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1 Disentangling the effect of dietary restriction on mitochondrial function using
2 recombinant inbred mice

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15

16 **Abstract**

17 Dietary restriction (DR) extends lifespan and healthspan in many species, but
18 precisely how it elicits its beneficial effects is unclear. We investigated the impact of
19 DR on mitochondrial function within liver and skeletal muscle of female ILSXISS
20 mice that exhibit strain-specific variation in lifespan under 40% DR. Strains TejJ89
21 (lifespan increased under DR), TejJ48 (lifespan unaffected by DR) and TejJ114
22 (lifespan decreased under DR) were studied following 10 months of 40% DR (13
23 months of age). Oxygen consumption rates (OCR) within isolated liver mitochondria
24 were unaffected by DR in TejJ89 and TejJ48, but decreased by DR in TejJ114. DR
25 had no effect on hepatic protein levels of PGC-1 α , TFAM, and OXPHOS complexes
26 I-V. Mitonuclear protein imbalance (nDNA:mtDNA ratio) was unaffected by DR, but
27 HSP90 protein levels were reduced in TejJ114 under DR. Surprisingly hepatic
28 mitochondrial hydrogen peroxide (H₂O₂) production was elevated by DR in TejJ89,
29 with total superoxide dismutase activity and protein carbonyls increased by DR in
30 both TejJ89 and TejJ114. In skeletal muscle, DR had no effect on mitochondrial
31 OCR, OXPHOS complexes or mitonuclear protein imbalance, but H₂O₂ production
32 was decreased in TejJ114 and nuclear PGC-1 α increased in TejJ89 under DR. Our
33 findings indicate that hepatic mitochondrial dysfunction associates with reduced
34 lifespan of TejJ114 mice under 40% DR, but similar dysfunction is not seen in
35 skeletal muscle mitochondria. We highlight tissue-specific differences in the
36 mitochondrial response in ILSXISS mice to DR, and underline the importance and
37 challenges of exploiting genetic heterogeneity to help understand mechanisms of
38 ageing.

39

40 **Keywords:** Dietary restriction, ILSXISS, mitochondria, PGC1- α , mitochondrial

41 unfolded protein response, aging

42 1.1 Introduction

43 Dietary restriction (DR), in its most general sense; defined here as reductions
44 in energy intake, reductions in specific macro or micronutrients or as intermittent
45 fasting, is the most extensively applied experimental intervention employed to
46 manipulate ageing and longevity [1], [2]. Since the first study demonstrating that DR
47 extended the reproductive period and lifespan of female rats almost one century ago
48 [3], an extensive body of research has studied the effects of DR in a wide number of
49 organisms (reviewed in [1], [2], [4]–[6]). In addition to its effects on lifespan, DR also
50 attenuates and/or postpones a broad-spectrum of age-associated pathologies,
51 including obesity, insulin resistance, cognitive decline, immune dysfunction, stem cell
52 ageing, sarcopaenia and cataracts [1], [2], [4], [5]. DR in rodents confers protection
53 against a number of spontaneous and experimentally-induced cancers [6], and
54 delays several age-associated pathologies, including metabolic and cardiovascular
55 disease, cancer and brain atrophy in non-human primates [7]–[9]. Similarly, DR
56 elicits numerous beneficial metabolic effects in humans including weight loss, lower
57 visceral and intramuscular adiposity, insulin sensitivity and lowers several risk factors
58 linked to cancer and cardiovascular disease [10], [11].

59 However, contrary to the belief that the effect of DR on longevity is universal,
60 several studies have reported a lack of, or even a detrimental effect of, DR on
61 lifespan [9], [12]–[16] (reviewed in [17]). In Rhesus macaques (*Macaca mulatta*), DR
62 extended lifespan in a study conducted by the National Primate Research Center at
63 the University of Wisconsin [7], but did not extend lifespan in a study undertaken by
64 the National Institute of Health [9]. The precise reasons for the differing outcomes
65 between these studies appears complex but may reflect inter-study differences in
66 diet, animal husbandry and geographical origin [1], [7], [18]. In addition, a potentially

67 critical factor that may help explain the unresponsiveness of particular organisms to
68 DR-induced longevity is genetic background [1], [7], [18], [19]. For example, the
69 effect of DR on survival in DBA/2 mice has been a source of debate for many years,
70 with DR reported to extend [20], [21], have no effect [22], or shorten lifespan [14].
71 However, a recent study examining lifespan in male and female DBA/2 and C57BL/6
72 mice under 20% and 40% DR revealed that DBA/2 mice are indeed responsive to
73 DR, although the impact of sex, strain and the magnitude of DR on survival outcome
74 appears important [23]. In two independent studies undertaken by the Universities of
75 Texas [24] and Colorado [15], survival was determined in multiple strains of
76 heterogeneous ILSXISS recombinant inbred mice maintained on 40% DR, with
77 distinct strain-specific effects on survival observed following 40% DR. The first study
78 [24] reported that across 39 female and 41 male strains studied, only 21% of female
79 strains showed life extension under DR, and only 5% of male strains. Surprisingly, a
80 greater number of strains (27% and 26% for females and males respectively)
81 showed reduced lifespan under DR. The latter study [15], which assayed 42 female
82 ILSXISS strains, similarly reported a significant strain-specific response to DR, with
83 only 21% of strains showing life extension and 19% showing life shortening effects of
84 DR.

85 Despite DR being the primary experimental intervention used to study
86 ageing, it is still unclear as to precisely how DR acts mechanistically to induce its
87 effects, although a multitude of mechanisms have been proposed [4], [5], [25]–[27].
88 Mitochondrial dysfunction is a key hallmark of ageing and disease [28], with ageing
89 associated with altered mitochondrial morphology, reduced mitochondrial oxidative
90 capacity and ATP production, increased mitochondrial-derived reactive oxygen
91 species (ROS) generation and greater oxidative damage [29]. Consequently,

92 significant research effort has investigated whether DR can induce beneficial effects
93 on the mitochondrial phenotype, such as maintaining mitochondrial function during
94 ageing, reducing ROS production and attenuating oxidative damage. While it is
95 generally assumed that DR reduces ROS production, a recent meta-analysis
96 involving 157 rodent DR studies in which ROS levels (primarily hydrogen peroxide)
97 were assayed, highlighted that 62% of studies actually reported no change in ROS
98 levels relative to *ad libitum* (AL) fed controls [30]. Ambiguity also exists regarding the
99 effect of DR on mitochondrial respiration in rodents, with DR reported to increase
100 [31]–[34], decrease [35], or have no effect relative to AL controls [36]. Similarly, the
101 impact of DR on various antioxidants and markers of oxidative damage appears
102 fairly inconclusive, although as with all these parameters tissue-specific effects, the
103 duration and intensity of DR, the sex and the age of the animals at the point of study
104 may affect experimental outcomes [30]. The concept that DR induces mitochondrial
105 biogenesis, that is the production of new mitochondrial proteins, has been proposed
106 as a critical mechanism underlying the beneficial effects of DR [37], with DR reported
107 to induce mitochondrial biogenesis in a number of tissues including liver [33].
108 Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1 α) is
109 described as the master regulator of mitochondrial biogenesis [38]. DR slows age-
110 related declines in *Pgc-1 α* expression in rat skeletal muscle and heart, and it is
111 proposed that mitochondrial function adapts to DR through PGC1 α regulation [33],
112 [39], [40]. However, there is considerable debate in the literature as to how best to
113 quantify biogenesis. Indeed, several studies suggest that DR may increase
114 mitochondrial efficiency in order to maintain an ‘optimally efficient’ electron transport
115 system, potentially driven by the nuclear localisation of PGC1- α [39], [41] without any
116 increase in mitochondrial number *per se* [31], [34], [42]. Hancock et al [42] examined

117 a range of tissues, including liver and skeletal muscle, in rats and suggested that
118 rather than altering rate of protein synthesis in the mitochondria that DR affords
119 protection to mitochondria through defending against DNA damage. Similarly, Lanza
120 et al [34] reported that DR did not stimulate the synthesis of new mitochondrial
121 proteins in mouse skeletal muscle, but rather minimised damage to existing cellular
122 components, through decreased mitochondrial oxidant emission and upregulated
123 antioxidant defences. Indeed, mitochondrial protein synthesis within liver, muscle
124 and heart of mice was unaffected by DR, although cellular proliferation was
125 decreased [43], with a recent proteomic approach reporting that mitochondrial
126 biogenesis may actually be reduced within liver of DR mice [44]. Consequently, such
127 findings question the concept that DR increases mitochondrial biogenesis, and
128 further challenge the somewhat counterintuitive idea of expending energy on protein
129 synthesis at a time of energy and/or nutrient restriction [34].

130 Recently, the role of mitonuclear protein imbalance, that is a stoichiometric
131 imbalance between OXPHOS subunits encoded by nuclear DNA (nDNA) and
132 mitochondrial DNA (mtDNA) which activates the cytoprotective mitochondrial
133 unfolded protein response (UPR^{mt}), in longevity control has gained significant
134 coverage (see[45]–[48]). Both rapamycin and resveratrol induced mitonuclear protein
135 imbalance and UPR^{mt} in mouse hepatocytes *in vitro* [45]. UPR^{mt} induction in *C.*
136 *elegans* also appeared necessary for longevity in developing worms exposed to high
137 glucose, but shortened lifespan when induced in adulthood [49]. Longevity in BXD
138 mice is associated with reduced mitochondrial translation as determined by reduced
139 expression of mitochondrial protein 5 (*Mrps-5*); *Mrps-5* expression in skeletal muscle
140 decreases with age and this is attenuated by DR [45]. In addition, skeletal muscle
141 from long-lived *Surf1*^{-/-} mice display evidence of UPR^{mt} [50]. However, whether

142 mitonuclear imbalance and UPR^{mt} are important in DR-induced longevity in mice
143 remains to be elucidated.

144 Given the ongoing quest to identify the mechanistic drivers of DR, it has been
145 suggested that employing a comparative-type approach which takes advantage of
146 the variability in the DR response reported in certain rodent strains may help
147 delineate the mechanisms underpinning DR [17], [19]. Indeed, several groups have
148 already undertaken such approaches using DBA/2 mice [51]–[53], showing for
149 example that this strain are hyperinsulinaemic and insulin resistant compared to
150 C57BL/6 mice [54]. In the present study we investigated the potential linkage
151 between DR-induced longevity and mitochondrial function within both liver and
152 skeletal muscle by exploiting the highly variable effect of DR on lifespan in ILSXISS
153 mice. To this end we compared females mice from three ILSXISS strains showing
154 repeatable responses to DR across two independent studies [15], [24]; TejJ89
155 (lifespan extended under DR relative to AL controls), TejJ48 (lifespan unaffected by
156 DR), and TejJ114 (lifespan shortened under DR). We predicted that DR would
157 positively impact on a number of parameters associated with mitochondrial function
158 in strain TejJ89, that DR would have no impact on these parameters in strain TejJ48,
159 and that DR would induce mitochondrial dysfunction in the negative responding
160 TejJ114 (see Figure 1 for schematic outlining our original predictions).

161

162 **2. Materials and methods**

163 ***Animals***

164 ILSXISS recombinant inbred (RI) mouse strains are derived from a cross
165 between inbred long sleep (ILS) and inbred short sleep (ISS) mice [55]. Mice from
166 three strains; TejJ89, TejJ48 and TejJ114 were purchased from a commercial
167 breeder (The Jackson Laboratory, Bar Harbour, Maine, URL:
168 <http://www.informatics.jax.org>). The rationale for studying these particular strains
169 was that in addition to repeatable effect of DR on lifespan across two independent
170 studies [15], [24], no strain-specific differences in median lifespan were observed
171 upon *ad libitum* (AL) feeding. Mice were maintained in groups of 4 post-weaning in
172 shoebox cages (48cm×15cm×13cm), with AL access to water and standard chow
173 (CRM(P), Research Diets Services, LBS Biotech, UK; Atwater Fuel Energy- protein
174 22%, carbohydrate 69%, fat 9%) and maintained on a 12L/12D cycle (lights on
175 0700–1900h) at 22±2°C. At 9 weeks of age, cages were assigned to either an AL or
176 DR group, with no difference in body mass observed between treatment groups at
177 this time (TejJ89 AL vs. DR $t=0.056$, $p=0.583$, TejJ48 AL vs. DR $t=0.677$, $p=0.509$,
178 TejJ114 AL vs. DR $t=0.289$, $p=0.777$). Mice were introduced to DR in a graded
179 fashion; at 10 weeks of age mice were exposed to 10% DR (90% of AL feeding), at
180 11 weeks this was increased to 20% DR, and from 12 weeks of age until the
181 termination of the experiment mice were exposed to 40% DR, relative to their
182 appropriate strain-specific AL controls. Total food intake of AL mice from each strain
183 was measured weekly (± 0.01 g) and food intake of the DR cohort calculated from the
184 average AL intake per mouse over the preceding week [54]. DR mice were fed daily
185 at 1800hrs and fed directly into the cage. Following 10 months of 40% DR
186 (equivalent to 13 months of age), mice were fasted overnight and then culled the

187 following morning by cervical dislocation. One lobe of liver, one gastrocnemius
188 muscle and the heart were dissected out and immediately snap-frozen in liquid
189 nitrogen and stored at -80°C until use. The remaining liver tissue and gastrocnemius
190 muscle were subsequently used for the mitochondrial functional studies. A total of 4
191 mice per day were culled, with the time between dissection, mitochondrial isolation
192 and mitochondrial analysis kept as uniformed as possible, with the particular tissue
193 (liver or skeletal muscle) processed and analysed alternated each day. All
194 experiments were carried out under a licence from the UK Home Office (Project
195 Licence 60/4504) and followed the “principles of laboratory animal care” (NIH
196 Publication No. 86-23, revised 1985).

197 ***Mitochondrial Respiration***

198 ***Isolation of Mouse Liver and Skeletal Muscle Mitochondria***

199 Liver mitochondria were isolated using previously published protocols [56],
200 [57]. Briefly the liver was weighed and washed in MSHE+BSA buffer (210 mM
201 mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EGTA and 0.5% (w/v) fatty acid free
202 bovine serum albumin (pH 7.2)). The liver was rapidly minced with scissors in 10
203 volumes of MSHE+BSA buffer before homogenisation (2-3 strokes) using a glass-
204 glass homogeniser (Fisher Scientific, Loughborough, UK). As with the liver,
205 gastrocnemius muscle was harvested, weighed, washed and then minced in ice-cold
206 isolation buffer (100 mM sucrose, 100mM KCL, 50mM Tris HCl, 1mM KH₂PO₄,
207 0.1mM EGTA, 0.2% BSA (pH 7.4)), as set out in protocols previously described [58].
208 The muscle was subsequently rinsed three times in 1ml of fresh isolation buffer, and
209 then 1ml of 2% Proteinase Type XXIV (Sigma Aldrich, Dorset, UK) was added and
210 the sample vortexed for 1 min⁻¹ at room temperature, followed by 1 min⁻¹ incubation
211 on ice. Samples were subsequently added to a glass homogeniser and 4ml of

212 isolation buffer added before homogenisation (10 strokes). Using differential
213 centrifugation for both liver [56] and muscle [58] a mitochondrial pellet was isolated
214 and subsequently re-suspended (liver; MSHE buffer with no BSA, skeletal muscle;
215 suspension buffer (225mM Mannitol, 75mM sucrose, 10mM Tris, 0.1mM EDTA (pH
216 7.4)). Total liver and skeletal muscle mitochondrial protein (mg/ml) were determined
217 using the Bradford assay (Sigma Aldrich, Dorset, UK).

218 ***XF Assay – Plate Preparation***

219 Isolated mitochondria were diluted 10X in mitochondrial assay solution (MAS;
220 70 mM sucrose, 220 mM mannitol, 10 mM KH_2PO_4 , 5 mM MgCl_2 , 2 mM HEPES, 1.0
221 mM EGTA and 0.2% (w/v) fatty acid-free BSA, pH 7.2) containing substrate (10 mM
222 pyruvate, 2 mM malate), and subsequently diluted to produce a 10 μg mitochondrial
223 suspension. Mitochondria were added to wells of a Seahorse XF assay plate (Agilent
224 Technologies, CA, USA) at a concentration of 10 μg per well. The plate was
225 centrifuged at 2000g for 20 minutes at 4°C. A XF cartridge (Agilent Technologies)
226 was then prepared as described by Brand *et al.* [56]. The plate was then transferred
227 to a XF24 Analyser (Agilent Technologies) and the experiment initiated as previously
228 described [57]. Basal Oxygen consumption rate (OCR) was measured in substrate
229 (10 mM pyruvate, 2 μM malate). Following this OCR was sequentially recorded for
230 state 3 (addition of ADP (4mM)), state 4 (addition of oligomycin (2.5 $\mu\text{g}/\text{ml}$)), state 3u
231 (FCCP (4 μM Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazine)), and finally in
232 the presence of Antimycin A and rotenone (40 μM), inhibitors of Complex III and I
233 respectively, to determine non-mitochondrial respiratory capacity [57]. Analysis was
234 carried out using Seahorse XF software (www.seahorsebio.com). Respiratory control
235 ratio, expressed as the ratio between state 3u (FCCP-induced maximal uncoupled-
236 stimulated respiration) and state 4o (respiration in the absence of ADP) did not differ

237 by treatment within a strain or between strains for either liver or muscle mitochondria
238 (Fig. S1A&B).

239 **Protein extraction**

240 Liver, skeletal muscle and heart tissue were suspended in 1ml of ice cold
241 RIPA buffer (Radio Immuno Precipitation Assay buffer, 150 mM sodium chloride,
242 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl
243 sulphate), 50 mM Tris, pH 8.0) containing protease inhibitors (Halt™ Protease and
244 Phosphatase Inhibitor Cocktail, Thermo Fisher Scientific, UK). The liver and heart
245 were rapidly minced on ice with scissors and homogenised. A stainless steel bead
246 (Catalogue # 69989, QIAGEN, Manchester, UK), was added to the eppendorf
247 containing the skeletal muscle tissue and RIPA buffer, and then homogenised at
248 maximum speed (30Hz) for 4 min⁻¹ on the RETSCH MM 400 mixer mill (Catalogue#
249 10573034, Fisher Scientific). Following homogenisation all lysates were incubated
250 on ice for 40-60 min⁻¹ before being centrifuged at 16,000 x g for 10 min⁻¹ at 4°C, and
251 total protein levels subsequently determined using the BCA protein assay (G
252 Biosciences, MO, USA). Nuclear and cytoplasmic fractionation of liver and skeletal
253 muscle was undertaken using the ReadyPrep™ Protein Extraction
254 (Cytoplasmic/Nuclear) Kit (Catalogue #163-2089, Bio-Rad, UK). Briefly, ~50 mg of
255 tissue was homogenised together with 0.75ml of cold cytoplasmic protein extraction
256 buffer containing protease inhibitors (Halt™ Protease and Phosphatase Inhibitor
257 Cocktail,) using a chilled Wheaton Dounce tissue homogeniser (Catalogue #62400-
258 595, VWR, West Sussex, UK), with protein concentration of each fraction determined
259 using the BCA protein assay.

260 **Western Blot Analysis**

261 Equal volumes of tissue protein extract (50µg) in Laemmli sample buffer were
262 loaded onto 4-12% Bis-Tris pre-cast polyacrylamide gels (Life Technologies,
263 Paisley, UK). Following this, proteins were transferred to polyvinylidene difluoride
264 membranes (BioRad). Ponceau staining was used to ensure equal loading of
265 protein and for normalisation purposes. Membranes were incubated in Tris-buffered
266 saline Tween (1X TBST) containing 5% BSA for 1h⁻¹. Blots were then washed in
267 TBST (5×5min), incubated with primary antibody for 24h⁻¹ (4°C), washed again
268 (TBST) and incubated with secondary antibody for 1h⁻¹ at room temperature. Blots
269 were visualised using Clarity™ Western ECL Substrate (BioRad) and a
270 ChemiDoc™XRS system (BioRad). Antibodies for peroxisome proliferator-activated
271 receptor-gamma co-activator 1 alpha (PGC-1α) and the oxidative phosphorylation
272 complex antibody (OXPHOS cocktail; CI subunit NDUFB8, CII-30kDa (SDH), CIII-
273 Core protein 2 (UQCRC2), CIV subunit I (MTCO1) and CV alpha subunit (ATP5A)
274 were from Abcam, Cambridge, UK, mitochondrial transcription factor A (TFAM) and
275 secondary (anti-rabbit) antibodies from Santa Cruz Biotechnology Inc. (Santa Cruz,
276 CA, USA), and HSP60 and HSP90 from BD Biosciences (BD Biosciences, Oxford,
277 UK. Mitonuclear protein imbalance (nDNA:mtDNA ratio) was determined by the
278 ratio of nuclear encoded SDHB (Complex II) to mitochondrial-encoded MTCO1
279 (Complex IV) as described previously [45].

280 ***Mitochondrial ROS production***

281 Fresh respiratory medium (MAS; 70 mM sucrose, 220 mM mannitol, 10 mM
282 KH₂PO₄, 5 mM MgCl₂, 2 mM HEPES, 1.0 mM EGTA and 0.2% (w/v) fatty acid-free
283 BSA, pH 7.2) was supplemented with 1U/ml horseradish peroxidase and 10 µM
284 Amplex® Red reagent (ThermoFisher Scientific, UK) as previously described [59].
285 90µl of this medium was then added to each well of a standard 96 well plate

286 (Costar®, Sigma Aldrich, UK) and heated for two min⁻¹ at 37°C. Fluorescence was
287 then measured at 15 second intervals for 2-3 min using a spectrophotometer
288 (Clariostar microplate reader, BMG Labtech) at excitation and emission wavelength
289 of 563 and 587nm respectively. A sequential protocol was then run simultaneously
290 for each sample in triplicate and completed within 15 min⁻¹. First the baseline
291 fluorescence was measured by adding 10ul of mitochondrial suspension, and then
292 mitochondrial hydrogen peroxide production from all complexes was determined by
293 adding a saturating concentration of succinate (10mM), and then rotenone added
294 (0.5uM; an inhibitor of complex I). The fluorescence signal was calibrated using 176
295 nM of hydrogen peroxide, and then hydrogen peroxide production was calculated
296 following background correction.

297 ***Oxidative damage and antioxidant levels***

298 Protein carbonyl (PC), total glutathione (GSH) and total superoxide dismutase
299 (SOD) activity were measured in liver samples using commercially available kits and
300 following the manufacturer's protocols (Catalogue#10005020 Carbonyl assay kit,
301 #706002 Total SOD activity assay kit, #703002 Glutathione assay kit, Cayman
302 Chemical Company, Estonia). Hepatic 4-Hydroxynonenal (HNE)-protein adduct
303 levels were determined using an anti-HNE-His mouse IgG protein binding plate
304 (Catalogue#STA-838, Cell Biolabs Inc., CA, USA). All assays were read on a plate
305 reader (BMG Labtech, UK).

306 ***Statistical Analysis***

307 All statistical analyses were performed using R and figures were produced
308 using GraphPad Prism (GraphPad Inc., La Jolla, CA, USA, version 5) software. Data
309 were checked for normality using the Shapiro–Wilks test and a logarithmic

310 transformation was undertaken if data not normally distributed. Data was analysed
311 using linear modelling (LM) with treatment (AL or DR) and strain (TejJ89, TejJ48 and
312 TejJ114) introduced as fixed factors. Following transformation, if the data was not
313 normally distributed an appropriate non-parametric test was applied. Results are
314 reported as mean±standard error of the mean (SEM), with $p<0.05$ regarded as
315 statistically significant.

316

317 **3. Results**

318 Mitochondrial oxygen consumption rates (OCR) within isolated liver
319 mitochondria under all conditions studied (Fig. 2A) were unaffected by 10 months of
320 40% DR in strain TejJ89 relative to AL controls. A similar lack of a DR effect on
321 hepatic respiratory capacities was observed in strain TejJ48 (Fig. 2B). In contrast,
322 DR in strain TejJ114 (Fig. 2C) significantly reduced State 3 ($t = 2.860$, $p = 0.006$) and
323 State 3u ($t = 2.950$, $p=0.006$) mitochondrial OCR relative to AL controls. While no
324 differences in mitochondrial OCR were observed across ILSXISS strains under AL
325 feeding (Fig. 2D), State 3 OCR in TejJ89 was significantly increased ($t= 2.24$,
326 $p=0.039$) relative to the other two strains under DR (Fig. 2E).

327 Mitochondrial hydrogen peroxide (H_2O_2) production within liver was increased
328 significantly ($t=3.555$, $p=0.002$) by DR in strain TejJ89 relative to its appropriate
329 control, but DR had no effect on H_2O_2 production in strains TejJ48 and TejJ114 (Fig.
330 3A). While no difference between strains in H_2O_2 production was observed under AL
331 feeding (Fig. 3B), strain TejJ89 had significantly higher ROS levels compared to
332 TejJ48 and TejJ114 under DR, and TejJ48 had significantly higher ROS levels
333 ($t=2.339$, $p=0.039$) compared to TejJ114 (Fig. 3C). Total hepatic SOD activity was
334 increased by DR treatment in strains TejJ89 ($t = 3.776$, $p=0.004$) and TejJ114 ($t =$
335 2.845 , $p = 0.010$) relative to their respective AL controls (Fig. 4A), but no strain-
336 specific effect on SOD activity was observed under either AL or DR feeding (Fig.
337 S2A & B). Liver total glutathione (Fig. 4B; Fig. S2C and D) and 4-HNE (Fig. 4C; Fig.
338 S2E & F) were unaffected by either treatment or strain. However, hepatic protein
339 carbonyl (PC) levels (Fig. 4D) were significantly increased in strains TejJ89 ($t=2.420$,
340 $p=0.037$) and TejJ114 ($t=2.440$, $p=0.040$) under DR when compared to their
341 respective AL controls. In addition, while PC levels were not different between

342 strains under AL feeding (Fig. S2G), hepatic PC levels were significantly lower in
343 TejJ48 compared to both TejJ89 ($t=6.860$, $p<0.001$) and TejJ114 ($t=3.220$, $p=0.010$)
344 under DR (Fig. S2H).

345 Total hepatic protein levels of PGC-1 α (Fig. 5A), a key transcriptional co-
346 activator linked to mitochondrial metabolism and biogenesis, was unaltered by
347 treatment or strain, with both nuclear (Fig. 5B) and cytoplasmic (Fig. 5C) PGC-1 α
348 levels likewise unaffected. Similarly, mitochondrial transcription factor A (TFAM), a
349 key activator of mitochondrial transcription, was unaffected by DR or strain (Fig. 5D).
350 We then examined various OXPHOS complexes within liver, but again observed no
351 treatment effect or observed any differences between strains under AL or DR
352 feeding (Figure S3A-C). Given the evidence of mitochondrial dysfunction in TejJ114,
353 we then went on to investigate whether DR induced mitonuclear protein imbalance
354 and UPR^{mt} by firstly calculating the ratio of nuclear encoded SDHB to mitochondrially
355 encoded MTCO1 as previously described [45]. We observed no effect of either
356 treatment or strain on mitochondrial nuclear imbalance (Fig. 6A-C). No differences
357 were observed between either treatment groups or strains in the mitochondrial
358 chaperone HSP60 (Fig. 6D), but a significant reduction in hepatic HSP90 (Fig. 6E)
359 was observed in strain TejJ114 under DR ($t=2.267$, $p=0.045$) relative to AL controls.

360 In order to determine whether mitochondrial dysfunction in strain TejJ114 was
361 specific to liver, we also examined a number of mitochondrial parameters in isolated
362 skeletal muscle from these same mice. No treatment or strain-specific differences in
363 mitochondrial OCR were observed (Fig. 7A-E). Skeletal muscle mitochondrial H₂O₂
364 production significantly reduced under DR in strain TejJ114 (Mann Whitney $p=0.009$)
365 (Fig. 9A) compared to AL controls, but unaffected by DR in the other two strains. No
366 differences in mitochondrial H₂O₂ production was observed between strains under

367 AL feeding (Fig. 9B), but was significantly reduced in TejJ114 under DR relative to
368 TejJ89 ($t=2.903$, $p=0.044$) and TejJ48 (Mann Whitney $p=0.009$). Similar to liver, total
369 and cytosolic PGC1 α levels (Fig. 8A & B) were unaffected by DR in all strains,
370 although nuclear PGC1 α levels were increased in TejJ89 ($t=3.174$, $p=0.034$). Protein
371 levels of various OXPHOS complexes and mitonuclear protein imbalance within
372 skeletal muscle were unaffected by treatment and strain (Figure S3D-F; Fig. 10A), as
373 was also the case in heart (Fig. S3G-I; Fig. 10B).

374 **4. Discussion**

375 The phenotypic plasticity of the mitochondria is crucial to allow energetic
376 demands to be met and in order to sustain bioenergetic efficiency [42], [60].
377 Consequently impairments in this dynamic system can lead to profound health
378 consequences, with mitochondrial dysfunction widely proposed as a hallmark of
379 ageing [61], [62]. As discussed earlier, enhanced mitochondrial function has been
380 put forward as a candidate mechanism underlying the beneficial effects of DR on
381 lifespan and healthspan [63], [64]. Here we sought to investigate mitochondrial
382 function in using a comparative approach in ILSXISS mice which are known to show
383 significant strain-specific variation in lifespan under 40% DR [15], [24].

384 Contrary to our initial predictions, 40% DR did not alter hepatic mitochondrial
385 respiratory capacity in strain TejJ89, which is reported to show lifespan extension
386 under DR. While several studies have reported that DR increases mitochondrial
387 respiratory capacity in rodents [31]–[34], other studies have reported a DR-induced
388 decrease [65], [35], [66], or no effect of DR on mitochondrial respiratory capacity
389 [36], [67] relative to AL controls. The precise reasons for this ambiguity appears
390 complex but the tissue studied, the preparation used (isolated mitochondria vs.
391 permeabilised tissue vs. tissue homogenate), the duration, age of onset and level of
392 DR imposed, and the sex, age and the genetic background of the animal may all be
393 important [31], [64], [68], [69]. Strain TejJ48 under DR, as predicted, displayed
394 essentially no mitochondrial phenotype relative to its AL control. However, in strain
395 TejJ114, mitochondrial respiratory capacity associated with State 3 and State 3u
396 (maximal uncoupled oxygen consumption rates) were significantly reduced under
397 DR. Both ageing and many pathologies associated with ageing reduce mitochondrial
398 respiratory capacities in a range of tissues [70], [71], and the apparent DR-induced

399 hepatic mitochondrial dysfunction may offer new insights in to why DR truncates
400 lifespan in this particular ILSXISS strain. To determine whether the hepatic
401 mitochondrial dysfunction in TejJ114 was liver-specific, we determined mitochondrial
402 respiratory capacity in isolated skeletal muscle mitochondria in these same ILSXISS
403 mice under AL and DR feeding. Despite DR known to preserve mitochondrial
404 function during ageing in certain mouse strains, such as C57BL/6 and male B6D2F1
405 [31], [34], we saw no effect of DR on skeletal muscle mitochondrial respiratory
406 capacity in any strain. In view of the well-defined role of PGC1 α in modulating
407 mitochondrial function, and given that that mitochondrial adaptations to DR may be
408 driven through a PGC1 α -induced transcriptional program [39], [41], [64], [72], we
409 investigated protein levels of PGC1 α within liver in each strain under AL and DR. In
410 line with the absence of any effect of DR on mitochondrial respiratory capacity, we
411 observed no increase in hepatic PGC1 α (total, cytosolic, nuclear) or in mitochondrial
412 transcription factor A (TFAM) protein levels of strain TejJ89 under DR. Similarly,
413 PGC-1 α and TFAM levels were unaffected by DR in strains TejJ48 or TejJ114.
414 However, a tissue-specific response was observed with 40% DR significantly
415 increasing nuclear PGC1 α within skeletal muscle of strain TejJ89, in line with what
416 has been reported in other studies (for review see[64]).

417 We then employed an immunoblot approach to examine respiratory chain
418 complexes I, II, III, IV and V, and we again observed no treatment effect in any
419 strains for liver, skeletal muscle or in heart. Indeed, it has been reported that DR-
420 induced attenuation of ageing-associated declines in mitochondrial function within
421 skeletal muscle appears to be independent of any effect on mitochondrial respiratory
422 chain protein levels [34] However, this approach enabled us to examine mitonuclear
423 protein imbalance; that is the ratio of nuclear encoded SDHB to mitochondrially

424 encoded MTCO1. An increase in this ratio is associated with an induction in the
425 cytoprotective mitochondrial unfolded protein response (UPR^{mt}), a recently proposed
426 conserved lifespan determinant [45]. We found no evidence that DR increased
427 mitonuclear protein imbalance within the liver, skeletal muscle or heart of ILSXISS
428 mice under DR feeding. The UPR^{mt} invokes a transcriptional program in response to
429 a number of processes, including mitochondrial dysfunction, resulting in the induction
430 of various chaperones and proteases that help facilitate mitochondrial proteostasis
431 [73]. We therefore, then examined the molecular chaperones HSP60 and HSP90,
432 and similarly showed a lack of any treatment effect across our strains, except for a
433 significant reduction in HSP90 levels within liver of TejJ114 mice under DR. HSP90
434 engages with a large number of 'client' proteins through co-chaperones, plays a
435 major role in signal transduction, protects the 20S proteasome against oxidative
436 inactivation and may actively regulate mitochondrial metabolism [74]–[76]. Whilst it is
437 difficult to disentangle cause and effect here, our data indicates that in strain TejJ114
438 40% DR leads to hepatic mitochondrial dysfunction and that this is correlated with
439 reduced HSP90 levels.

440 It is evident that both ageing and several disease states are associated with
441 greater ROS-induced oxidative damage, but it is equivocal as to whether ROS-
442 induced oxidative damage is the mechanism underpinning ageing and disease [30],
443 [77], [78]. A large number of studies have investigated whether DR can reduce ROS,
444 induce various antioxidants and attenuated oxidative damage in model organisms. In
445 an excellent recent meta-analysis, the Van Remmen laboratory [30] examined
446 several hundred studies that have investigated the effect of DR in rodents on ROS
447 production, various antioxidants and on oxidative damage. Their approach found that
448 DR had remarkably little impact on ROS production or antioxidant activity overall, but

449 53% of studies reported that DR reduced oxidative damage. Perhaps surprisingly we
450 found that DR in strain TejJ89, which shows lifespan extension under 40% DR, had
451 significantly higher hepatic mitochondrial H₂O₂ production, alongside greater hepatic
452 total SOD activity and higher protein carbonyl levels relative to its appropriate AL
453 control. In addition, TejJ89 had significantly higher hepatic mitochondrial H₂O₂
454 production relative to the other two strains under DR feeding. Strain TejJ114 had
455 increased hepatic total SOD activity and protein carbonyl levels relative to its AL
456 controls, but mitochondrial ROS levels were not significantly altered. Similarly, the
457 hepatic protein carbonyl levels in line TejJ114 under DR could not be explained by
458 differences in hepatic NADPH oxidase levels (Fig. S4). The mitochondrial ROS
459 profile in skeletal muscle was similar in skeletal muscle but H₂O₂ production was
460 significantly reduced by DR in TejJ114 compared to its appropriate control and when
461 compared to the other two strains under DR. Consequently, our findings further
462 question the precise role of ROS-induced oxidative damage being the mechanism
463 underpinning ageing. We can speculate that the increased ROS under DR in strain
464 TejJ89 elicited a beneficial mitohormesis-like effect [79] resulting in DR-induced
465 longevity, although this remains to be determined and does not appear to involve the
466 UPR^{mt}. As a small aside, it is particularly sobering to note that in the comprehensive
467 meta-analysis on DR and oxidative stress in rodents undertaken by Walsh *et al.* [30],
468 96% of all observations were made in male rodents, with the remaining 4%
469 undertaken using females alone or mixed sex populations.

470 In conclusion, our findings do not completely support our initial predictions (Fig.
471 1), in which we forecast a clear continuum from the stimulatory and beneficial effects
472 of DR on the mitochondrial phenotype in positive responding strain TejJ89 through to
473 predicted mitochondrial dysfunction in strain TejJ114 under DR. In the positive

474 responding strain TejJ89 we saw no evidence of a DR-induced increase in
475 mitochondrial respiratory capacity in liver and muscle mitochondria or in PGC-1 α
476 levels within liver, although nuclear PGC-1 α levels were induced in muscle.
477 Paradoxically, TejJ89 had increased hepatic mitochondrial ROS production, greater
478 SOD activity and higher hepatic protein carbonyl levels under DR, further highlighting
479 the complexity between mitochondrial respiratory capacity, mitochondrial ROS and
480 oxidative damage [80]. While strain TejJ114 under DR did show evidence of
481 mitochondrial dysfunction within liver relative to AL controls, this effect was tissue-
482 specific as was not observed in skeletal muscle. What is clear is that it will now be
483 important to investigate whether the liver-specific mitochondrial dysfunction observed
484 in strain TejJ114 under 40% DR is evident under less restrictive feedings conditions,
485 i.e. 10-30% DR, as for all these strains we do not currently know where exactly the
486 optimal longevity 'sweet-spot' for these lines under DR sits. Our data also raises the
487 possibility that DR-induced longevity in ILSXISS mice does not appear to involve
488 mitonuclear imbalance and UPR^{mt}, which also appears to be the case in worm DR
489 (*eat-2*) mutants [81]. What is also clear is that almost all we know about the potential
490 mechanisms underlying DR in rodents is based almost exclusively on C57BL/6 mice
491 [19]. Consequently, there is a need for more studies using mouse strains in addition
492 to C57BL/6 mice, e.g. ILSXISS, DBA/2, UM-HET3, as these models may help
493 provide new insights in to ageing mechanisms that are public, i.e. shared, across all
494 mouse strains rather than mechanisms that are private, i.e. specific to C57BL/6
495 mice.

496

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502

503

504 **Figure legends**

505 **Figure 1:** Schematic showing the predicted mitochondrial functional response to 10
506 months of 40% dietary restriction (DR) in three strains of female ILSXISS mice that
507 show a differential response of DR on longevity (**TejJ89 lifespan extension; TejJ48**
508 **no change in lifespan; TejJ114 lifespan shortening**).

509 **Figure 2:** Mitochondrial respiration (Oxygen consumption rate, OCR) was unaltered
510 by 10 months of 40% DR in isolated liver mitochondria from strains TejJ89 (A) and
511 TejJ48 (B). In strain TejJ114 (C), a significant treatment effect was observed with
512 State 3 and State 3u OCR significantly reduced under DR relative to AL mice. No
513 strain differences on mitochondrial functional was observed in AL mice (D). (E) State
514 3 respiratory capacity was significantly increased in strain TejJ89 under DR when
515 compared with the other strains under DR. Values are expressed as mean \pm SEM,
516 with n = 8 per group. * denotes $p < 0.05$.

517 **Figure 3:** Hydrogen peroxide (H_2O_2) production within isolated liver mitochondria,
518 expressed as fold change relative to respective AL controls. (A) H_2O_2 production was
519 increased by DR only in strain TejJ89. H_2O_2 production was unaltered between
520 strains under AL feeding (B), but under DR H_2O_2 production was elevated in strain
521 TejJ89 compared to both TejJ48 and TejJ114, and strain TejJ48 produced more
522 H_2O_2 than TejJ114. Values are expressed as mean \pm SEM, with n = 6 per group. **
523 $p < 0.001$, * $p < 0.05$.

524 **Figure 4:** Hepatic antioxidant defence and oxidative damage markers. (A) Total
525 SOD activity was significantly increased by DR in strains TejJ89 and TejJ114 relative
526 to their respective AL controls. (B) Total glutathione (GSH) and (C) 4-
527 Hydroxynonenal (HNE) levels were unaffected by treatment or strain. (D) Protein

528 carbonyl (PC) levels were significantly increased by DR in strains TejJ89 and
529 TejJ114 relative to AL controls. Values are expressed as mean \pm SEM, where n= 6
530 per group. ** p<0.001, * p<0.05.

531 **Figure 5:** Total (A), nuclear (B), and cytosolic (C) hepatic PGC-1 α protein levels. No
532 treatment or strain differences in hepatic PGC-1 α protein levels were observed (D)
533 DR or strain similarly had no effect on hepatic TFAM levels. Values are expressed as
534 arbitrary units (AU) relative to total protein (determined by Ponceau staining). All
535 values are expressed as means \pm SEM, where n = 6 per group.

536 **Figure 6:** Hepatic mitonuclear protein imbalance, expressed as the ratio between the
537 nuclear DNA (SDHB) relative to mitochondrial DNA (MTCO1). (A-C) Mitochondrial
538 protein imbalance was unaffected by treatment or strain. No treatment or strain
539 effects were detected in HSP60 levels, however hepatic HSP90 was significantly
540 reduced by DR in strain TejJ114 compared to its AL counterpart. No differences in
541 HSP90 levels were observed between strains within the AL or DR treatment groups.
542 Values are expressed as arbitrary units (AU) relative to total protein (determined by
543 Ponceau staining). All values are expressed as means \pm SEM, where n = 6 per
544 group. * p< 0.05

545 **Figure 7:** Mitochondrial respiration (Oxygen consumption rate, OCR) in isolated
546 skeletal muscle mitochondria was unaltered by 10 months of 40% DR in all strains (A
547 –C). Similarly no differences were observed between strains within the AL (D) or DR
548 groups (E). Values are expressed as mean \pm SEM, with n = 8 per group

549 **Figure 8:** Total (A), nuclear (B), and cytosolic (C) PGC-1 α protein levels in skeletal
550 muscle. No differences in PGC-1 α protein levels were observed by treatment in total
551 (A) or cytosolic (C) skeletal muscle fractions. An increase in PGC-1 α was observed

552 in nuclear PGC-1 α protein fraction with DR (B). Strain was not found to alter PGC-1 α
553 levels in total, nuclear or cytosolic proteins within either the AL or DR treatment
554 group. Values are expressed as arbitrary units (AU) relative to total protein
555 (determined by Ponceau staining). All values are expressed as means \pm SEM, where
556 n = 6 per group. * p < 0.05

557 **Figure 9:** Skeletal muscle mitochondrial hydrogen peroxide (H₂O₂) production,
558 expressed as fold change relative to respective AL controls. (A) H₂O₂ production was
559 significantly decreased by DR in strain TejJ114. H₂O₂ production was unaltered
560 between strains under AL feeding (B), but under DR feeding H₂O₂ production was
561 reduced in strain TejJ114 relative to the other two strains. Values are expressed as
562 mean \pm SEM, with n = 6 per group. ** p < 0.001, * p < 0.05.

563 **Figure 10:** Mitonuclear protein imbalance in gastrocnemius (A) and heart (B),
564 expressed as the ratio between the nuclear DNA (SDHB) relative to mitochondrial
565 DNA (MTCO1). No treatment or strains effects were detected. Values are expressed
566 as arbitrary units (AU) relative to total protein (determined by Ponceau staining). All
567 values are expressed as means \pm SEM, where n = 6 per group.

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