Prior reproduction alters how mitochondria respond to an oxidative event

Wendy R. Hood[1], Yufeng Zhang[1,3], Halie A. Taylor[1], Noel R. Park[1,4], Abby E. Beatty[1], Ryan J. Weaver[1], Kang Nian Yap[1], and Andreas N. Kavazis[2]

[1]Department of Biological Sciences, Auburn University

[2]School of Kinesiology, Auburn University

[3]Current address: School of Health Sciences, University of Memphis

[4]Current address: Department of Molecular Biology, Princeton University

Corresponding author

Wendy R. Hood

Department of Biological Science

101 Rouse Life Science Building

Auburn University, Auburn, AL 36849

E-mail: wrh0001@auburn.edu

Summary statement

Interactions among oxidative stressors are predicted to impact performance. Two key findings are that reproduction prior to an oxidative stressor (x-irradiation) improved liver mitochondria coupling, while skeletal muscle mitochondrial density decreased.

© 2019. Published by The Company of Biologists Ltd.

Journal of Experimental Biology • Accepted manuscript

Abstract

An animal's pace of life is mediated by the physiological demands and stressors it experiences (e.g., reproduction) and one likely mechanism that underlies these effects is oxidative stress. Reproduction has been shown to increase or reduce oxidative stress under different conditions and modify mitochondrial performance. We hypothesized that the changes associated with reproduction can alter how animals respond to future oxidative stressors. We tested this theory by comparing the organ-specific mitochondrial response in female wild-derived house mice. Specifically, we compared mice that reproduced or were virgins to mice that were exposed to an oxidant (i.e., radiation) or not-exposed to radiation. We measured liver and skeletal muscle mitochondrial density, respiratory performance, enzyme activity, and oxidant production, as well as markers of oxidative damage to tissues. In the liver, prior reproduction prevented a radiation-induced reduction in mitochondrial density and increased mitochondrial respiratory performance. In skeletal muscle, prior reproduction resulted in a radiation-induced decline in mitochondrial density which could reduce the bioenergetic capacity of skeletal muscle mitochondria. Yet, electron transport chain complex I activity in skeletal muscle, which dropped with reproduction, returned to control levels following oxidant exposure. The results of this investigation indicate that prior reproduction alters the response of mitochondria to an oxidative challenge in an organ-specific manner. Such changes could have differential effects on future reproductive performance and risk of death.

Key words: reproduction; oxidative stress; life history; house mice; mitochondria

Journal of Experimental Biology • Accepted manuscript

INTRODUCTION

Mitochondrial energetics and reactive oxygen species (ROS) production play formative roles in variation in performance among individuals, populations, species, and major vertebrate taxa (Costantini, 2014; Costantini et al., 2010; Hood et al., 2018a; Speakman et al., 2015) and can alter specific life history traits. For example, differences in growth rate and body size between two populations of common frogs (*Rana temporaria*) are associated with differences in oxidative phosphorylation (OXPHOS) efficiency and the rate of ATP synthesis (Salin et al., 2012). Furthermore, high levels of oxidative damage markers in blood before reproduction, indicative of increased ROS production and/or reduced antioxidant production, have been shown to be correlated with lower litter sizes in laboratory mice (*Mus musculus domesticus*)(Stier et al., 2012), and experimental elevation of ROS induced oxidative damage in canaries (*Serinus canaria domestica*), via an inhibition of the antioxidant glutathione, delayed the onset of reproduction and reduced egg production, but did not affect hatching or fledging success (Costantini et al., 2016). Mitochondrial energetics and ROS production have also been suggested to underlie interactions among life history traits (Monaghan et al., 2009; Speakman and Garratt, 2014; Zhang and Hood, 2016). Such tradeoffs have been hypothesized to be driven by an accumulation of oxidative damage during reproduction, but emerging research suggests that this relationship is more complex.

The role of oxidative damage in life history tradeoffs has primarily been evaluated under the assumption that the performance of mitochondria, cells, and organs are negatively related to the amount of ROS to which they are exposed. Because mitochondria produce both the majority of the cell's ATP and cellular ROS, the oxidative

Journal of Experimental Biology • Accepted manuscript

cost of reproduction hypothesis proposes that high-energy expenditure during reproduction contributes to an accumulation of oxidative damage (Hood et al., 2018b; Monaghan et al., 2009; Speakman and Garratt, 2014). In turn, this somatic damage, if severe enough, could immediately impact survival or performance during the next reproductive bout. Moreover, this damage could also accumulate over several sequential reproductive events, ultimately curtailing the animal's lifespan (Costantini et al., 2010; Hood et al., 2018b; Monaghan et al., 2009). Yet, support for this hypothesis has been equivocal. In a review of 21 studies, Speakman and Garratt (2014) found no consistent impact of reproduction on oxidative stress in 7 species of birds and 8 species of mammals. Blount et al. (2016) came to a similar conclusion comparing reproductive versus non-reproductive animals in a meta-analysis which included 15 studies representing 11 species of mammals and 3 species of birds. Interestingly, Blount et al. (2016) found a positive correlation between the number of young and oxidative stress during reproduction that was not apparent in Speakman and Garratt's (2014) review.

Further, results from recent studies suggest that the response of cells to changes in ROS does not fall along the negative linear response curve, as is typically assumed in studies of ROS production or oxidative stress (Hood et al., 2018b; Zhang et al., 2018a; Zhang et al., 2018b). Instead, the response appears to be biphasic. Under the theory of mitochondrial hormesis (Ristow, 2014; Ristow and Schmeisser, 2011; Tapia, 2006), modest levels of ROS within cells stimulate an adaptive and beneficial signaling cascade that can improve mitochondrial energetics via an upregulation of antioxidants, repair molecules, and mitochondrial biogenesis (D'Autréaux and Toledano, 2007; Morimoto and Santoro, 1998; Sano and Fukuda, 2008). Under this framework, the

Journal of Experimental Biology • Accepted manuscript

threshold at which ROS exposure causes a beneficial vs negative response depends on the relative ROS levels within the cell before exposure and on the presence of any interacting stressors, such as inclement weather, stress from antagonistic interactions, and disease, which could have compounding effects on ROS levels (Hood et al., 2018b).

One approach that investigators have taken to better understand the role that ROS plays in animal performance is to evaluate how life history events are impacted by an induced change in ROS. The Costantini et al. (2016) canary study described above is an excellent example of this, where ROS induced damage was increased by experimentally inhibiting an antioxidant. Smith et al. (2014) also showed that exposure to a ROS-inducing compound (paraquat) can impact the interaction between life history events. These authors showed that mild increases in ROS production increased relative fitness (a measure that accounted for lifetime reproductive output and longevity) but when exposure was high, fitness decreased (Smith et al., 2014). Further, exercise is well known to induce a modest increase in ROS production (Guers et al., 2016; Powers and Jackson, 2008), and thus can alter endogenous ROS production. Zhang et al. (2018) found that female mice that exercised prior to reproduction gave birth to more young that were heavier at weaning compared to sedentary reproductive mice. Our laboratory has also reported that reproduction causes few lasting negative effects on mitochondrial physiology in mice and rats (Hyatt et al., 2017; Hyatt et al., 2018; Mowry et al., 2016). Instead, we found that reproduction has beneficial effects on respiratory performance of liver mitochondria in laboratory rats (*Rattus norvegicus*) (Hyatt *et al.*

Journal of Experimental Biology • Accepted manuscript

2017, 2018) and a comparable trend in the wild-derived house mouse (*Mus musculus musculus*) (Mowry et al., 2016).

With this investigation, we ask whether the change in redox environment following reproduction will alter how organs respond to a subsequent oxidative event. Based on the benefits described above, we predicted that reproduction would improve the capacity of cells to respond to a subsequent oxidative event. We used wild-derived house mice for this investigation because wild mice retain greater responsiveness to stressors than their laboratory counterparts (Abolins et al., 2017; Gaukler et al., 2015; Harper, 2008; Ruff et al., 2015; Williams et al., 2010) and they have not been actively selected for large litter sizes which could alter the energetic demand of the reproductive event. ROS exposure can be experimentally altered using a number of different methods (Koch and Hill, 2016). We selected radiation for this study because it can be applied with few extraneous side effects (Koch and Hill, 2016; Zhang et al., 2018b). To isolate the impact of prior reproduction on self-maintenance, and presumably future performance, we exposed adult female mice to x-irradiation 1-week after weaning, when reproductive tissues, and particularly the mammary tissue (Hood, pers. obs.), has regressed. Animals were euthanized four days after x-irradiation as we have shown that this time point elicits a mitohormetic response (i.e. drop in ROS, oxidative damage, and and increase in complex activity; Zhang et al., 2018b). We predicted that prior reproduction would improve the response of liver and skeletal muscle to x-irradiation relative to virgin control mice. These changes were predicted to include improved mitochondrial respiratory performance, improved mitochondrial density, improved enzymatic capacity of the electron transport chain complexes, and/or reduced oxidative

Journal of Experimental Biology • Accepted manuscript

damage. Further, within the reproductive group, we asked if number of young produced influenced this response.

MATERIALS AND METHODS

Experimental animals and procedures

Adult female wild-derived house mice (*Mus musculus* were used in this experiment. Mice were 3-4 months old when breeding was initiated and 6-8 months old at the termination of the study. These mice were descended from wild mice and were approximately 19 generations removed from the wild individuals. The experiment was conducted in August 2016-January 2017 and animals were maintained in standard polypropylene rodent boxes with a wire bar top. Paired adults were kept in 29x19x13 cm boxes. Late pregnancy females were moved to 48x27x16 cm breeder boxes until weaning. To maintain natural sensitivity to the environment, animals were kept in a building with open windows that exposed them to natural light-dark cycles and outdoor temperatures for Auburn, AL. All animals had a substantial amount of natural cotton bedding that buffered the mice and their young from low ambient temperatures. Standard rodent chow (Teklad Global Diet 2019) and water were provided *ad libitum* throughout the experimental period, and all animals were provided with a running wheel as enrichment. All husbandry and experimental procedures were approved by the Auburn University Institutional Animal Care and Use Committee (PRN 2015-2794, PRN 2015-2793).

Journal of Experimental Biology • Accepted manuscript

Female mice were randomly assigned to one of four groups (n = 10/group): 1) virgin control (no x-irradiation), 2) virgin and exposed to x-irradiation, 3) reproductive control, and 4) reproductive and exposed to x-irradiation. Age-matched mice in the virgin groups were euthanized at the same time as the reproductive mice to ensure that the age distribution was comparable between groups and that each group experienced the same changes in ambient temperatures. Mice within each group were from different parental lineages. Because cannibalism is relatively common during a female's first reproductive event in wild mice, we bred females in the reproductive groups twice to ensure that each had just successfully reared a litter at the time of x-irradiation. Males were removed when females neared parturition of their second litter Pups of both litters were weaned at 28 days. Two of the reproductive control mice were removed from the study because they failed to complete two full reproductive events.

Virgin and reproductive mice in the x-irradiation groups were x-irradiated 7 days after weaning was complete in the reproductive groups. Mice were held in a rodent plastic transport cage (37.3 x 23.4 x 14.0 cm; Innovive, San Diego, CA) and exposed to x-radiation using the PRIMUS linear accelerator (Siemens, Munich, Germany) at the Radiology Laboratory in the Auburn University College of Veterinary Medicine. To ensure an even dosage of radiation throughout the animal's body during irradiation, mice were gently restrained using a thin layer of plastic placed just above the backs of the mice and secured to the sides of the cage with tape. All x-irradiated mice were irradiated at a dose rate of 2 Gy/min for 2½ min to achieve a total dosage of 5 Gy following Zhang et al (2018b). Control mice were also taken to the Radiology Laboratory in the Auburn University College of Veterinary Medicine but were not placed

Journal of Experimental Biology • Accepted manuscript

in the linear accelerator. Four days after exposure or transport (controls), mice were anesthetized with isoflurane vapors and swiftly decapitated with a rodent guillotine.

After euthanasia, the liver and hind leg muscles (including the tibialis anterior, soleus, gastrocnemius, quadriceps, and hamstrings) were removed and weighed. The left lateral and right medial lobes of the liver and the entire right leg muscle were used for mitochondrial isolation. The left leg muscle and remaining liver were flash frozen in liquid nitrogen and stored at -80ºC for future analyses.

Mitochondrial isolation

Mitochondria were isolated following similar procedures outlined previously (Hyatt et al., 2017; Mowry et al., 2016; Zhang et al., 2018b). Excised leg muscles were trimmed to remove fat and connective tissues, weighed, and placed in 10 volumes of solution I (100 mM KCl, 40 mM Tris HCl, 10 mM Tris base, 1 mM $MgCl_2$, 1 mM EGTA, 0.2 mM ATP, and 0.15% (wt/vol) free fatty acid bovine serum albumin (BSA), pH 7.50). Muscles were minced with scissors and the mince was homogenized for 15 seconds with a polytron (Kinematica, Inc., Bohemia, NY). Protease (Trypsin) was added (5 mg/g wet muscle), and the digested mince was mixed continually for 7 minutes. Digestion was terminated by the addition of an equal volume of solution I. The homogenate was centrifuged (Heraeus Megafuge, Life Technologies Corporation, Grand Island, NY) at 500 g for 10 minutes at 4ºC and the supernatant was rapidly decanted through a double layer of cheesecloth and centrifuged at 3,500 g for 10 minutes. The supernatant was discarded, and the mitochondrial pellet was resuspended in solution I. The suspension was centrifuged at 3,500 g for 10 minutes. The supernatant was again discarded, and

Journal of Experimental Biology • Accepted manuscript

the pellet was resuspended in 10 volumes of solution II (similar to solution I, but without BSA). This resuspended pellet was subsequently centrifuged at 3,500 g for 10 minutes. The final mitochondrial pellet was suspended in 250 µl of a solution containing 220 mM mannitol, 70 mM sucrose, 10 mM Tris HCl, and 1 mM EGTA, pH 7.40. The liver was removed, weighed, and placed in 10 volumes of solution III (250 mM sucrose, 5 mM HEPES, and 1 mM EGTA), minced with scissors and the mince was homogenized with a Potter-Elvehjem PTFE pestle and glass tube (2 passes). The homogenate was centrifuged at 500 g for 10 minutes at 4ºC. The supernatant was rapidly decanted through a double layer of cheesecloth and centrifuged at 3,500 g for 10 minutes. The supernatant was discarded, and the mitochondrial pellet was resuspended in solution III. The suspension was centrifuged at 3,500 g for 10 minutes. The final mitochondrial pellet was suspended in 250 µl of a solution containing 220 mM mannitol, 70 mM sucrose, 10 mM Tris HCl, and 1 mM EGTA, pH 7.40.

Mitochondrial respiration

Mitochondria respiration was determined polarographically (Oxytherm, Hansatech Instruments, UK) following procedures outlined previously (Hyatt et al., 2017; Mowry et al., 2016; Zhang et al., 2018b). Respiration was measured using 2 mM pyruvate, 2 mM malate, and 10 mM glutamate as a substrate. Oxygen consumption was measured from isolated mitochondria under two conditions. Specifically, we report state 3 (Brand and Nicholls, 2011)(also referred to as P_{PMG} (Du et al., 2016) that is the mitochondrial oxygen consumption rate in the presence of substrates and added ADP (we added 5.0 µL of a 50 mM solution of ADP to raise the known concentration to 0.25

Journal of Experimental Biology • Accepted manuscript

mM) and state 4 (also refer to it as L_T) that is the mitochondrial oxygen consumption rate in the presence of high ATP and occurs following the phosphorylation of the added ADP in the chamber. We calculated and report respiratory control ratio (RCR) by dividing state 3 by state 4 (i.e., PPMG /LT).

Mitochondrial hydrogen peroxide

The measurement of hydrogen peroxide (H_2O_2) emission in isolated mitochondria was conducted using Amplex Red (Thermofisher, Waltham, MA) (Kavazis et al., 2009). Formation of resorufin (Amplex Red oxidation) by H_2O_2 was measured at an excitation wavelength of 545 nm and an emission wavelength of 590 nm using a Synergy H1 Hybrid plate reader (BioTek; Winooski, VT, USA), at 37ºC in a 96-well plate using succinate to initiate mitochondrial respiration. We quantified the rate of H_2O_2 production in our samples by comparing the slope from readings of resorufin formation taken every 5 minutes for 15 minutes to a standard curve of known H_2O_2 concentrations.

Electron transport chain (ETS) enzymatic activity and citrate synthase activity

Microplate spectrophotometric enzymatic assays of complex I, II, III, and IV and citrate synthase (CS) were performed as described previously (Hyatt et al., 2017; Kavazis et al., 2009; Zhang et al., 2018b) by utilizing the Synergy H1 Hybrid plate reader. Briefly, complex I (NADH dehydrogenase) enzyme activity (EC 1.6.5.3) was measured as a function of the decrease in absorbance from NADH oxidation by decylubiquinone before and after rotenone addition, complex II (succinate dehydrogenase) activity (EC 1.3.5.1) was measured as a function of the decrease in

Journal of Experimental Biology • Accepted manuscript

absorbance from 2,6-dichloroindophenol reduction, complex III (ubiquinol cytochrome c oxidoreductase) activity (EC 1.10.2.2) was determined as a function of the increase in absorbance from cytochrome c reduction, complex IV (cytochrome c oxidoreductase) activity was determined as a function of the decrease in absorbance from cytochrome c oxidation and its specificity was determined by monitoring changes in absorbance in the presence of KCN, and citrate synthase (EC 4.1.3.7) was measured as a function of the increase in absorbance from 5,5'-dithiobis-2-nitrobenzoic acid reduction (Trounce et al., 1996). Citrate synthase activity values obtained in isolated mitochondria were used as a normalizing factor for respiratory function measurements on isolated mitochondria.

Also, citrate synthase activity in whole tissue homogenate was used as a proxy for mitochondrial density (Hyatt et al., 2017; Trounce et al., 1996; Zhang et al., 2018b).

Markers of oxidative damage

Western blots were conducted as previously described (Hyatt et al., 2017; Mowry et al., 2016; Zhang et al., 2018b) on liver and skeletal muscle samples to analyze the relative levels of the following targets of oxidative damage: a marker of lipid peroxidation (4-Hydroxynonenal; 4-HNE; ab46545; Abcam, Cambridge, MA), and a marker of protein oxidation (protein carbonyls; OxyBlot; s7150; EMD Millipore, Billerica, MA). Each membrane was stained by Ponceau and was used as the loading and transfer control. A chemiluminescent system was used to visualize marked proteins (GE Healthcare Life Sciences, Pittsburgh, PA). Images were taken and analyzed with the ChemiDocIt Imaging System (UVP, LLC, Upland, CA).

Journal of Experimental Biology • Accepted manuscript

Statistical analysis

We used generalized least squares models to compare the mean effect of x-irradiation exposure, reproduction, and their interaction on eleven markers of mitochondrial physiology and oxidative stress in the liver and skeletal muscle of mice. We calculated parameters that were derived from the raw response data, such as RCR, after removing the outliers. Preliminary analyses revealed that simple linear models, which assume homoscedastic error distributions, fit the data poorly in some cases (Supplemental Figure S1). We found that the variance among reproductive and x-ray groups was not equal (Fligner Killeen test: $X^2 = 16.9$, $p = 0.0007$), which could lead to biased estimates of the standard errors. We then fit generalized least squares models with unequal variances among groups and found that the residuals were homoscedastic (Supplemental Fig 2), suggesting this model structure better fit the data. We used the results from the generalized least squares models to make statistical inferences about the effect of x-irradiation exposure and radiation and their combined effects on mitochondrial performance. However, we note that the results from the linear model are categorically similar to those of the generalized least squares models (Supplemental data).

The number of pups weaned by each individual in the reproductive groups ranged from 5 to 19 (Supplemental Figure 3). We tested for the effect of pup number on the mitochondrial response to x-ray using generalized least squares models. We estimated the mean mitochondrial response by fitting models with pup number, x-ray treatment, and the interaction between pup number and x-ray treatment as fixed effects while allowing for different variances between reproductive control and reproductive x-

Journal of Experimental Biology • Accepted manuscript

ray individuals. The interaction term between number of pups and x-ray treatment tests whether the effect of x-ray treatment depended on the number of pups weaned.

To aid in visualizing the relative effect size of x-irradiation and reproduction on markers of mitochondrial physiology we calculated the standardized mean difference, Hedges' *g*, and 95% confidence intervals (95% CI) using the control, virgin mice as the baseline for comparison. Hedges' *g* is defined as the mean difference of the response between groups divided by the pooled standard deviation (Hedges, 1981). The values are centered around zero (no difference between groups) where an absolute value of 0.2, 0.5, 0.8, 1.2, and 2 are considered, small, medium, large, very large, and huge differences in units of standard deviation, respectively (Cohen, 1988; Sawilowsky, 2009). Confidence intervals that do not include zero are considered statistically different than no difference between groups at $\alpha = 0.05$ (Nakagawa and Cuthill, 2007). The Hedges' *g* and 95% CI results are generally consistent with the results from the generalized least squares models described above (Supplemental data).

We conducted these analyses in R (version 3.3.1, R Core Team 2016). All data and corresponding code generated to perform these analyses are available from the online data repository (Dryad:XXXX).

RESULTS

All means are reported ± standard deviation. The full model results, means, standard deviations, and sample sizes are provided in Supplementary Table 1.

Journal of Experimental Biology • Accepted manuscript

Liver

The generalized least squares models included comparisons of the virgin and exposed to x-irradiation, reproductive control, and reproductive and exposed to x-irradiation groups to the virgin control group (Figure 1, Supplementary Table 1). In addition, we evaluated the interactions among these groups (Figure 2, Supplementary Table 1). While each of the comparisons were included in the omnibus test, we describe the effects of each treatment independently. First, we found that liver mitochondrial density was lower in reproductive control compared to virgin control (CS activity, virgin control: 613 ± 197 nmol/min/mg protein; reproductive control: 423± 164 nmol/min/mg protein, $t = -2.23$ $p = 0.032$, Figure 1A). No other robust effects of reproduction were detected (Figure 1, Supplementary Data 1). We also report that the liver mitochondria of virgin x-irradiated mice tended to have slightly lower state 3 respiration compared to virgin control (virgin control: 52.8 ± 27.9, virgin x-ray: 35.6 ± 11.5 pmol/min/CS activity, $t = -1.80$, $p = 0.081$, Fig. 1a) and state 4 respiration (virgin control: 12.6 ± 7.3, virgin x-ray: 7.80 ± 2.64 pmol/min/CS activity $t = -1.95$, $p = 0.060$, Fig. 1a). We found that mitochondrial density decreased by nearly half in mice that were x-irradiated (virgin control: 613 ± 197 nmol/min/mg protein, virgin x-ray: 300 ± 135 nmol/min/mg protein, $t = -4.14$ $p < 0.001$, Figure 1A). However, complex II activity was higher in virgin x-irradiated mice than in virgin control mice (virgin control: 172 ± 99 nmol/min/mg protein, virgin x-ray: 299 ± 96 nmol/min/mg protein, $t = 2.89$ $p = 0.007$, Figure 1B). ROS production from liver mitochondria of virgin x-irradiated mice was lower than that of

Journal of Experimental Biology • Accepted manuscript

virgin control mice (virgin control: 4790 ± 1784, virgin x-ray: 3197 ± 826 pmol/min/mg protein, $t = -2.56$ $p = 0.015$, Figure 1C).

Comparing the mice that reproduced and were exposed to x-irradiation to the virgin control mice, we found that the liver mitochondria RCR was higher than controls (virgin control: 4.48 ± 1.25, repro-x-ray: 6.61 ± 1.02 RCR $t = -4.17$ $p < 0.001$, Figure 1A). In addition, we also observed a trend suggesting that state 4 respiration is lower in reproductive x-irradiation mice than virgin control (virgin control: 12.6 ± 7.3, repro-x-ray: 8.01 ± 2.66 pmol/min/CS activity $t = -1.95$ $p = 0.070$, Figure 1A).

Finally, when we examined the interaction between reproduction and x-irradiation exposure we found that the effect of x-irradiation exposure on mitochondrial density depended on the reproductive status of the female (reproductive * x-ray, $t = 3.18$, $p = 0.003$). X-irradiation reduced CS activity of virgins but did not change CS activity of reproductive mice (Figure 2).

Skeletal muscle

Like the models for the liver, generalized least squares models for skeletal muscle compared the reproductive group to the virgin x-ray, reproductive control, and reproductive-x-ray group to the virgin control mice (Figure 3, Supplementary Table 1). In addition, we evaluated the interaction among these groups (Figure 4, Supplementary Table 1). Skeletal muscle mitochondria of reproductive controls had lower complex I and III activity (complex I: virgin control: 945 ± 450 nmol/min/mg protein, reproductive control: 440 ± 282 nmol/min/mg protein, $t = -2.84$ $p = 0.008$, complex III: virgin control: 1640 ± 306 nmol/min/mg protein, reproductive control: 1173 ± 504 nmol/min/mg protein,

Journal of Experimental Biology • Accepted manuscript

$t = -2.30$ $p = 0.029$, Figure 3B). X-irradiation also lowered complex III activity (virgin control: 1640 ± 306 nmol/min/mg protein, virgin x-ray: 1261 ± 360 nmol/min/mg protein, $t = -2.53$ $p = 0.016$. Figure 3B). Female mice that reproduced and were exposed to x-ray also had lower complex III activity than virgin control mice (virgin control: 1640 ± 306 nmol/min/mg protein, reproductive x-ray: 904 ± 288 nmol/min/mg protein, $t = -5.53$ $p < 0.001$, Figure 3B) and mitochondrial density that was more than twice as low as the controls (CS activity, virgin control: 1179 ± 944 nmol/min/mg protein, reproductive x-ray: 735 ± 899 nmol/min/mg protein, $t = -2.253$ $p = 0.016$, Figure 3A).

We found two instances in which the effect of x-irradiation depended on the reproductive history of the mouse. X-irradiation significantly decreased mitochondrial density in females that reproduced previously (reproductive * x-ray, $t = -3.98$ $p = <0.001$, Figure 4A). We also found and interaction effect for complex I activity reproductive * x-ray, $t = 2.54$ $p = 0.016$, Figure 4B).

The effect of litter size on mitochondrial performance

Females in the reproductive control and reproductive x-ray groups did not differ in the mean number of pups they weaned prior to the experiment (mean ± sd, reproductive control: 12.8 ± 4.2 pups, reproductive control x-ray: 11.8± 3.7 pups, $z = -0.6$, $p = 0.57$; Supplemental Figure 3). We tested for an effect of litter size on mitochondrial physiology and found several significant relationships. We plotted the data from both the reproductive control group and the reproductive animals that were x-irradiated; the interactions between females that were and were not irradiated were not significant for any of variables measured ($p > 0.12$). In the reproductive control group,

Journal of Experimental Biology • Accepted manuscript

for every 1 pup weaned, liver RCR values increased by 0.32 (± 0.12 se, $p = 0.019$) and ROS production increased by 397 (± 190 pmol/min/mg protein se, $t = 2.68$, $p = 0.055$, Figure 5A,B). In addition, for every 1 pup weaned, skeletal muscle RCR values increased by 0.15 (± 0.02 se, $p < 0.001$, Figure 5C) while CS decreased by 84.0 ± 32.8 nmol/min/mg protein (t = -2.56. p = 0.023, Figure 5D).

In addition, we did note non-linear patterns in some response measures, such as complex IV activity of liver mitochondria of x-ray mice (Supplementary Fig 4). These patterns could be interpreted as a hormetic response, but we don't feel we have enough data to use the generalized additive model necessary to test this response.

DISCUSSION

The capacity for and efficiency of OXPHOS within mitochondria is plastic (Adhihetty et al., 2003; Brand, 2005). As a consequence, an organism's mitochondria are predicted to respond differently to a comparable oxidative event at different points in their life cycle. Because it has been proposed that reproduction is associated with an oxidative cost that could impact future performance (Monaghan et al., 2009; Speakman and Garratt, 2014), we hypothesized that reproduction could alter how a female's mitochondria respond to a subsequent oxidative event. Our results indicate that prior reproduction improves the ability of mitochondria in the liver to respond to an oxidative event by increasing respiratory coupling (RCR, Figure 1A) and conferring protection against a reduction in mitochondrial density (Figure 2). In contrast, prior reproduction reduced mitochondrial density and complex III activity in skeletal muscle mitochondria when coupled with an oxidative challenge (Figure 4A). At the same time x-irradiation

Journal of Experimental Biology • Accepted manuscript

appears to recover the post-reproduction drop in complex I (Figure 4B). These results suggest that response to oxidant exposure is both organ-specific and varies with the life history traits of the individual.

Effects on liver

While the focus of this investigation is on the interaction between prior reproduction and oxidant exposure, we also evaluated the effects of reproduction, x-irradiation, and reproduction and x-irradiation on the bioenergetic performance of individual organs and the effects of prior reproductive output (total pups weaned) on each of these variables. Reproduction is a period of high metabolic demand and morphological change. By measuring the impact of reproduction on the bioenergetic capacity of organs after reproduction has ended and the reproductive tissues have regressed, we can evaluate the impact of reproduction on future performance. Prior reproduction had a negative impact on the density of mitochondria in the liver of female mice (Figure 1A), but no other independent impacts of treatment groups were found. In contrast, the respiratory performance of mitochondria (RCR, Figure 5A) and ROS production in the liver (Figure 5B) were positively correlated with the reproductive output of females.

During reproduction and lactation, in particular, the liver increases in size and alters its metabolic processes to support the high demand for glucose and lipids for milk synthesis by active mammary glands (Hollister et al., 1987; Zhang et al., 2017). Prior studies suggest that when a female is relatively young and experiences abundant resources and minimal stress, prior reproduction can result in persistent improvement in

Journal of Experimental Biology • Accepted manuscript

the metabolic capacity of the liver (Hyatt et al., 2017; Hyatt et al., 2018; Mowry et al., 2016). The effect size was not strong enough to determine if this was also true for females in this study. The observed drop in mitochondrial density must then be consider a residual cost of reproduction, as it should reduce the energetic capacity of the liver relative to virgin mice.

Perhaps even more interesting were the positive relationships between the number of pups females weaned and respiratory performance (RCR) and ROS production by liver mitochondria (Figure 5A,B). There are two likely reasons that positive correlations between RCR and ROS and reproductive output were observed. It is possible that individual mice had inherent differences in the physiological capacity of their liver mitochondria which increased their reproductive capacity and thus, higher reproductive output was a consequence of greater RCR and ROS. Alternatively, the intensity of reproduction altered the performance of the liver mitochondria and as a consequence, RCR and ROS increases in response to reproductive output. ROS act as signaling molecules that can increase mitochondria performance, but it can also contribute to the accumulation of oxidative damage (Hood et al., 2018b; Ristow and Schmeisser, 2011; Zhang and Hood, 2016). In a meta-analysis, Blount et al (2016) described a similar positive relationship between reproductive performance and oxidative damage (a consequence of high ROS) which suggested that higher reproductive output is associated with greater oxidative stress costs. The positive relationship between reproductive output and RCR, combine with no effect of reproductive output on lipid peroxidation or protein carbonyls, suggests the animals in this study were unlikely have experienced a high cost to high reproductive performance.

Journal of Experimental Biology • Accepted manuscript

The modest oxidant exposure used for this experiment also had beneficial effects on liver mitochondria. X-irradiation increased complex II activity, in addition to reducing ROS production (Figure 1B,C). These findings are consistent with the patterns observed by Zhang *et al.* (2018b) for the sample variable at the same level of x-irradiation exposure. Yet despite these benefits, the virgin mice in this study experienced a reduction in mitochondrial density following x-irradiation. Zhang *et al.* (2018b) did not find any changes in CS activity between across the 1h, 1, 4 and 10 day time points post-irradiation.

Finally, we evaluted the impact of prior reproduction the response of liver to an oxidant in two different ways, by comparing the repro-xray group to the control mice and by testing for an interaction between the reproduction and x-ray groups. We show that when females that had reproduced and experienced an oxidative event, the RCR in the liver was nearly 50% greater than virgin control mice (Figure 1A). This finding suggests a postive syngery bewteen prior breeding and how the liver mitochondria respond to an oxidative event. Further, we found that prior reproduction appeared to protect the liver from the drop in mitochondrial density observed after x-irradiation in virgin mice (Figure 2) and did so without an apparent effect of prior reproduction output. It is feasible that the physiological changes associated with reproduction could improve the hepatic cellular response to subsequent stressors. These effects could allow females to maintain high metabolic function during subsequent reproductive events.

Journal of Experimental Biology • Accepted manuscript

Effects on skeletal muscle

Prior reproduction and x-irradiation reduced the activities of complexes I (reproduction only) and III (reproduction and x-irradiation) in skeletal muscle (Figure 3B). Complex I and III are the primary sites of ROS production within the electron transport system (Brand, 2016; Murphy, 2009). When each of these complexes is inhibited, either pharmacologically (Kwong & Sohal, 1998) or in response to ischemia (Kavazis et al., 2009), ROS production is increased. Despite these observed responses in prior studies, we found no change in ROS or oxidative damage with prior reproduction or x-irradiation. It is possible that our methods or timing of sample collection prevented us from detecting a change in ROS associated with drops in complex I and III activities. If so, we have no evidence that these changes had a negative impact on the condition or performance of the mitochondria. Using the same dose of radiation described here, Zhang et al. (2018b) found that complex I activity of mitochondria in skeletal muscle had dropped 1 h after exposure but was in the process of rebounding to levels that exceeded the controls by day 7, but on day 4, the response was not different from the controls. In a prior study, we found that rats displayed a reduction in ROS emission in skeletal muscle following lactation (Hyatt et al., 2018).

Despite the fact that number of reproductive events did not directly impact mitochondrial respiratory function, we did find a significant positive relationship between prior reproductive output and RCR, as observed in liver, but this was concurrent with a negative correlation between reproductive output and mitochondrial density (Figure 5C).

Journal of Experimental Biology • Accepted manuscript

Combined, these disparate findings suggest that the response of skeletal muscle to an oxidative event may be highly context dependent.

Furthermore, complex III activity in the mice that reproduced and were later subjected an oxidant was lower signicantly lower than control group suggestive of an additive effect of prior reproduction and ROS exposure (Figure 3B). Futher, we found that mitochondrial density in skeletal muscle dropped following oxidant exposure in females that reproduced, and that effect was greater in females that had given birth to more young. Interestingly, mitochondrial density was not impacted by the oxidative event in females that had not bred. Exposure to an oxidative event following reproduction reduce the bioenergetic capacity of muscle tissue. Despite this negative impact, oxidant exposure increased the activity of complex I in skeletal muscle, stimulating a possible compensation for the reproduction-induced drop in mitochondrial density (Figure 4).

Conclusions

The goal of this study was to understand how prior reproduction impacts an animal's response to a oxidative event, such as a subsequent reproductive event, stress, or pathogen exposure (Costantini & Moller 2009; Costantini 2014; Speakman & Garratt 2014; Blount et al. 2016; Leonardo et al. 2016; Salmón et al. 2018). It is particularly interesting there were more positive effects on the interaction between prior reproduction and ROS exposure on the liver than skeletal muscle. The liver plays an important role in producing glucose and fatty acids that will be made available to the young during pregnancy and lactation and further, the cells of the liver are mitotic and

Journal of Experimental Biology • Accepted manuscript

have substantial opportunity for renewal. In contrast, skeletal muscle cells are largely post-mitotic, suggesting the opportunity to for tissue renewal is substantially reduced. It is feasible that repeated reproduction and repeated oxidative effects could ultimately compromise skeletal muscle performance, making a prey species, such as a mouse more susceptible to predation. Yet, it is important to note that x-irradiation is an acute oxidant and many ecologically relative oxidative stressors that alter individual performance in the wild are chronic. While our findings confirm that the physiological changes that occur with reproduction alter how a female responds to oxidative stress, differences in the intensity and duration of the oxidative event will undoubtedly impact the outcome.

Here we provide evidence that the effects of ROS on mitochondrial performance are mediated by prior reproduction. Our results support the findings of prior studies by Stier et al. (2012), Costantini et al. (2016), and Zhang et al. (2018) which suggest that increased ROS production not only alters reproduction but also acts as a feedback signal that alters mitochondrial physiology and subsequent life-history events. Further work is needed to evaluate the fitness consequences of the observed changes in mitochondrial performance. It would be particularly interesting to evaluate the interaction between reproduction and oxidant exposed males where variation in performance among individuals can be especially high.

Journal of Experimental Biology • Accepted manuscript

Acknowledgements

We would like to thank the Hood lab undergraduates who assisted in maintaining the mice and the members of the Hood and Hill labs for their comments on an earlier version of this manuscript.

Competing interests

The authors declare no completing or financial interests.

Author Contributions

WRH, YZ, ANK conceived the ideas and designed methodology;

WRH, YZ, HT, NP, AB, ANK collected the data;

RJW, WRH, KNY analyzed the data;

WRH, ANK led the writing of the manuscript.

Funding

This work was supported by the National Science Foundation grants IOS1453784 and OIA1736150. The authors declare no conflict of interest.

Journal of Experimental Biology • Accepted manuscript

References

Abolins, S., King, E. C., Lazarou, L., Weldon, L., Hughes, L., Drescher, P., Raynes, J. G., Hafalla, J. C. R., Viney, M. E. and Rile, E. M. (2017). The comparative immunology of wild and laboratory mice, Mus musculus domesticus. *Nat. Commun.* **8**, 1–13.

Adhihetty, P. J., Irrcher, I., Joseph, A.-M., Ljubicic, V. and Hood, D. A. (2003). Plasticity of skeletal muscle mitochondria in response to contractile activity. *Exp. Physiol.* **88**, 99–107.

Blount, J. D., Vitikainen, E. I. K., Stott, I. and Cant, M. A. (2016). Oxidative shielding and the cost of reproduction. *Biol. Rev.* **91**, 483–497.

Brand, M. D. (2005). The efficiency and plasticity of mitochondrial energy transduction. *Biochem. Soc. Trans.* **33**, 897.

Brand, M. D. (2016). Mitochondrial generation of superoxide and hydrogen peroxide as the source of mitochondrial redox signaling. *Free Radic. Biol. Med.* **100**, 1–18.

Brand, M. D. and Nicholls, D. G. (2011). Assessing mitochondrial dysfunction in cells. *Biochem. J.* **435**, 297–312.

Cohen, J. (1988). *Statistical power analysis for the behavioral sciences.:* 2nd ed. Hillsdale, NJ: Lawrence Erlbaum Associates.

Costantini, D. (2014). Oxidative stress and hormesis in evolutionary ecology and physiology. In *A marriage between mechanistic and evolutionary approaches*, p. 362. Berllin: Springer.

Costantini, D., Metcalfe, N. B. and Monaghan, P. (2010). Ecological processes in a hormetic framework. *Ecol. Lett.* **13**, 1435–1447.

Costantini, D., Casasole, G., AbdElgawad, H., Asard, H., Eens, M. and Hopkins, W. (2016). Experimental evidence that oxidative stress influences reproductive decisions. *Funct. Ecol.* **30**, 1169–1174.

D'Autréaux, B. and Toledano, M. B. (2007). ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat. Rev. Mol. cell Biol.* **8**, 813–824.

Du, S. N. N., Mahalingam, S., Borowiec, B. G. and Scott, G. R. (2016). Mitochondrial physiology and reactive oxygen species production are altered by hypoxia acclimation in killifish (Fundulus heteroclitus). *J. Exp. Biol.* 1130–1138.

Gaukler, S. M., Ruff, J. S., Galland, T., Kandaris, K. A., Underwood, T. K., Liu, N. M., Young, E. L., Morrison, L. C., Yost, G. S. and Potts, W. K. (2015). Low-dose paroxetine exposure causes lifetime declines in male mouse body weight, reproduction and competitive ability as measured by the novel organismal performance assay. *Neurotoxicol Teratol* **47**, 46–53.

Guers, J. J., Kasecky-Lardner, L., Farquhar, W. B., Edwards, D. G. and Lennon-Edwards, S. (2016). Voluntary exercise attenuates reactive oxygen species mediated endothelial eysfunction in rodents red a high sodium diet independent of blood pressure. *FASEB J.* **30**, 1210–1287.

Harper, J. M. (2008). Wild-derived mouse stocks: An underappreciated tool for aging research. *Age (Omaha).* **30**, 135–145.

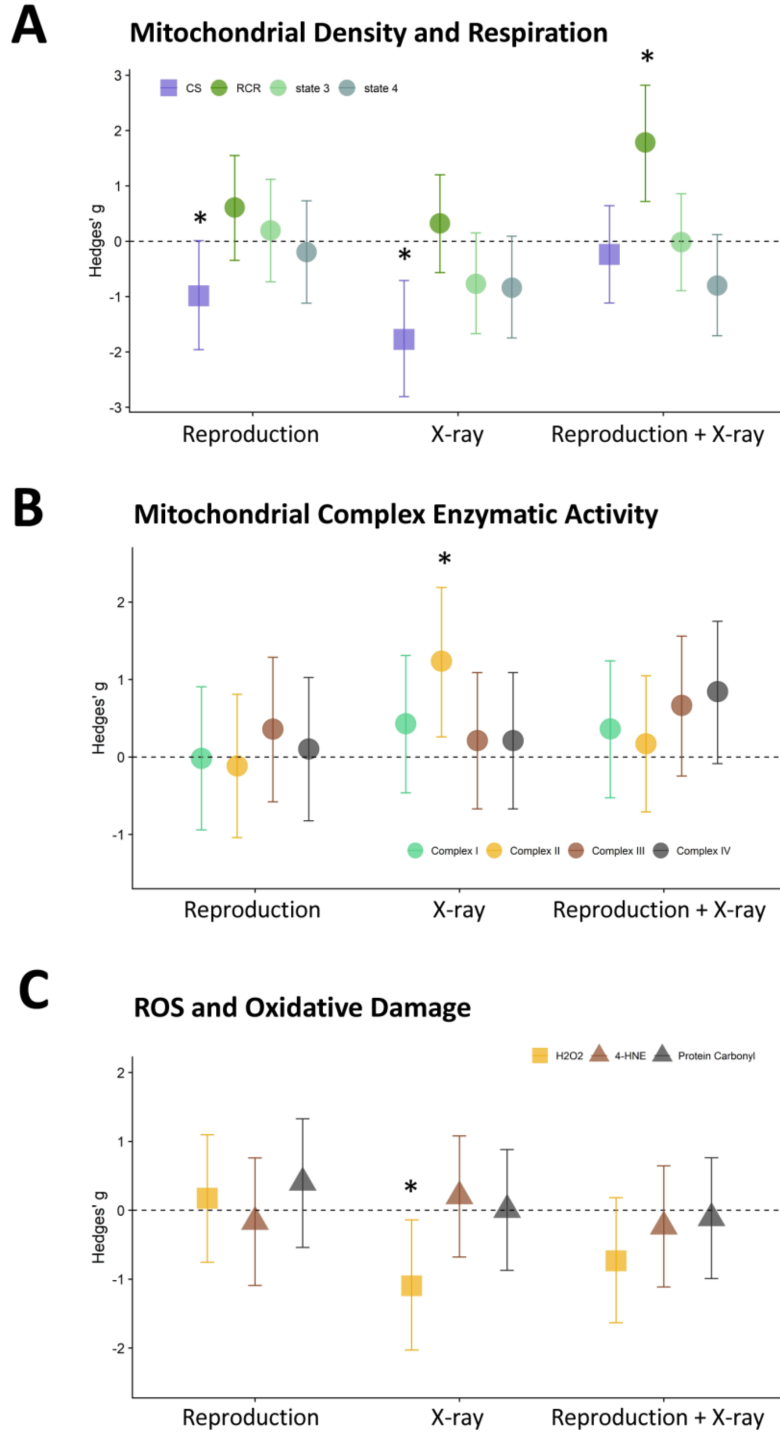
Hedges, L. V. (1981). Distribution Theory for Glass's Estimator of Effect size and

Related Estimators. *J. Educ. Stat.* **6**, 107–128.
Hollister, A., Okubara, P., Watson, J. G. and Chaykin, S. (1987). Reproduction in mice: Liver enlargement in mice during pregnancy and lactation. *Life Sci.* **40**, 11–18.
Hood, W. R., Austad, S. N., Bize, P., Jimenez, A. G., Montooth, K. L., Schulte, P. M., Scott, G. R., Sokolova, I., Treberg, J. R. and Salin, K. (2018a). The Mitochondrial Contribution to Animal Performance, Adaptation, and Life-History Variation. *Integr. Comp. Biol.* **58**, 480–485.
Hood, W. W. R., Zhang, Y., Mowry, A. A. V., Hyatt, H. W. H. and Kavazis, A. N. (2018b). Life history trade-offs within the context of mitochondrial hormesis. *Integr. Comp. Biol.* **58**, 567–577.
Hyatt, H. W., Zhang, Y., Hood, W. R. and Kavazis, A. N. (2017). Lactation has persistent effects on a mother's metabolism and mitochondrial function. *Sci. Rep.* **7**, 17118.
Hyatt, H. W. H. W., Zhang, Y., Hood, W. R. W. R. and Kavazis, A. N. A. N. (2018). Physiological, mitochondrial, and oxidative stress differences in the presence or absence of lactation in rats. *Reprod. Biol. Endocrinol.* **16**, 2–14.
Kavazis, A. N., Talbert, E. E., Smuder, A. J., Hudson, M. B., Nelson, W. B. and Powers, S. K. (2009). Mechanical ventilation induces diaphragmatic mitochondrial dysfunction and increased oxidant production. *Free Radic. Biol. Med.* **46**, 842–850.
Koch, R. E. and Hill, G. E. (2016). An assessment of techniques to manipulate oxidative stress in animals. *Funct. Ecol.* **31**, 9–21.
Kwong, L. K. and Sohal, R. S. (1998). Substrate and site specificity of hydrogen peroxide generation in mouse Mitochondria. *Arch. Biochem. Biophys.*
Monaghan, P., Metcalfe, N. B., Torres, R. and Dev, S. (2009). Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. *Ecol. Lett.* **12**, 75–92.
Morimoto, R. I. and Santoro, M. G. (1998). Stress–inducible responses and heat shock proteins: New pharmacologic targets for cytoprotection. *Nat. Biotechnol.* **16**, 833–838.
Mowry, A. V., Kavazis, A. N., Sirman, A. E., Potts, W. K. and Hood, W. R. (2016). Reproduction Does Not Adversely Affect Liver Mitochondrial Respiratory Function but Results in Lipid Peroxidation and Increased Antioxidants in House Mice. *PLoS One* **11**, e0160883.
Murphy, M. P. (2009). How mitochondria produce reactive oxygen species. *Biochem. J.* **417**, 1–13.
Nakagawa, S. and Cuthill, I. C. (2007). Effect size, confidence interval and statistical significance: a practical guide for biologists. *Biol. Rev.* **82**, 591–605.
Powers, S. K. and Jackson, M. J. (2008). Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. *Physiol. Rev.* **88**,.
Ristow, M. (2014). Unraveling the truth about antioxidants: mitohormesis explains ROS-induced health benefits. *Nat Med* **20**, 709–711.
Ristow, M. and Schmeisser, S. (2011). Extending life span by increasing oxidative stress. *Free Radic. Biol. Med.* **51**, 327–336.
Ruff, J. S., Hugentobler, S. A., Suchy, A. K., Sosa, M. M., Tanner, R. E., Hite, M. E., Morrison, L. C., Gieng, S. H., Shigenaga, M. K. and Potts, W. K. (2015).

Compared to sucrose, previous consumption of fructose and glucose monosaccharides reduces survival and fitness of female mice. *J Nutr* **145**, 434–441.
Salin, K., Roussel, D., Rey, B. and Voituron, Y. (2012). David and Goliath: A mitochondrial coupling problem? *J. Exp. Zool. Part a-Ecological Genet. Physiol.* **317A**, 283–293.
Sano, M. and Fukuda, K. (2008). Activation of mitochondrial biogenesis by hormesis. *Circ. Res.* **103**,.
Sawilowsky, S. S. (2009). New Effect Size Rules of Thumb. *J. Mod. Appl. Stat. Methods*.
Smith, S. W., Latta, L. C., Denver, D. R. and Estes, S. (2014). Endogenous ROS levels in C. elegans under exogenous stress support revision of oxidative stress theory of life-history tradeoffs. *BMC Evol. Biol.* **14**, 161.
Speakman, J. R. and Garratt, M. (2014). Oxidative stress as a cost of reproduction: Beyond the simplistic trade-off model. *BioEssays* **36**, 93–106.
Speakman, J. R., Blount, J. D., Bronikowski, A. M., Buffenstein, R., Isaksson, C., Kirkwood, T. B. L. L., Monaghan, P., Ozanne, S. E., Beaulieu, M., Briga, M., et al. (2015). Oxidative stress and life histories: unresolved issues and current needs. *Ecol. Evol.* **5**, 5745–5757.
Stier, A., Reichert, S., Massemin, S., Bize, P. and Criscuolo, F. (2012). Constraint and cost of oxidative stress on reproduction: correlative evidence in laboratory mice and review of the literature. *Front Zool* **9**, 37.
Tapia, P. C. (2006). Sublethal mitochondrial stress with an attendant stoichiometric augmentation of reactive oxygen species may precipitate many of the beneficial alterations in cellular physiology produced by caloric restriction, intermittent fasting, exercise and dietary p. *Med. Hypotheses* **66**, 832–843.
Trounce, I. A., Kim, Y. L., Jun, A. S. and Wallace, D. C. (1996). Assessment of mitochondrial oxidative phosphorylation in patient muscle biopsies, lymphoblasts, and transmitochondrial cell lines. *Methods Enzym.* **264**, 484–509.
Williams, J. B., Miller, R. A., Harper, J. M. and Wiersma, P. (2010). Functional linkages for the pace of life, life-history, and environment in birds. *Integr. Comp. Biol.* **50**, 855–868.
Zhang, Y. and Hood, W. R. (2016). Current versus future reproduction and longevity: a re-evaluation of predictions and mechanisms. *J. Exp. Biol.* **219**, 3177–3189.
Zhang, Y., Kallenberg, C., Hyatt, H. W., Kavazis, A. N. and Hood, W. R. (2017). Change in the lipid transport capacity of the liver and blood during reproduction in rats. *Front. Physiol.* **8**, https://doi.org/10.3389/fphys.2017.00517.
Zhang, Y., Brasher, A. L., Park, N. R., Taylor, H. A., Kavazis, A. N. and Hood, W. R. (2018a). High activity before breeding improves reproductive performance by enhancing mitochondrial function and biogenesis. *J. Exp. Biol.* **221**, jeb.177469.
Zhang, Y., Humes, F., Almond, G., Kavazis, A. N. and Hood, W. R. (2018b). A mitohormetic response to pro-oxidant exposure in the house mouse. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* **314**, R122–R134.

Journal of Experimental Biology • Accepted manuscript

Figures



Journal of Experimental Biology • Accepted manuscript

Fig. 1. The relative effects of prior reproduction and oxidant exposure via x-radiation on the liver of wild-derived mice. Data include mitochondrial density and respiration (A), the enzymatic activity of the mitochondrial complexes (B) and ROS and oxidative damage in wild-derived house mice (C). Reproductive measurements were taken 11 days after weaning. Animals were exposed to radiation 7 days after weaning and measurements were taken 4 days later. All virgin and non-irradiated control mice were age-matched to the experimental mice. Points and error bars are showing Hedges' *g* and 95% confidence intervals compared to the virgin control group. '*' indicates that the response was significantly different from the virgin controls from generalized least squares models. Sample sizes per group were virgin-control n=10, virgin-xray n=10, reproduction-control n=8, and reproduction-xray n=10. Absolute values and the results of the generalized least squares models are provided in supplementary table 1.

Journal of Experimental Biology • Accepted manuscript

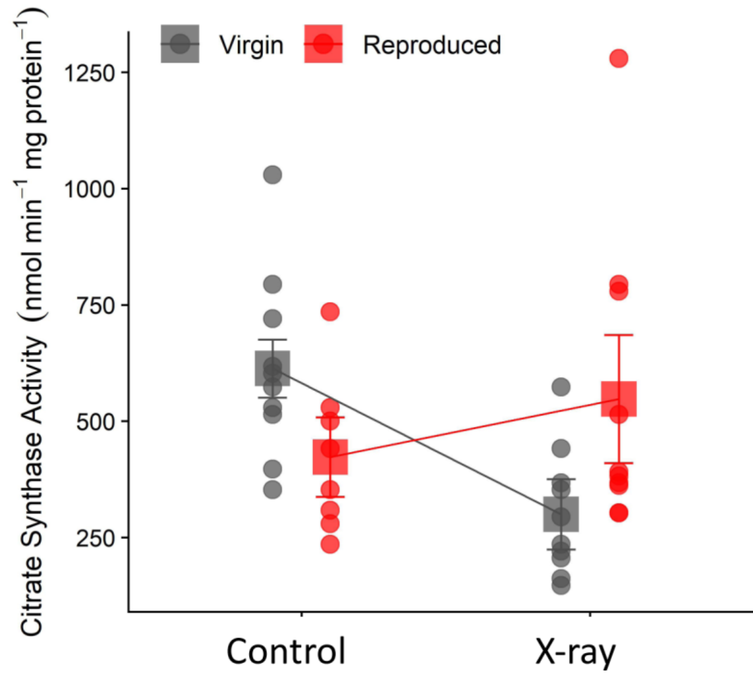
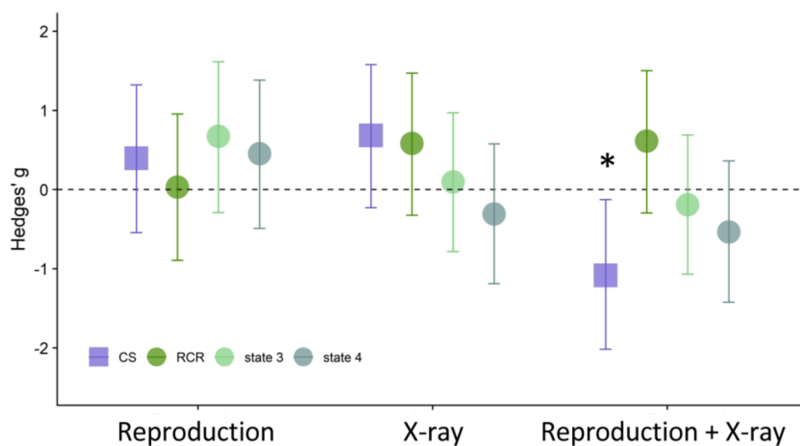


Fig. 2. The effect of prior reproduction on mitochondrial density in the liver of wild-derived mice following an oxidative event. Citrate synthase (CS) activity of virgin mice (gray) and mice that had reproduced (red) after exposure to x-ray irradiation and unexposed control mice (t = 3.18, p = 0.003). Reproductive measurements were taken 11 days after weaning. Animals were exposed to radiation 7 days after weaning and measurements were taken 4 days later. All virgin and non-irradiated control mice were age-matched to the experimental mice. Interactions were evaluated using generalized least squares models. Squares and error bars are showing the least-squares estimated mean and s.e. while points represent the individual samples within each group. Sample sizes per group were virgin-control n=10, virgin-xray n=10, reproduction-control n=8, and reproduction-xray n=10. Absolute values are provided in supplementary table 1.

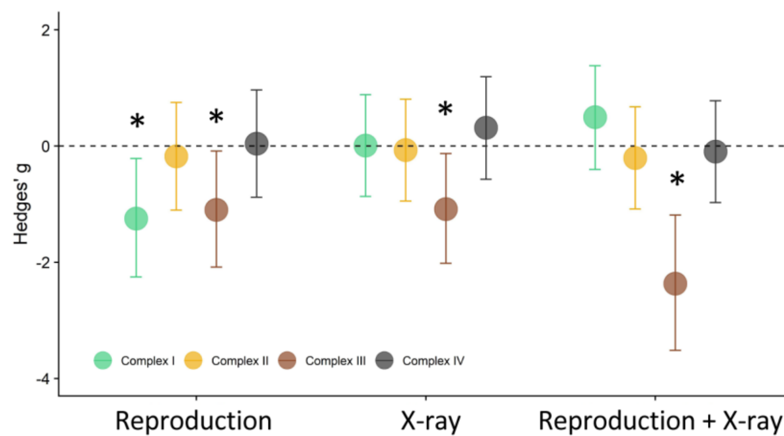
Journal of Experimental Biology • Accepted manuscript

Skeletal Muscle

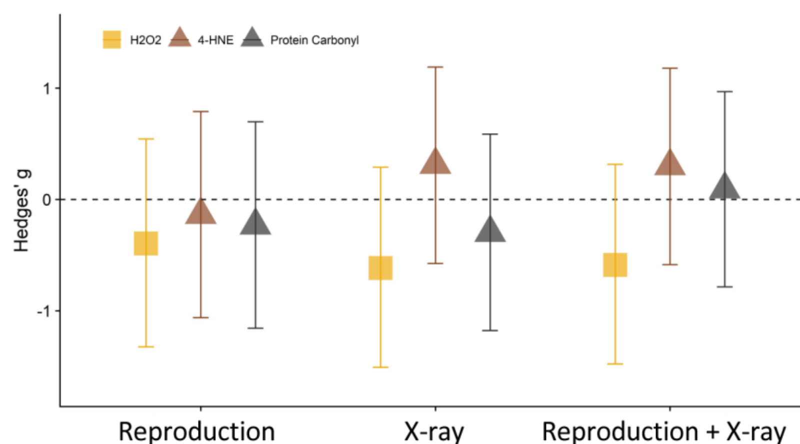
A **Mitochondrial Density and Respiration**



B **Mitochondrial Complex Enzymatic Activity**



C **ROS and Oxidative Damage**



Journal of Experimental Biology • Accepted manuscript

Fig. 3. The relative effects of prior reproduction and oxidant exposure via x-radiation on the skeletal muscle of wild-derived mice. Data include mitochondrial density and respiration (A), the enzymatic activity of the mitochondrial complexes (B) and ROS and oxidative damage in age-matched wild-derived house mice (C). Reproductive measurements were taken 11 days after weaning. Animals were exposed to radiation 7 days after weaning and measurements were taken 4 days later. All virgin and non-irradiated control mice were age-matched to the experimental mice. Points and error bars are showing Hedges g and 95% confidence intervals compared to the virgin control group. '*' indicates that the response was significantly different from the virgin controls from generalized least squares models. Sample sizes per group were virgin-control n=10, virgin-xray n=10, reproduction-control n=8, and reproduction-xray n=10. Absolute values and the results of the generalized least squares models are provided in supplementary table 1.

Journal of Experimental Biology • Accepted manuscript

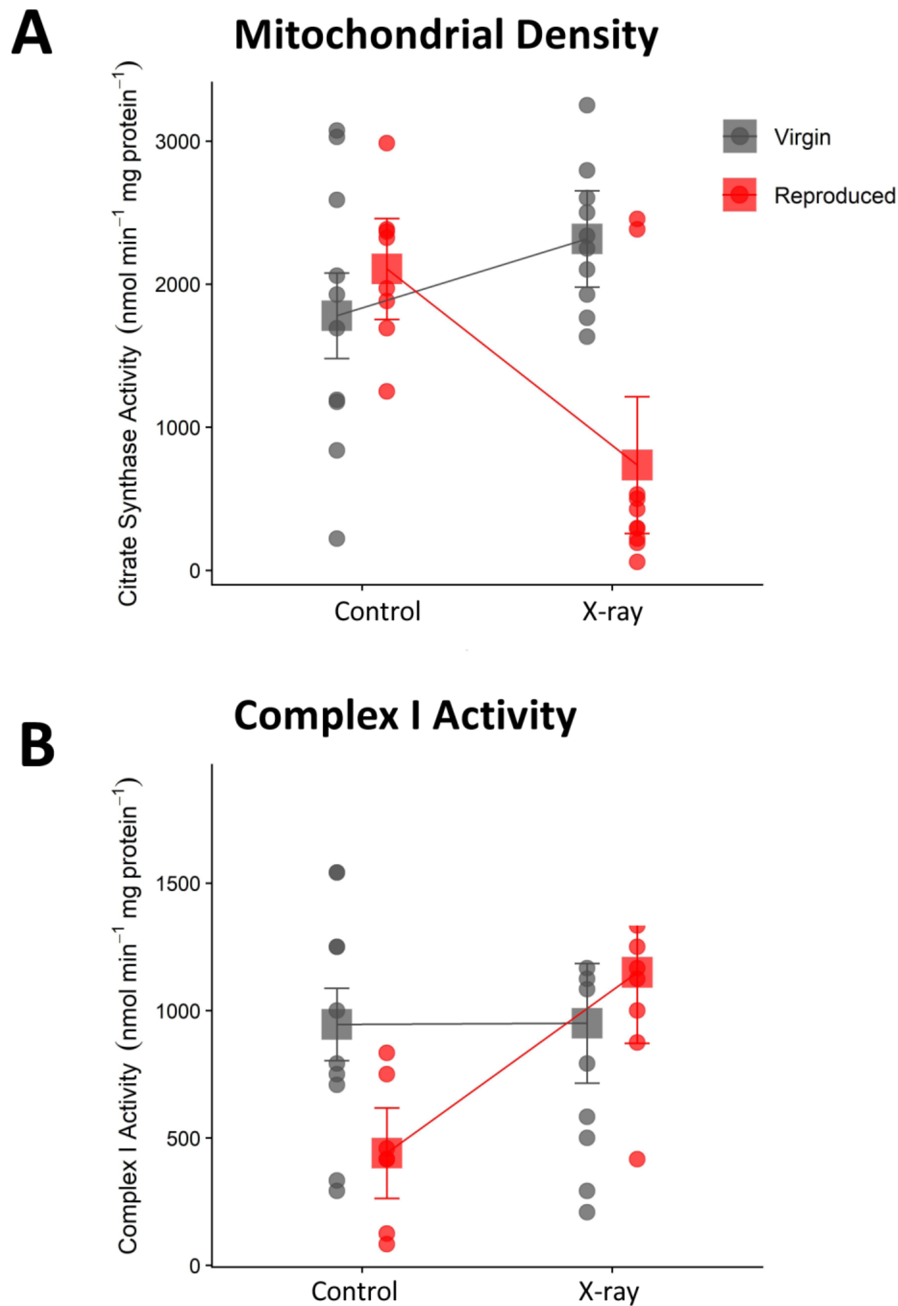


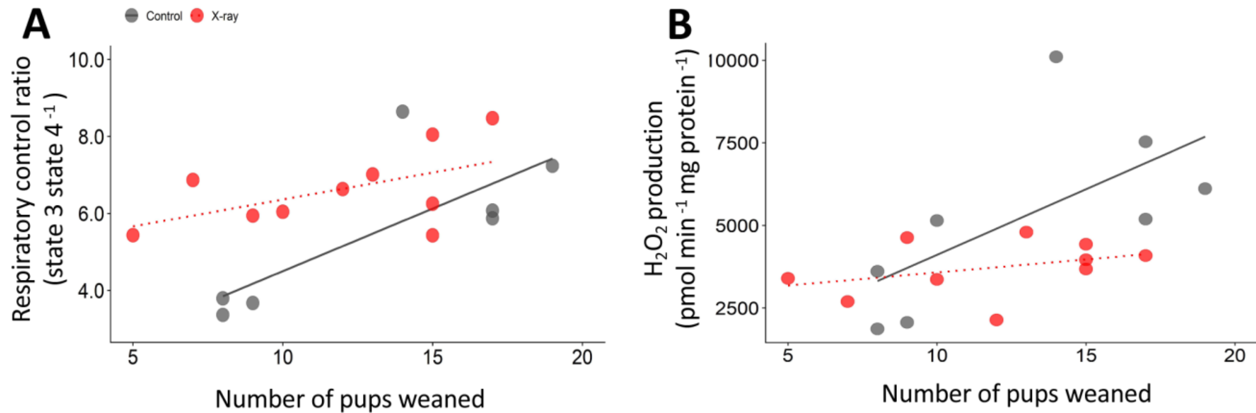
Figure 4. The effect of prior reproduction on the response of skeletal muscle to an oxidative event in wild-derived mice. Citrate synthase (CS; $t = -3.98$ $p = < 0.001$) and complex I activity ($t = 2.54$ $p = 0.016$) of virgin mice (gray) and mice that reproduced (red) after exposure to x-ray irradiation and unexposed control mice. Difference between the responses of control and irradiated mice are given, both for

Journal of Experimental Biology • Accepted manuscript

animals that reproduced and those that remained virgin. Reproductive measurements were taken 11 days after weaning. Animals were exposed to radiation 7 days after weaning and measurements were taken 4 days later. All virgin and non-irradiated control mice were age-matched to the experimental mice. Interactions were evaluated using generalized least squares models. Squares and error bars are showing the least-squares estimated mean and s.e. while points represent the individual samples within each group. Sample sizes per group were virgin-control n=10, virgin-x-ray n=10, reproduction-control n=8, and reproduction-x-ray n=10. Absolute values are provided in supplementary table 1.

Journal of Experimental Biology • Accepted manuscript

Liver



Skeletal Muscle

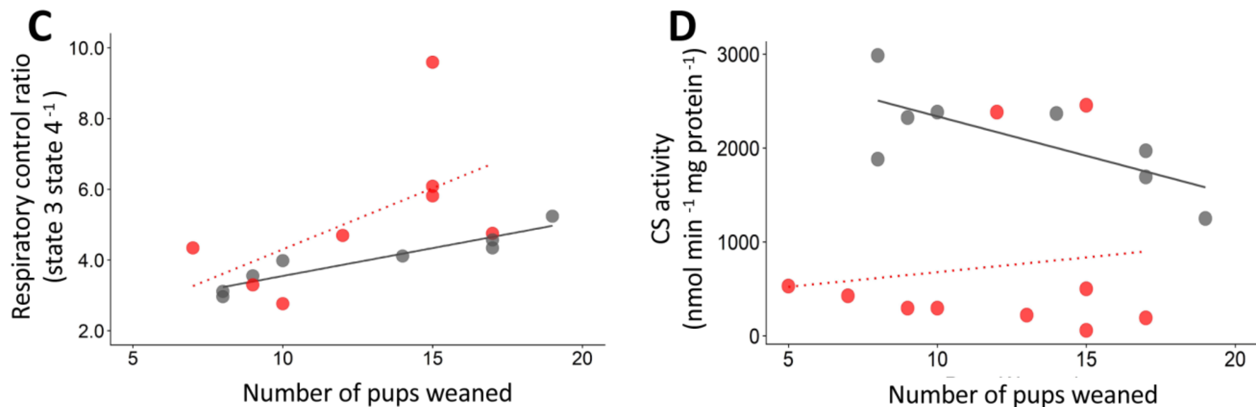


Figure 5. The relationship between total number of pups weaned and mitochondrial function in wild-derived mice. Using generalized least squares models, we found that the number of pups weaned significantly increased respiratory control ratio (A) and hydrogen peroxide production (B) in liver mitochondria from non-irradiated mice (gray circles and line). We also found a positive relationship between number of pups weaned and respiratory control ratio in skeletal muscle of non-irradiated mice (C) but a negative relationship in mitochondrial density (D) (gray circles and lines) . Sample sizes per group were reproduction-control n=8 and reproduction-x-ray n=10. Absolute values are provided in supplementary table 1. In irradiated reproductive mice, all measures tended to increase, but the model estimates of these effects are not robust ($p > 0.12$; red circles and dashed lines). Number of pups weaned is the total of two reproductive bouts.

Journal of Experimental Biology • Accepted manuscript

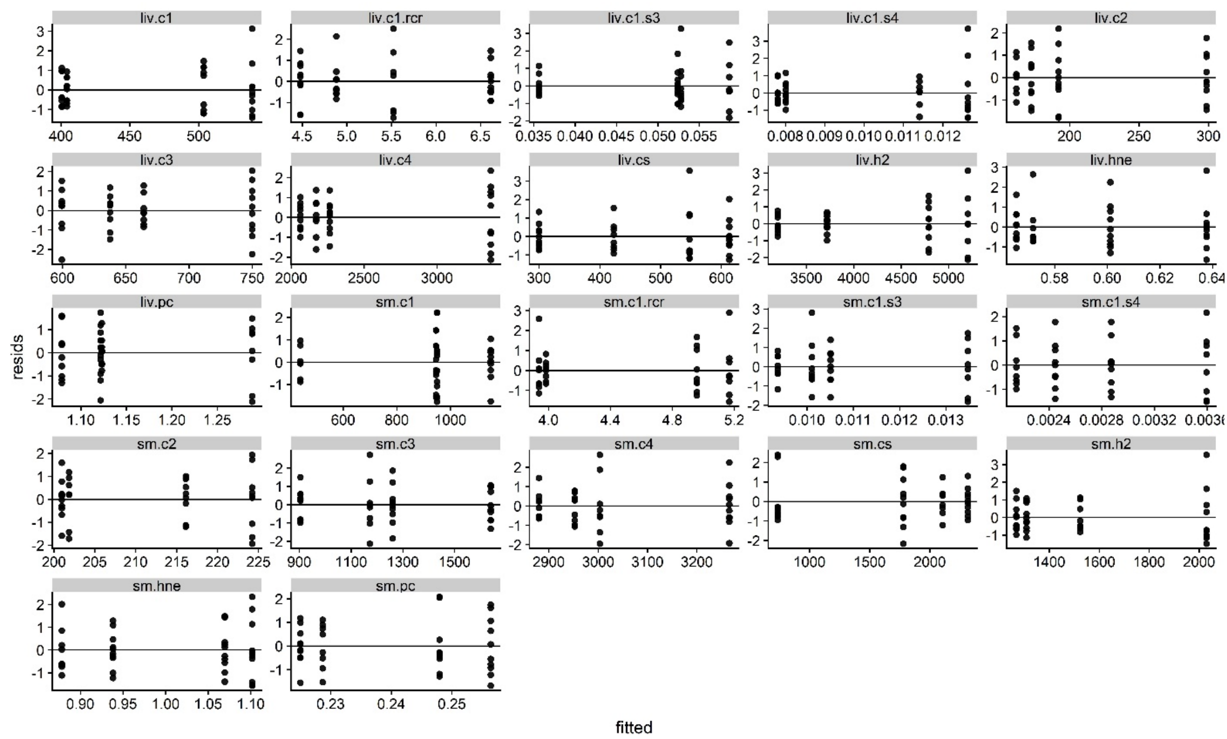


Figure S1. Standardized residuals plotted against the fitted values (estimated means from the model) from linear models. The header over each model result follows the conventions of the abbreviations described below.

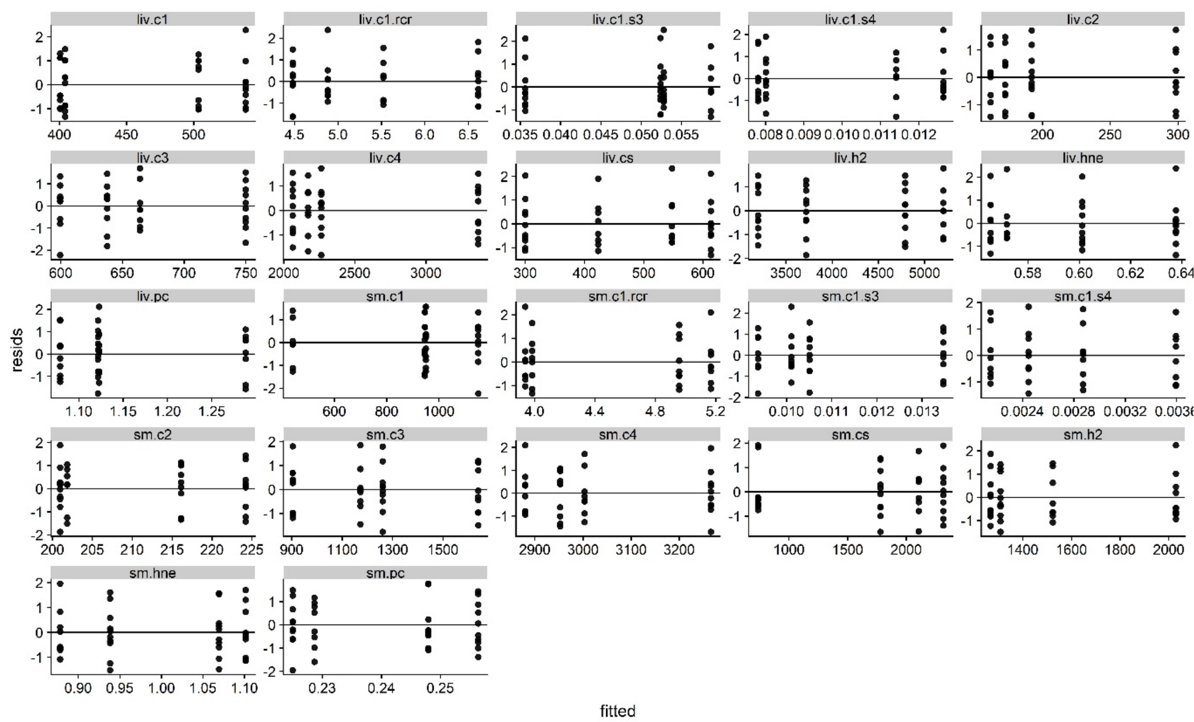


Figure S2. Standardized residuals plotted against the fitted values (estimated means from the model) from generalized least squares models. The header over each model result follows the conventions of the abbreviations described below.

Journal of Experimental Biology • Supplementary information

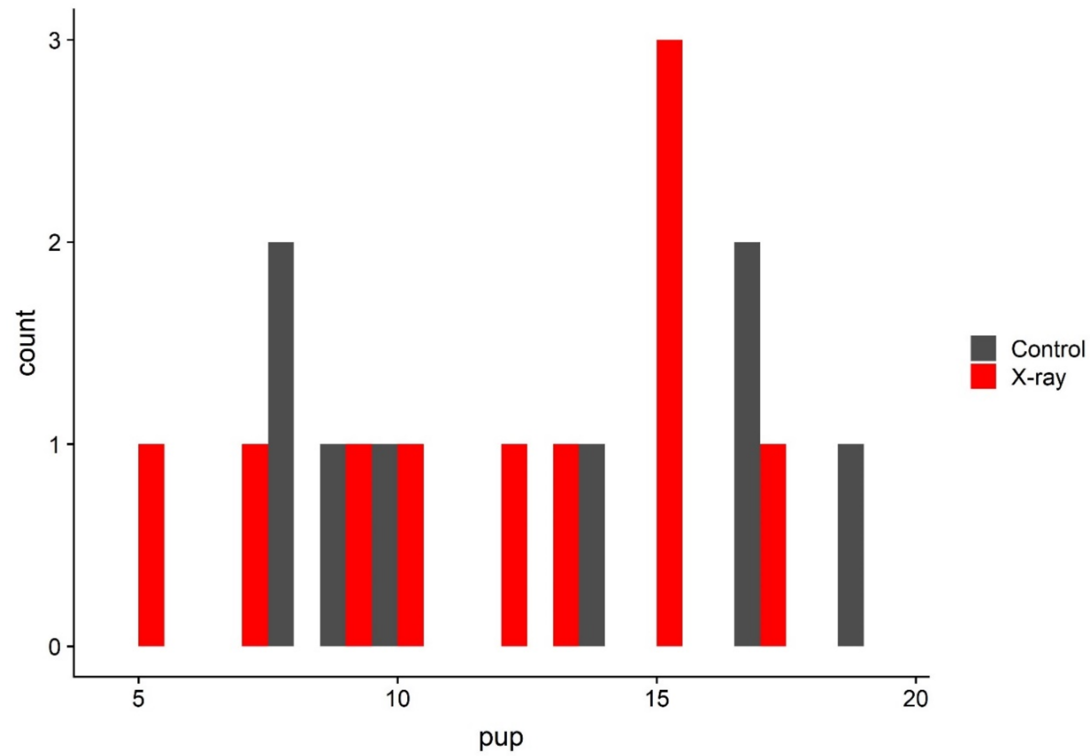


Figure S3. The number of pups weaned by x-irradiated (red) and non-irradiated (gray) female mice.

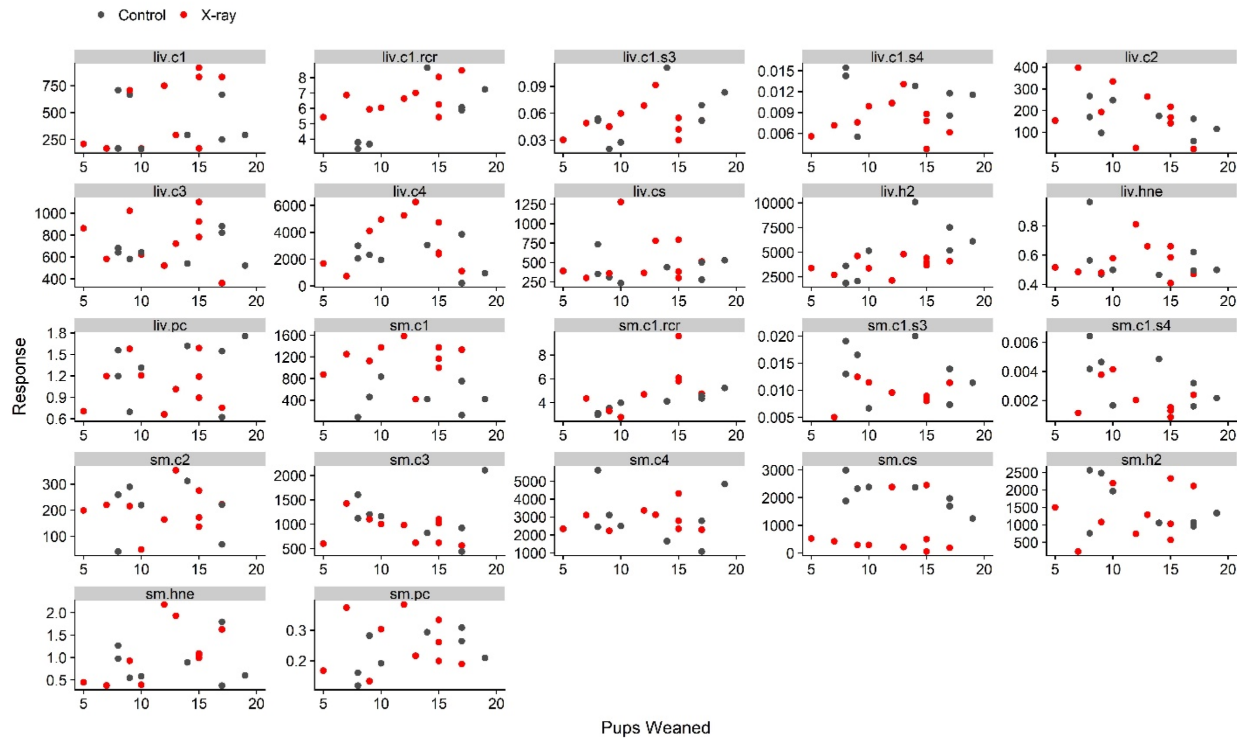


Fig S4. The effect of number of pups weaned on each measure of mitochondrial performance and oxidative stress for x-irradiated mice (red dots) and non-irradiated mice (gray dots). Results are from a generalized least squares model. The y-axis units for each model are presented in the main text. The header over each model result follows the conventions of the abbreviations described below.

Abbreviations

nr = non-reproductive, virgin mice

r = reproductive mice

x = x-irradiated mice

con = non-irradiated mice

liv = liver

sm = skeletal muscle

c1 = complex I of the electron transport system (ETS)

c2 = complex II activity of ETS

c3 = complex III activity of ETS

c4 = complex IV activity of ETS

cs = citrate synthase activity

s3 = state 3 respiration

s4 = state 4 respiration

rcr = respiratory control ratio

h2 = H2O2 production

hne = 4-Hydroxynonenal

pc = protein carbonyl

The header above each model result represents a specific measure taken from the tissue listed. For example, liv.c1.s3 is state 3 respiration of liver mitochondria using complex I substrates. sm.c2 is complex II activity of skeletal muscle, and so on.

Table S1. Raw means and standard deviations for mitochondrial measurements and p-value results of the effect of reproduction, x-irradiation (x-ray) exposure, and their interaction from Generalized Least Squares linear models. Significant values are in bold.

	Virgin females		Reproductive females		P-values			
	Control (A) *n* = 10	X-ray (B) *n* = 10	Control (C) *n* = 8	X-ray (D) *n* = 10	Repro (C vs. A) *n* = 18	X-ray (B vs. A) *n* = 20	Repro + X-ray (D vs. A) *n* = 20	Interaction (all data) *n* = 38
Liver								
Mitochondrial density - CS activity (nmol/min/mg protein)	613±197	300±135	423±164	548±314	**0.032**	**0.0002**	0.580	**0.003**
Respiration via complex 1 substrates								
RCR (state 3/state 4 respiration)	4.48±1.25	4.88±1.14	5.52±2.01	6.61±1.02	0.233	0.468	**< 0.001**	0.493
state 3 respiration (pmol/min/CS activity)	52.8±27.9	35.6±11.5	58.6±29.3	52.4±18.3	0.670	0.081	0.971	0.478
state 4 respiration (pmol/min/CS activity)	12.6±7.3	7.80±2.64	11.4±3.40	8.01±2.66	0.647	0.060	0.070	0.626
Complex activity								
complex I (nmol/min/mg protein)	404±177	539±385	400±237	504±327	0.971	0.322	0.403	0.868
complex II (nmol/min/mg protein)	172±99	299±96	162±72	192±120	0.791	**0.007**	0.691	0.138
complex III (nmol/min/mg protein)	599±197	638±141	664±129	750±234	0.408	0.623	0.129	0.680
complex IV (nmol/min/mg protein)	2063±813	2263±983	2171±1176	3368±1931	0.827	0.623	0.057	0.240
ROS indicator - H_2O_2 (pmol/min/mg protein)	4790±1784	3197±826	5203±2772	3718±847	0.717	**0.015**	0.095	0.928
Oxidative damage								

Lipids - 4HNE adducts (arbitrary units)	0.601+0.166	0.638+0.179	0.572+0.167	0.565+0.119	0.711	0.639	0.584	0.683
Proteins - Protein carbonyls (arbitrary units)	1.12+0.37	1.12+0.19	1.29+0.43	1.08+0.33	0.388	0.991	0.789	0.359
Muscle								
Mitochondrial density - CS activity (nmol/min/mg protein)	1779+944	2316+493	2107+526	735+899	0.359	0.120	**0.016**	**< 0.001**
Respiration via complex 1 substrates								
RCR (state 3/state 4 respiration)	3.94+1.71	4.96+1.65	3.98+0.76	5.17+2.11	0.944	0.208	0.201	0.884
state 3 respiration (pmol/min/CS activity)	10.1+4.62	10.5+3.39	13.5+4.95	9.37+2.40	0.150	0.830	0.665	0.102
state 4 respiration (pmol/min/CS activity)	2.87+1.34	2.44+1.28	3.59+1.73	2.15+1.22	0.347	0.497	0.255	0.302
Complex activity								
complex I (nmol/min/mg protein)	945+450	950+592	440+282	1150+328	**0.008**	0.986	0.254	**0.016**
complex II (nmol/min/mg protein)	224+129	216+85	202+106	201+81	0.705	0.874	0.645	0.920
complex III (nmol/min/mg protein)	1640+306	1261+360	1173+504	904+288	**0.029**	**0.016**	**< 0.001**	0.660
complex IV (nmol/min/mg protein)	2952+734	3266+1136	3003+1525	2880+686	0.932	0.469	0.825	0.550
ROS indicator - H_2O_2 (pmol/min/mg protein)	2029+1493	1267+754	1524+714	1309+720	0.351	0.159	0.178	0.391
Oxidative damage								
Lipids - 4HNE adducts (arbitrary units)	0.938+0.371	1.069+0.433	0.878+0.467	1.102+0.635	0.768	0.472	0.487	0.772

Journal of Experimental Biology • Supplementary information

Proteins - Protein carbonyls (arbitrary units)	0.248±0.086	0.225±0.057	0.229±0.069	0.256±0.089	0.601	0.489	0.830	0.314

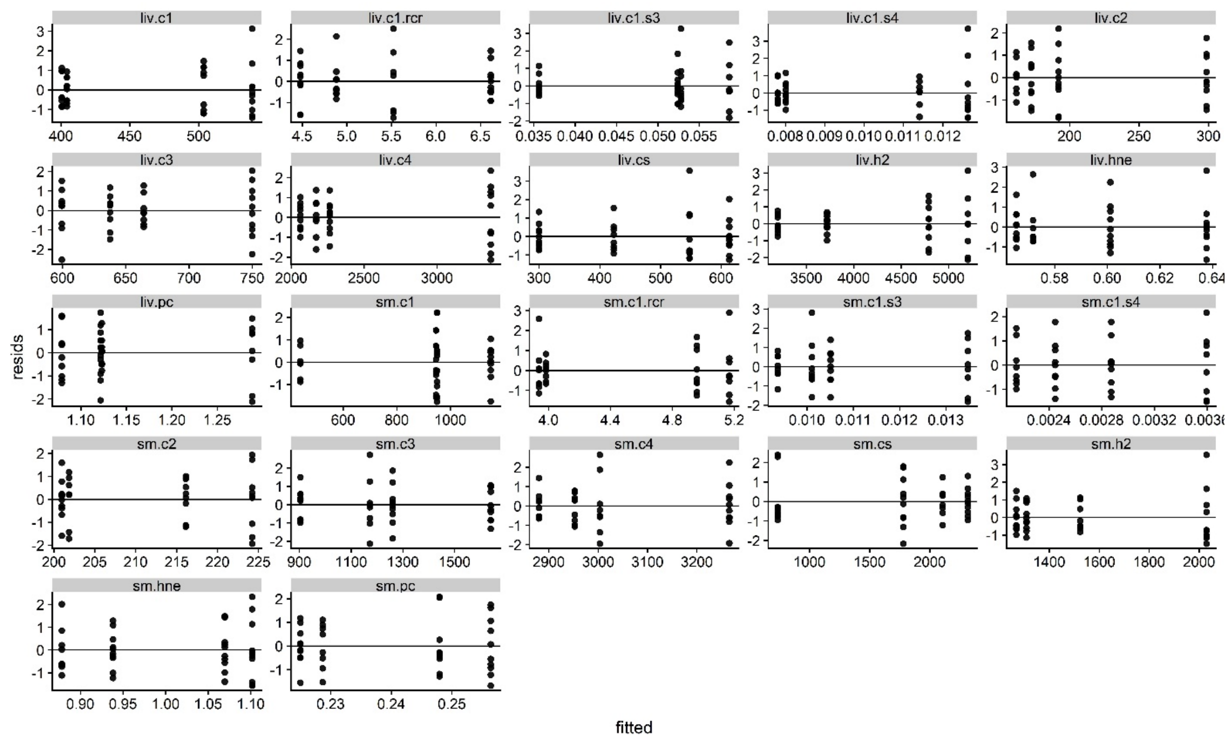


Figure S1. Standardized residuals plotted against the fitted values (estimated means from the model) from linear models. The header over each model result follows the conventions of the abbreviations described below.

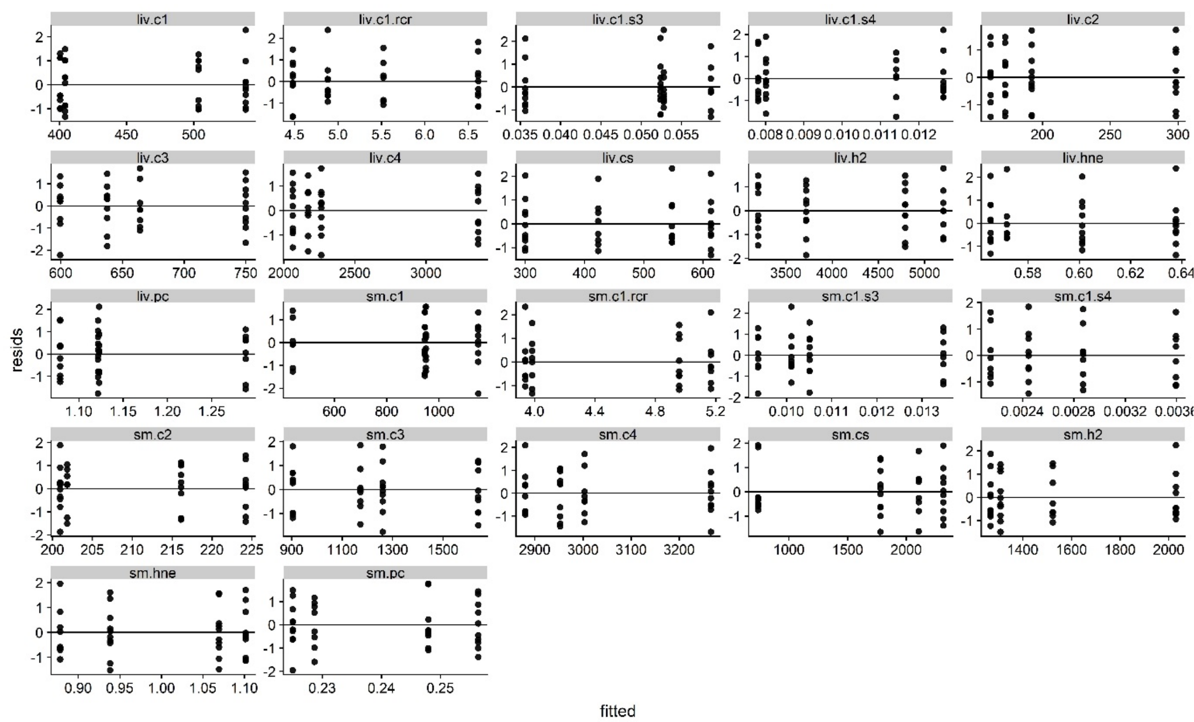


Figure S2. Standardized residuals plotted against the fitted values (estimated means from the model) from generalized least squares models. The header over each model result follows the conventions of the abbreviations described below.

Journal of Experimental Biology • Supplementary information

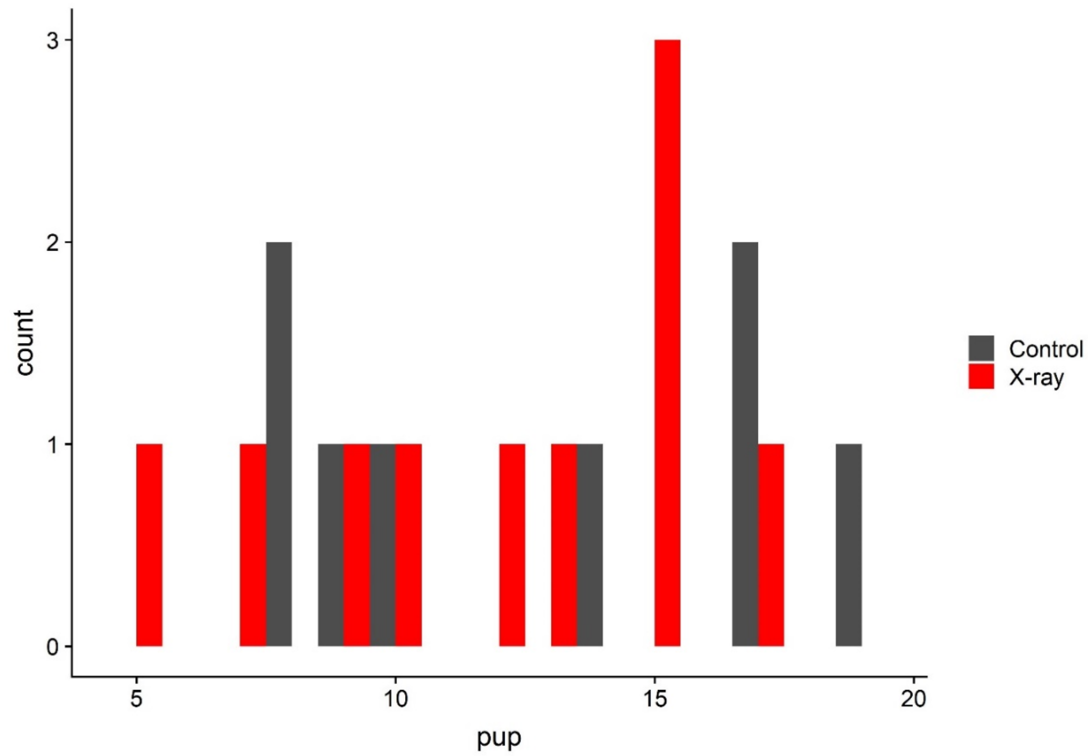


Figure S3. The number of pups weaned by x-irradiated (red) and non-irradiated (gray) female mice.

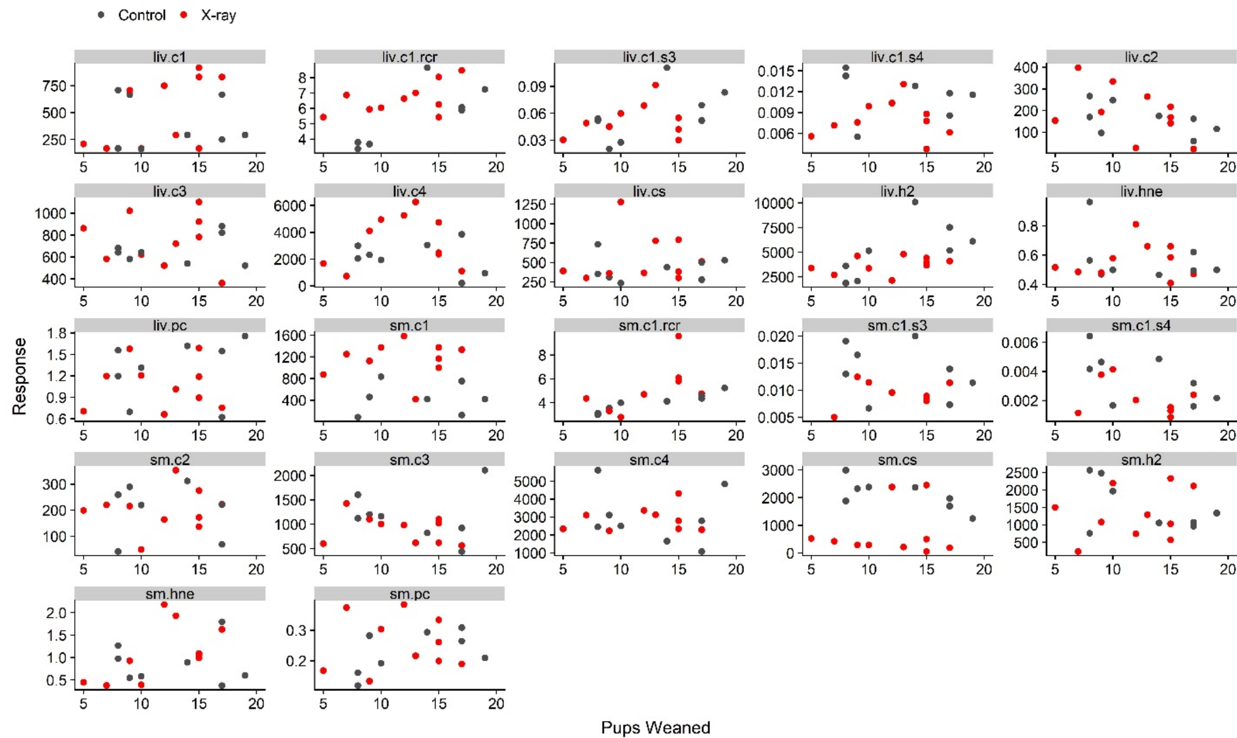


Fig S4. The effect of number of pups weaned on each measure of mitochondrial performance and oxidative stress for x-irradiated mice (red dots) and non-irradiated mice (gray dots). Results are from a generalized least squares model. The y-axis units for each model are presented in the main text. The header over each model result follows the conventions of the abbreviations described below.

Abbreviations

nr = non-reproductive, virgin mice

r = reproductive mice

x = x-irradiated mice

con = non-irradiated mice

liv = liver

sm = skeletal muscle

c1 = complex I of the electron transport system (ETS)

c2 = complex II activity of ETS

c3 = complex III activity of ETS

c4 = complex IV activity of ETS

cs = citrate synthase activity

s3 = state 3 respiration

s4 = state 4 respiration

rcr = respiratory control ratio

h2 = H2O2 production

hne = 4-Hydroxynonenal

pc = protein carbonyl

The header above each model result represents a specific measure taken from the tissue listed. For example, liv.c1.s3 is state 3 respiration of liver mitochondria using complex I substrates. sm.c2 is complex II activity of skeletal muscle, and so on.

Table S1. Raw means and standard deviations for mitochondrial measurements and p-value results of the effect of reproduction, x-irradiation (x-ray) exposure, and their interaction from Generalized Least Squares linear models. Significant values are in bold.

	Virgin females		Reproductive females		P-values			
	Control (A) *n* = 10	X-ray (B) *n* = 10	Control (C) *n* = 8	X-ray (D) *n* = 10	Repro (C vs. A) *n* = 18	X-ray (B vs. A) *n* = 20	Repro + X-ray (D vs. A) *n* = 20	Interaction (all data) *n* = 38
Liver								
Mitochondrial density - CS activity (nmol/min/mg protein)	613±197	300±135	423±164	548±314	**0.032**	**0.0002**	0.580	**0.003**
Respiration via complex 1 substrates								
RCR (state 3/state 4 respiration)	4.48±1.25	4.88±1.14	5.52±2.01	6.61±1.02	0.233	0.468	**< 0.001**	0.493
state 3 respiration (pmol/min/CS activity)	52.8±27.9	35.6±11.5	58.6±29.3	52.4±18.3	0.670	0.081	0.971	0.478
state 4 respiration (pmol/min/CS activity)	12.6±7.3	7.80±2.64	11.4±3.40	8.01±2.66	0.647	0.060	0.070	0.626
Complex activity								
complex I (nmol/min/mg protein)	404±177	539±385	400±237	504±327	0.971	0.322	0.403	0.868
complex II (nmol/min/mg protein)	172±99	299±96	162±72	192±120	0.791	**0.007**	0.691	0.138
complex III (nmol/min/mg protein)	599±197	638±141	664±129	750±234	0.408	0.623	0.129	0.680
complex IV (nmol/min/mg protein)	2063±813	2263±983	2171±1176	3368±1931	0.827	0.623	0.057	0.240
ROS indicator - H_2O_2 (pmol/min/mg protein)	4790±1784	3197±826	5203±2772	3718±847	0.717	**0.015**	0.095	0.928
Oxidative damage								

Journal of Experimental Biology • Supplementary information

Lipids - 4HNE adducts (arbitrary units)	0.601±0.166	0.638±0.179	0.572±0.167	0.565±0.119	0.711	0.639	0.584	0.683
Proteins - Protein carbonyls (arbitrary units)	1.12±0.37	1.12±0.19	1.29±0.43	1.08±0.33	0.388	0.991	0.789	0.359
Muscle								
Mitochondrial density - CS activity (nmol/min/mg protein)	1779±944	2316±493	2107±526	735±899	0.359	0.120	**0.016**	**< 0.001**
Respiration via complex 1 substrates								
RCR (state 3/state 4 respiration)	3.94±1.71	4.96±1.65	3.98±0.76	5.17±2.11	0.944	0.208	0.201	0.884
state 3 respiration (pmol/min/CS activity)	10.1±4.62	10.5±3.39	13.5±4.95	9.37±2.40	0.150	0.830	0.665	0.102
state 4 respiration (pmol/min/CS activity)	2.87±1.34	2.44±1.28	3.59±1.73	2.15±1.22	0.347	0.497	0.255	0.302
Complex activity								
complex I (nmol/min/mg protein)	945±450	950±592	440±282	1150±328	**0.008**	0.986	0.254	**0.016**
complex II (nmol/min/mg protein)	224±129	216±85	202±106	201±81	0.705	0.874	0.645	0.920
complex III (nmol/min/mg protein)	1640±306	1261±360	1173±504	904±288	**0.029**	**0.016**	**< 0.001**	0.660
complex IV (nmol/min/mg protein)	2952±734	3266±1136	3003±1525	2880±686	0.932	0.469	0.825	0.550
ROS indicator - H_2O_2 (pmol/min/mg protein)	2029±1493	1267±754	1524±714	1309±720	0.351	0.159	0.178	0.391
Oxidative damage								
Lipids - 4HNE adducts (arbitrary units)	0.938±0.371	1.069±0.433	0.878±0.467	1.102±0.635	0.768	0.472	0.487	0.772

Proteins - Protein carbonyls (arbitrary units)	0.248±0.086	0.225±0.057	0.229±0.069	0.256±0.089	0.601	0.489	0.830	0.314