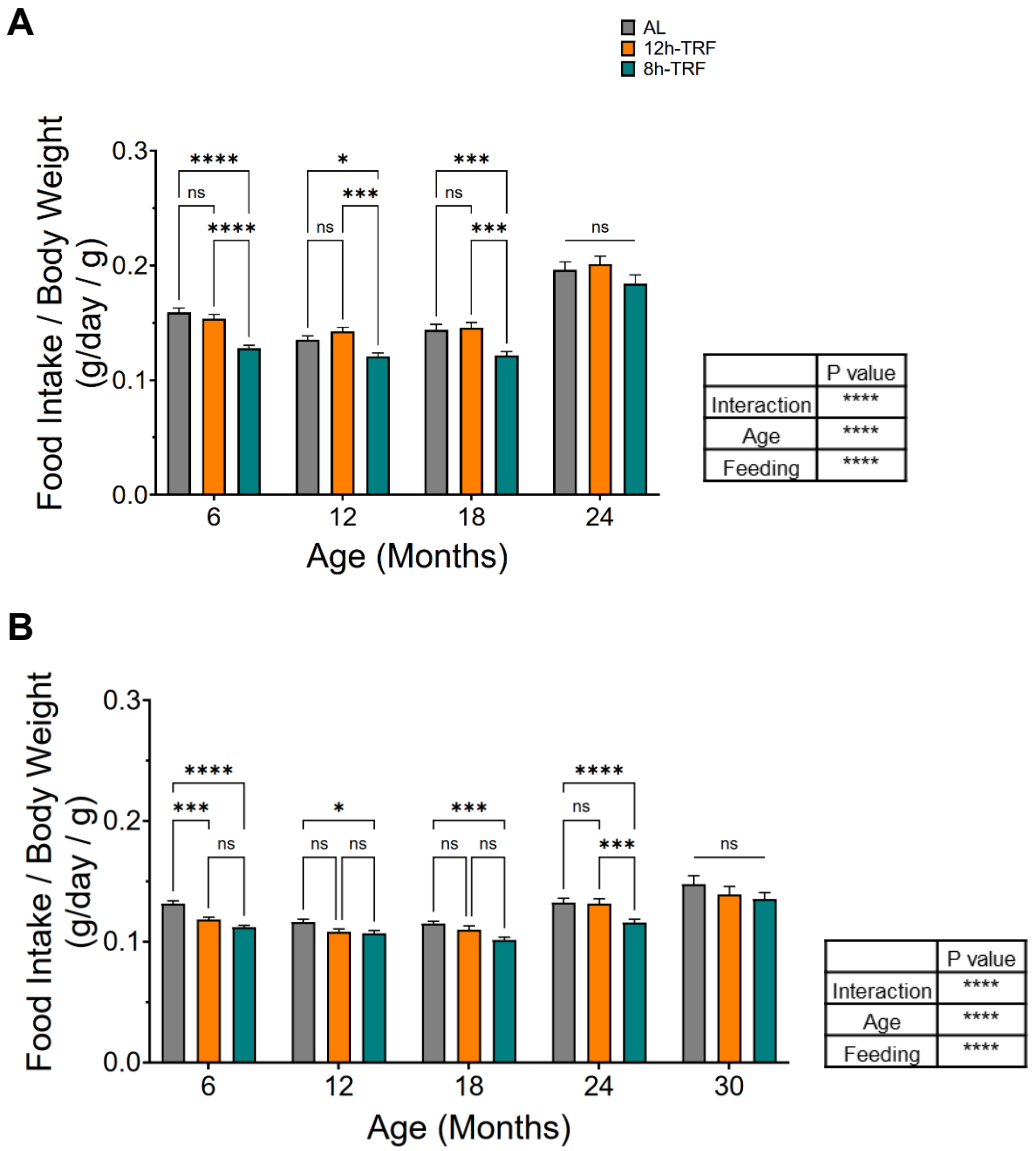


Figure S3: Food intake relative to body weight by monthly age epochs: (A) In females and (B) males, comparison of mean food intake (g/day) with hoarding removed relative to body weight (g) ± SEM at 3 months of age and then every 6 months. Two-way ANOVA, Tukey's post-hoc.

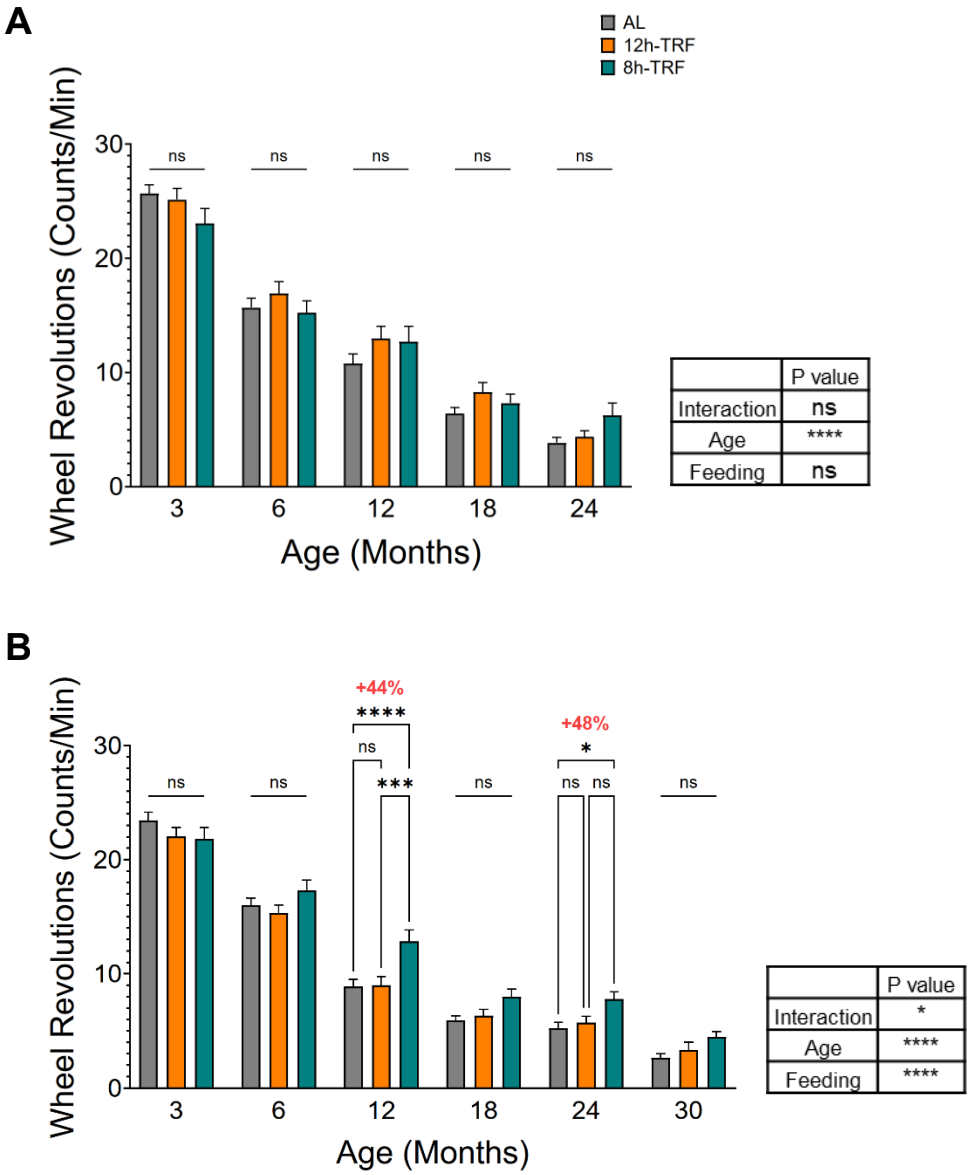


Figure S4: Wheel activity by monthly age epochs: (A) In females and (B) males, comparison of daily wheel revolutions (Counts/Min) at 3 months of age and then every 6 months. Mean of 21 days at each age point ± SEM. Two-way ANOVA, Tukey's post-hoc.

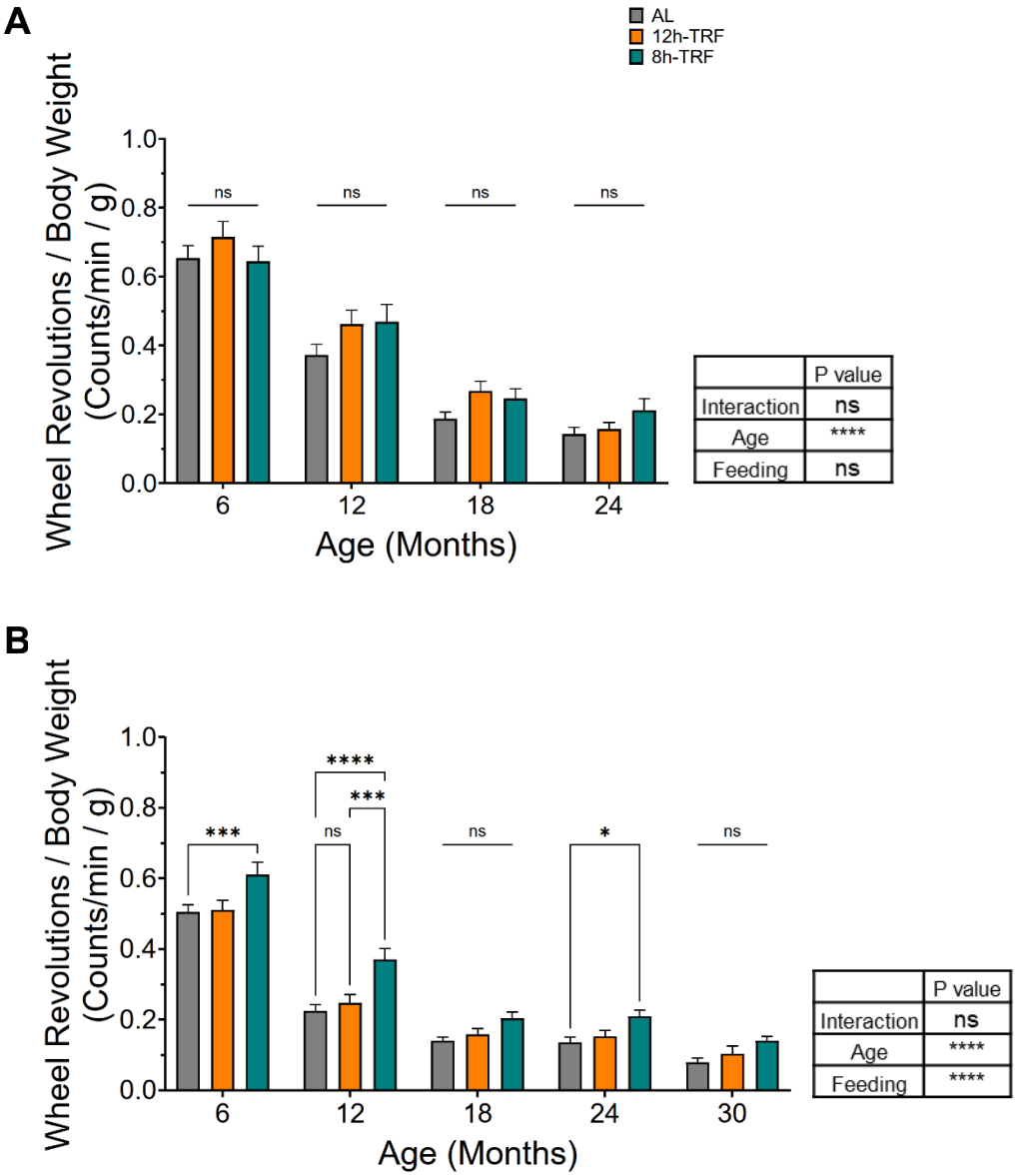


Figure S5: Wheel activity relative to body weight by monthly age epochs: (A) In females and (B) males, comparison of mean wheel revolutions (counts/min) relative to body weight (g) ± SEM at 3 months of age and then every 6 months. Two-way ANOVA, Tukey's post-hoc.

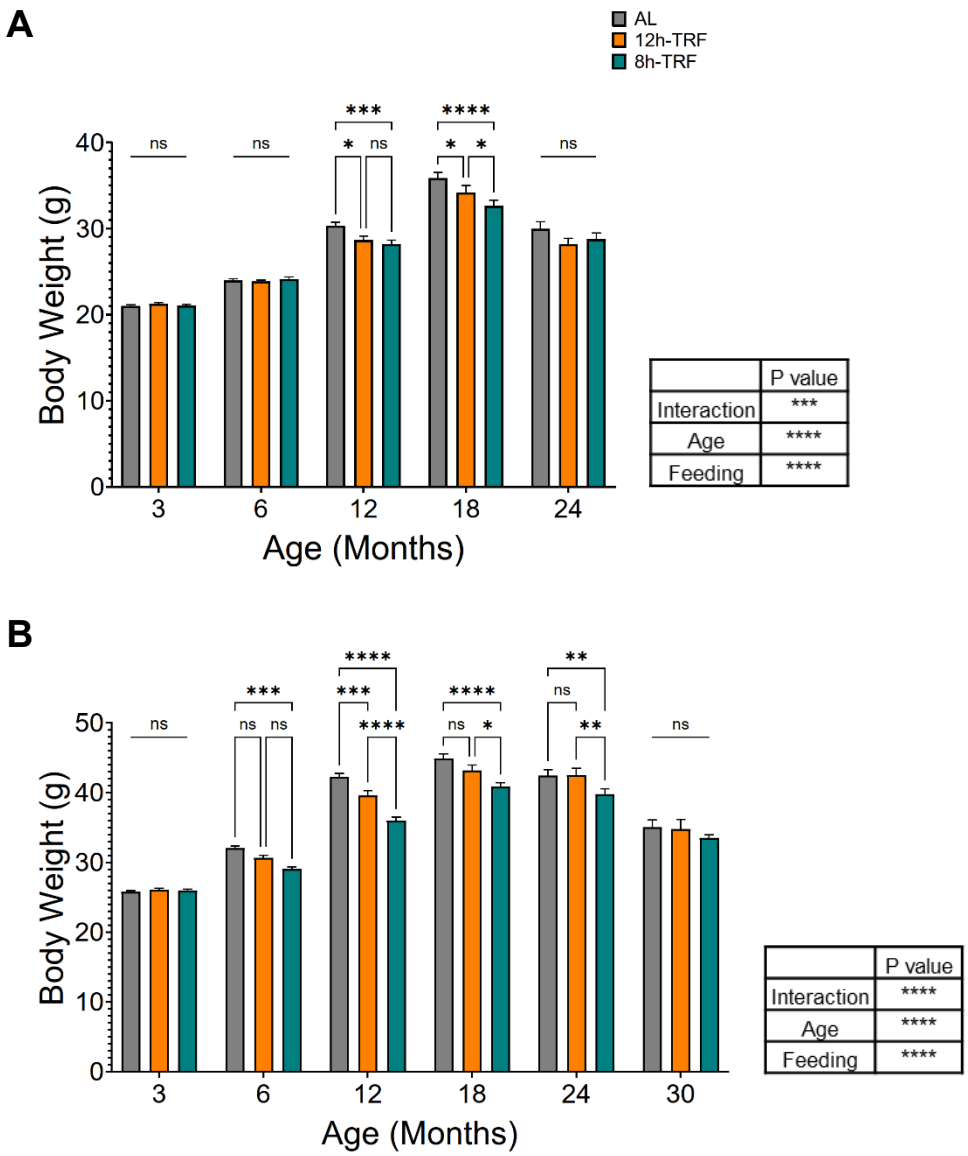


Figure S6: Body weight by monthly age epochs: (A) In females and (B) males, comparison of mean body weight (g) ± SEM at 3 months of age and then every 6 months. Two-way ANOVA, Tukey's post-hoc.

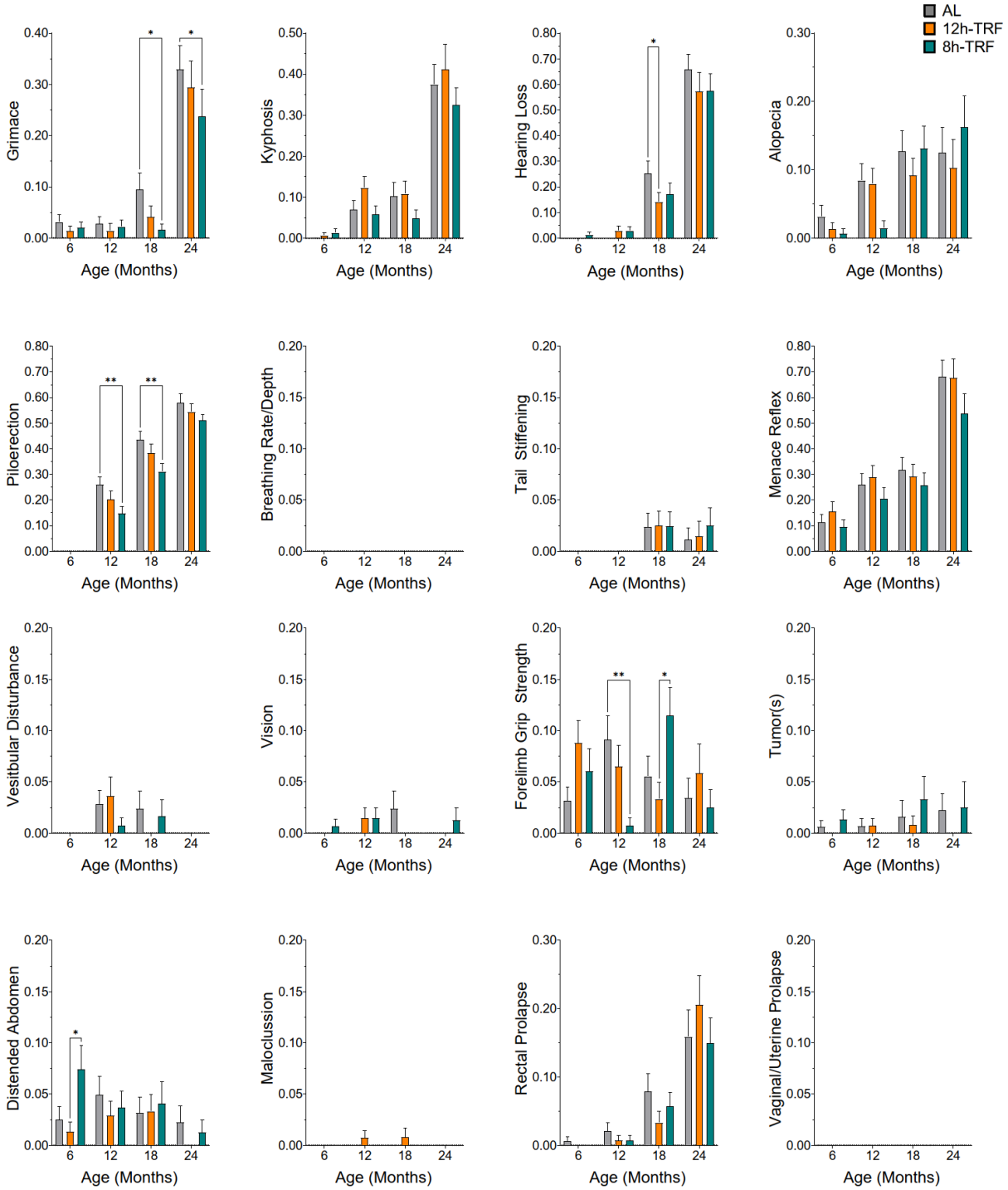
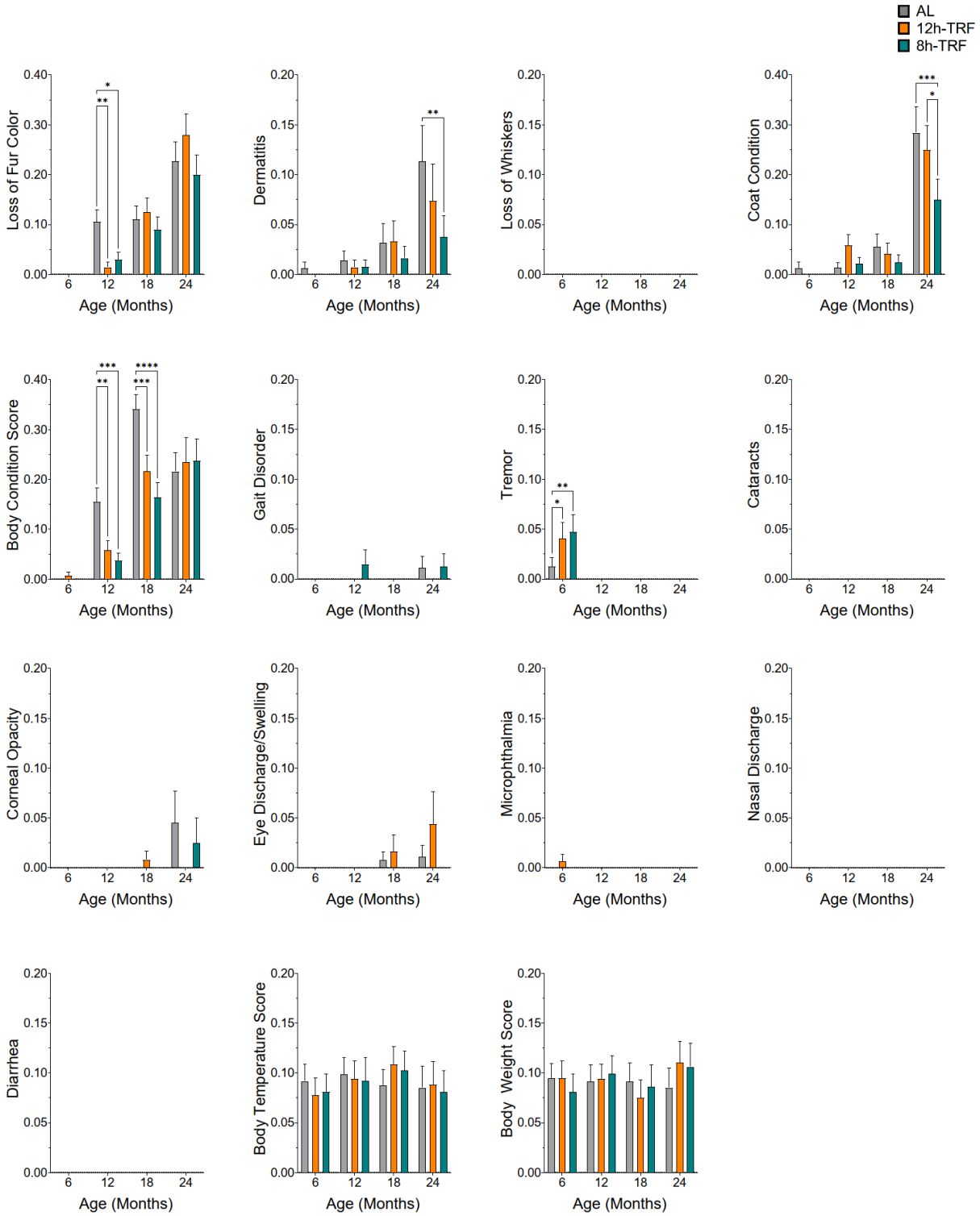


Figure S7: Individual frailty parameter scores in females. Mean frailty scores ± SEM every 6 months of age in females for each of the 31 parameters modified from (*47*). AL, N=44-79. 12h-TRF, N=34-74. 8h-TRF, N=40-74. Two-way ANOVA, Tukey's post-hoc.



806 **Figure S7: Individual frailty parameter scores in females (Continued).**

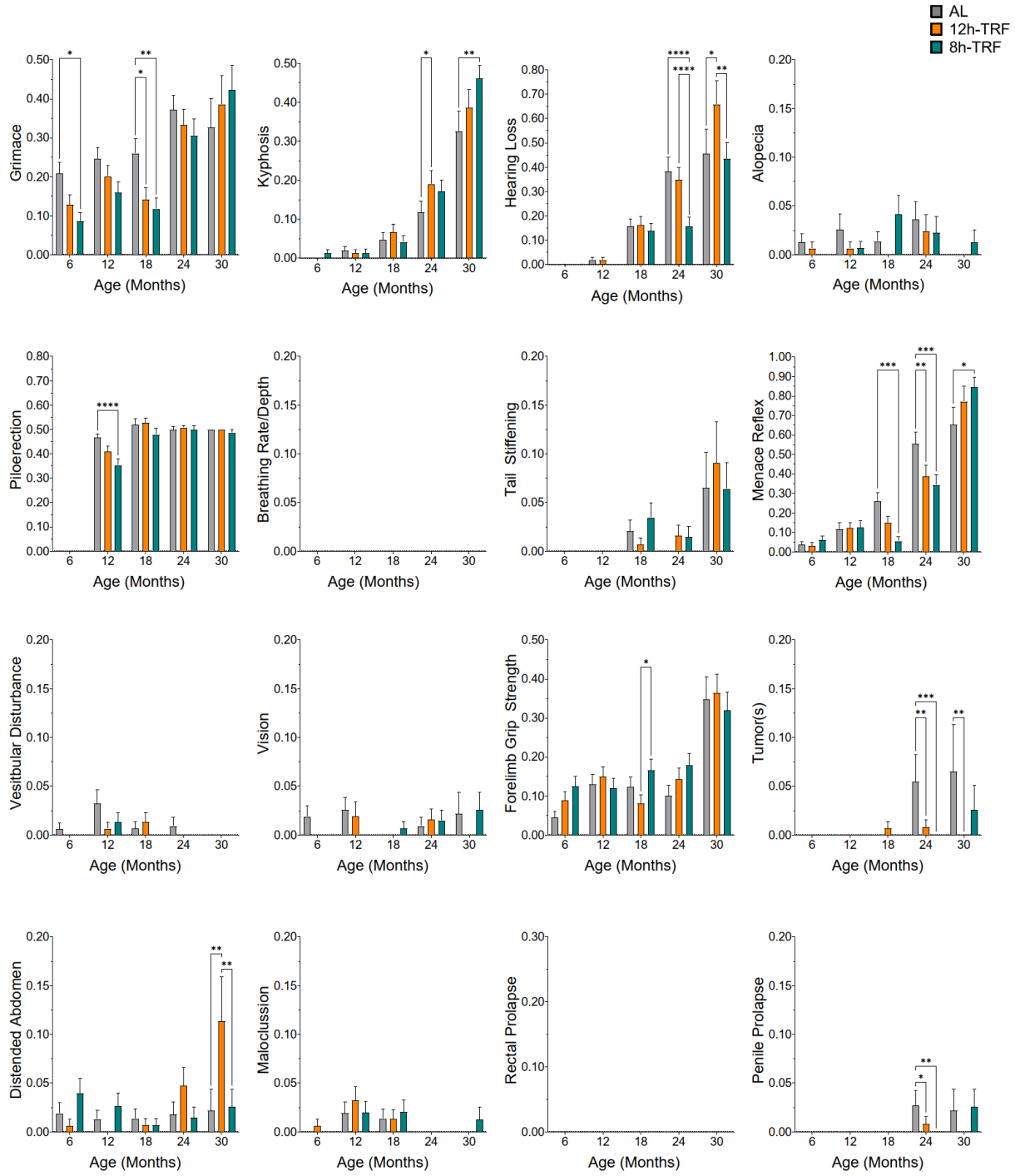


Figure S8: Individual frailty parameter scores in males. Mean frailty scores ± SEM every 6 months of age in males for each of the 31 parameters modified from (*47*). AL, N=23-79. 12h-TRF, N=22-78. 8h-TRF, N=39-76 Two-way ANOVA, Tukey's post-hoc.

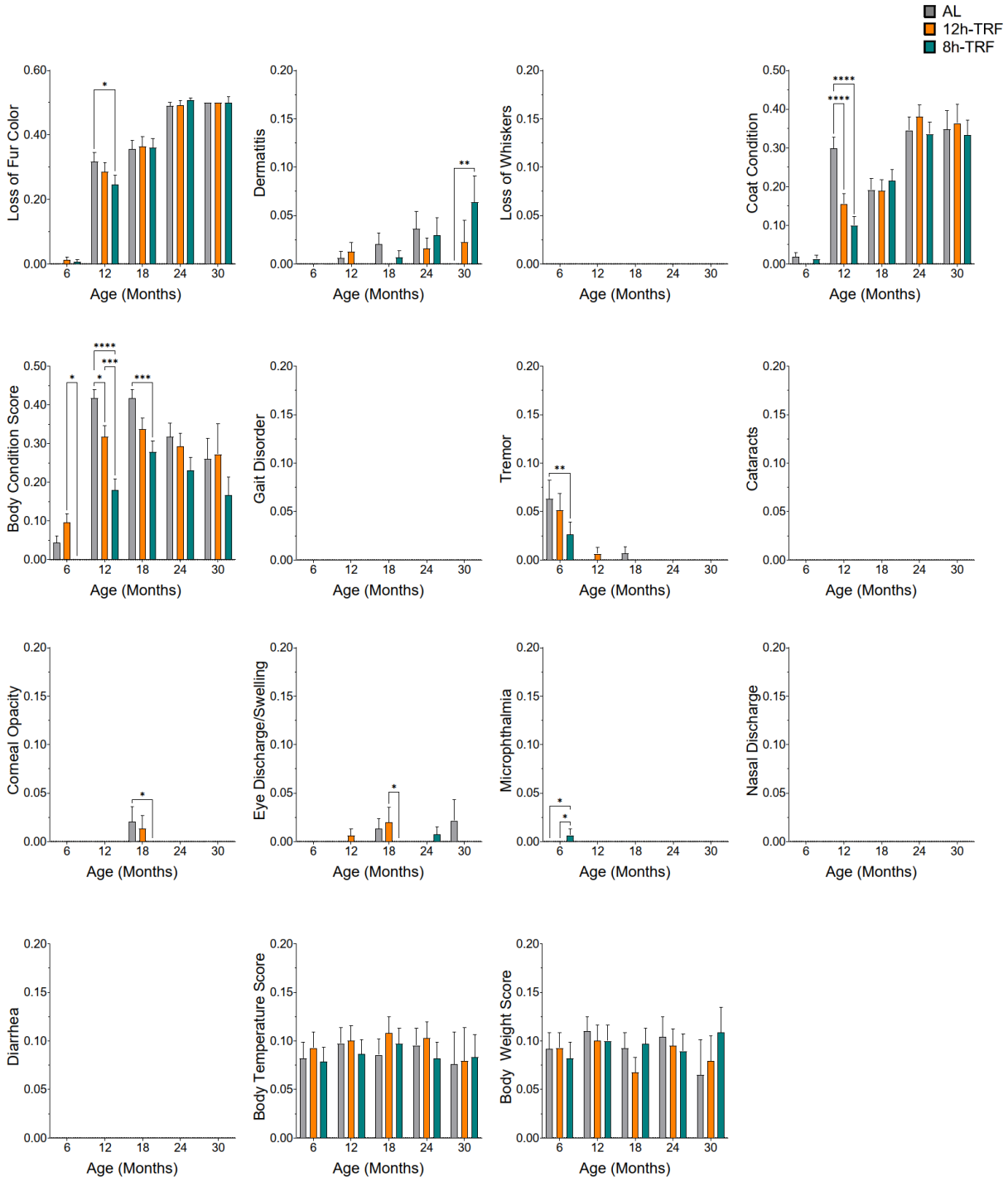


Figure S8: Individual frailty parameter scores in males (Continued).

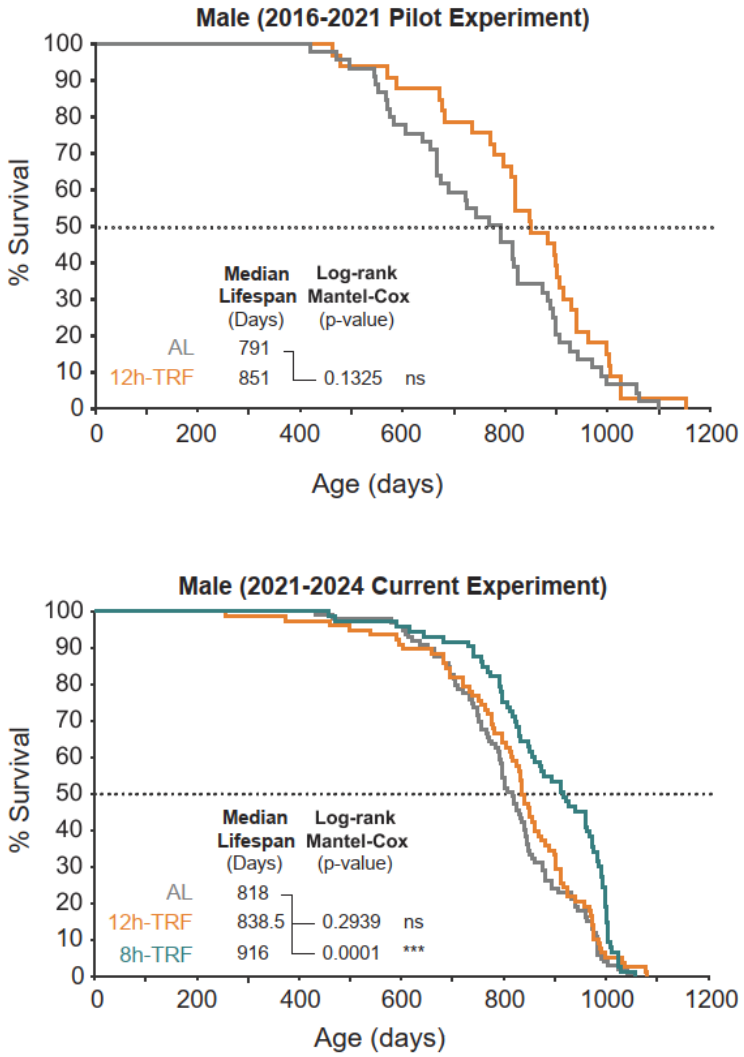


Figure S9: Repeat experiments show 12h-TRF does not extend lifespan in males: Kaplan-Meier survival curves and day median lifespan reached in a pilot experiment vs our current experiment. Log-Rank Mantel-Cox Test for significant difference in overall survival TRF vs AL. Fisher's exact test for median and maximal survival TRF vs AL. Pilot experiment: AL, N=43. 12h-TRF, N=33. Current experiment: AL, N=99. 12h-TRF, N=78. 8h-TRF, N=73.