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

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Abstract

Dietary restriction (DR) extends lifespan and healthspan in many species, but precisely how it elicits its beneficial effects is unclear. We investigated the impact of DR on mitochondrial function within liver and skeletal muscle of female ILSXISS mice that exhibit strain-specific variation in lifespan under 40% DR. Strains TejJ89 (lifespan increased under DR), TejJ48 (lifespan unaffected by DR) and TejJ114 (lifespan decreased under DR) were studied following 10 months of 40% DR (13 months of age). Oxygen consumption rates (OCR) within isolated liver mitochondria were unaffected by DR in TejJ89 and TejJ48, but decreased by DR in TejJ114. DR had no effect on hepatic protein levels of PGC-1a, TFAM, and OXPHOS complexes I-V. Mitonuclear protein imbalance (nDNA:mtDNA ratio) was unaffected by DR, but HSP90 protein levels were reduced in TejJ114 under DR. Surprisingly hepatic mitochondrial hydrogen peroxide (H_2O_2) production was elevated by DR in TejJ89, with total superoxide dismutase activity and protein carbonyls increased by DR in both TejJ89 and TejJ114. In skeletal muscle, DR had no effect on mitochondrial OCR, OXPHOS complexes or mitonuclear protein imbalance, but H_2O_2 production was decreased in TejJ114 and nuclear PGC-1a increased in TejJ89 under DR. Our findings indicate that hepatic mitochondrial dysfunction associates with reduced lifespan of TejJ114 mice under 40% DR, but similar dysfunction is not seen in skeletal muscle mitochondria. We highlight tissue-specific differences in the mitochondrial response in ILSXISS mice to DR, and underline the importance and challenges of exploiting genetic heterogeneity to help understand mechanisms of ageing.

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

41 unfolded protein response, aging

1.1 Introduction

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

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

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

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

significant research effort has investigated whether DR can induce beneficial effects on the mitochondrial phenotype, such as maintaining mitochondrial function during ageing, reducing ROS production and attenuating oxidative damage. While it is generally assumed that DR reduces ROS production, a recent meta-analysis involving 157 rodent DR studies in which ROS levels (primarily hydrogen peroxide) were assayed, highlighted that 62% of studies actually reported no change in ROS levels relative to *ad libitum* (AL) fed controls [30]. Ambiguity also exists regarding the effect of DR on mitochondrial respiration in rodents, with DR reported to increase [31]–[34], decrease [35], or have no effect relative to AL controls [36]. Similarly, the impact of DR on various antioxidants and markers of oxidative damage appears fairly inconclusive, although as with all these parameters tissue-specific effects, the duration and intensity of DR, the sex and the age of the animals at the point of study may affect experimental outcomes [30]. The concept that DR induces mitochondrial biogenesis, that is the production of new mitochondrial proteins, has been proposed as a critical mechanism underlying the beneficial effects of DR [37], with DR reported to induce mitochondrial biogenesis in a number of tissues including liver [33]. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) is described as the master regulator of mitochondrial biogenesis [38]. DR slows age-related declines in *Pgc-1α* expression in rat skeletal muscle and heart, and it is proposed that mitochondrial function adapts to DR through PGC1α regulation [33], [39], [40]. However, there is considerable debate in the literature as to how best to quantify biogenesis. Indeed, several studies suggest that DR may increase mitochondrial efficiency in order to maintain an ‘optimally efficient’ electron transport system, potentially driven by the nuclear localisation of PGC1-α [39], [41] without any increase in mitochondrial number *per se* [31], [34], [42]. Hancock et al [42] examined

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

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

2. Materials and methods

Animals

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

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

Mitochondrial Respiration

Isolation of Mouse Liver and Skeletal Muscle Mitochondria

Liver mitochondria were isolated using previously published protocols [56], [57]. Briefly the liver was weighed and washed in MSHE+BSA buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EGTA and 0.5% (w/v) fatty acid free bovine serum albumin (pH 7.2)). The liver was rapidly minced with scissors in 10 volumes of MSHE+BSA buffer before homogenisation (2-3 strokes) using a glass-glass homogeniser (Fisher Scientific, Loughborough, UK). As with the liver, gastrocnemius muscle was harvested, weighed, washed and then minced in ice-cold isolation buffer (100 mM sucrose, 100mM KCL, 50mM Tris HCl, 1mM KH_2PO_4, 0.1mM EGTA, 0.2% BSA (pH 7.4)), as set out in protocols previously described [58]. The muscle was subsequently rinsed three times in 1ml of fresh isolation buffer, and then 1ml of 2% Proteinase Type XXIV (Sigma Aldrich, Dorset, UK) was added and the sample vortexed for 1 min^{-1} at room temperature, followed by 1 min^{-1} incubation on ice. Samples were subsequently added to a glass homogeniser and 4ml of

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

XF Assay – Plate Preparation

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

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

Protein extraction

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

Western Blot Analysis

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

Mitochondrial ROS production

Fresh respiratory medium (MAS; 70 mM sucrose, 220 mM mannitol, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 2 mM HEPES, 1.0 mM EGTA and 0.2% (w/v) fatty acid-free BSA, pH 7.2) was supplemented with 1U/ml horseradish peroxidase and 10 uM Amplex® Red reagent (ThermoFisher Scientific, UK) as previously described [59]. 90µl of this medium was then added to each well of a standard 96 well plate

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

Oxidative damage and antioxidant levels

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

Statistical Analysis

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

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

3. Results

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

Mitochondrial hydrogen peroxide (H_2O_2) production within liver was increased significantly ($t=3.555$, $p=0.002$) by DR in strain TejJ89 relative to its appropriate control, but DR had no effect on H_2O_2 production in strains TejJ48 and TejJ114 (Fig. 3A). While no difference between strains in H_2O_2 production was observed under AL feeding (Fig. 3B), strain TejJ89 had significantly higher ROS levels compared to TejJ48 and TejJ114 under DR, and TejJ48 had significantly higher ROS levels ($t=2.339$, $p=0.039$) compared to TejJ114 (Fig. 3C). Total hepatic SOD activity was increased by DR treatment in strains TejJ89 ($t = 3.776$, $p=0.004$) and TejJ114 ($t = 2.845$, $p = 0.010$) relative to their respective AL controls (Fig. 4A), but no strain-specific effect on SOD activity was observed under either AL or DR feeding (Fig. S2A & B). Liver total glutathione (Fig. 4B; Fig. S2C and D) and 4-HNE (Fig. 4C; Fig. S2E & F) were unaffected by either treatment or strain. However, hepatic protein carbonyl (PC) levels (Fig. 4D) were significantly increased in strains TejJ89 ($t=2.420$, $p=0.037$) and TejJ114 ($t=2.440$, $p=0.040$) under DR when compared to their 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-1α (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-1α
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 H_2O_2
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 H_2O_2 production was observed between strains under

AL feeding (Fig. 9B), but was significantly reduced in TejJ114 under DR relative to TejJ89 ($t=2.903$, $p=0.044$) and TejJ48 (Mann Whitney $p=0.009$). Similar to liver, total and cytosolic PGC1α levels (Fig. 8A & B) were unaffected by DR in all strains, although nuclear PGC1α levels were increased in TejJ89 ($t=3.174$, $p=0.034$). Protein levels of various OXPHOS complexes and mitonuclear protein imbalance within skeletal muscle were unaffected by treatment and strain (Figure S3D-F; Fig. 10A), as was also the case in heart (Fig. S3G-I; Fig. 10B).

4. Discussion

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

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

hepatic mitochondrial dysfunction may offer new insights in to why DR truncates lifespan in this particular ILSXISS strain. To determine whether the hepatic mitochondrial dysfunction in TejJ114 was liver-specific, we determined mitochondrial respiratory capacity in isolated skeletal muscle mitochondria in these same ILSXISS mice under AL and DR feeding. Despite DR known to preserve mitochondrial function during ageing in certain mouse strains, such as C57BL/6 and male B6D2F1 [31], [34], we saw no effect of DR on skeletal muscle mitochondrial respiratory capacity in any strain. In view of the well-defined role of PGC1α in modulating mitochondrial function, and given that that mitochondrial adaptations to DR may be driven through a PGC1α-induced transcriptional program [39], [41], [64], [72], we investigated protein levels of PGC1α within liver in each strain under AL and DR. In line with the absence of any effect of DR on mitochondrial respiratory capacity, we observed no increase in hepatic PGC1α (total, cytosolic, nuclear) or in mitochondrial transcription factor A (TFAM) protein levels of strain TejJ89 under DR. Similarly, PGC-1α and TFAM levels were unaffected by DR in strains TejJ48 or TejJ114. However, a tissue-specific response was observed with 40% DR significantly increasing nuclear PGC1α within skeletal muscle of strain TejJ89, in line with what has been reported in other studies (for review see[64]).

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

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

It is evident that both ageing and several disease states are associated with greater ROS-induced oxidative damage, but it is equivocal as to whether ROS-induced oxidative damage is the mechanism underpinning ageing and disease [30], [77], [78]. A large number of studies have investigated whether DR can reduce ROS, induce various antioxidants and attenuated oxidative damage in model organisms. In an excellent recent meta-analysis, the Van Remmen laboratory [30] examined several hundred studies that have investigated the effect of DR in rodents on ROS production, various antioxidants and on oxidative damage. Their approach found that 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 H_2O_2 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 H_2O_2
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 H_2O_2 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

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

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Figure legends

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

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

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

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

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

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

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

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

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

552 in nuclear PGC-1α protein fraction with DR (B). Strain was not found to alter PGC-1α
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 ± SEM, where
556 n = 6 per group. * $p< 0.05$

557 **Figure 9**: Skeletal muscle mitochondrial hydrogen peroxide (H_2O_2) production,
558 expressed as fold change relative to respective AL controls. (A) H_2O_2 production was
559 significantly decreased by DR in strain TejJ114. H_2O_2 production was unaltered
560 between strains under AL feeding (B), but under DR feeding H_2O_2 production was
561 reduced in strain TejJ114 relative to the other two strains. Values are expressed as
562 mean ± 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 ± SEM, where n = 6 per group.

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