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ORIGINAL PAPER

Mitochondrial physiology varies with parity and body mass in the laboratory mouse (*Mus musculus*)

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Abstract

The life-history patterns that animals display are a product of their ability to maximize reproductive performance while concurrently balancing numerous metabolic demands. For example, the energetic costs of reproduction may reduce an animal's ability to support self-maintenance and longevity. In this work, we evaluated the impact of parity on mitochondrial physiology in laboratory mice. The theory of mitohormesis suggests that modest exposure to reactive oxygen species can improve performance, while high levels of exposure are damaging. Following this theory, we hypothesized that females that experienced one bout of reproduction (primiparous) would display improved mitochondrial capacity and reduced oxidative damage relative to non-reproductive (nulliparous) mice, while females that had four reproductive events (multiparous) would have lower mitochondrial performance and greater oxidative damage than both nulliparous and primiparous females. We observed that multiple reproductive events enhanced the mitochondrial respiratory capacity of liver mitochondria in females with high body mass. Four-bout females showed a positive relationship between body mass and mitochondrial capacity. In contrast, non-reproductive females showed a negative relationship between body mass and mitochondrial capacity and primiparous females had a slope that did not differ from zero. Other measured variables, too, were highly dependent on body mass, suggesting that a female's body condition has strong impacts on mitochondrial physiology. We also evaluated the relationship between how much females allocated to reproduction (cumulative mass of all young weaned) and mitochondrial function and oxidative stress in the multiparous females. We found that females that allocated more to reproduction had lower basal respiration (state 4), lower mitochondrial density, and higher protein oxidation in liver mitochondria than females that allocated less. These results suggest that, at least through their first four reproductive events, female laboratory mice may experience bioenergetic benefits from reproduction but only those females that allocated the most to reproduction appear to experience a potential cost of reproduction.

Keywords Reproduction · Life history · Oxidative stress · Mitochondrial function · RCR

Introduction

Reproduction is energetically demanding, particularly for female mammals (Kirkwood and Rose 1991). This high demand is thought to have important consequences for the performance of the individual, ultimately driving energetic tradeoffs (Williams 1966; Stearns 1989). When the demands

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of reproduction are high, it is predicted that less energy will be available for somatic maintenance and repair (Skogland 1989; Zera and Harshman 2001). In other words, an individual's capacity to support future reproductive events and longevity could be curtailed (Williams 1957, 1966). Despite numerous empirical studies and reviews focused on this phenomenon, the mechanisms that underlie tradeoffs remain poorly understood (Monaghan et al. 2009; Metcalfe and Monaghan 2013; Costantini 2014; Speakman and Garratt 2014; Blount et al. 2016).

There are two important challenges in understanding the mechanisms that govern life-history trade-offs. First, investigators must identify relevant variables. These variables should both play an important role in senescence and ultimately be negatively impacted by reproduction. Second, variance in the condition of individuals obscure tradeoffs. Thus, it is important to accurately identify variables that reflect individual variation in condition (van Noordwijk and de Jong 1986; Reznick et al. 2000). Several variables have been evaluated as possible contributors to the trade-off between reproduction and longevity, including but not limited to: body condition, bone density, metabolic rate, and telomere length (Dobson et al. 1999; Keech et al. 2000; Heidinger et al. 2012; Schmidt and Hood 2016). Because it is well established that the performance of mitochondria decline with age across organs (Yen et al. 1989; Ojaimi et al. 1999; Short et al. 2005), mitochondria have been a focus of interest for both biomedical research and ecologists interested in aging (Balaban et al. 2005; Monaghan et al. 2008; Flatt and Heyland 2011; Bratic and Larsson 2013).

Empirical tests of the hypothesis that reproduction leads to the accumulation of oxidative damage have yielded equivocal results with some studies showing an increase in oxidative damage; while, others show a reduction or no change in damage during reproduction (Speakman and Garratt 2014; Blount et al. 2016). There are several potential reasons for this disagreement. For example, oxidative damage is typically measured during reproduction, where physiological changes associated with the reproductive event could mask the impact of reproduction on somatic maintenance (Zhang and Hood 2016). Also, other processes such as mitochondrial replication error may have a greater contribution to the bioenergetic costs of reproduction than oxidative damage (Larsson et al. 2010; Itsara et al. 2014; Hood et al. 2019). Finally, the relationships between mitochondrial function and ROS production are complex (Powers and Jackson 2008; Speakman and Garratt 2014), as ROS have the potential to not only inhibit, but also to improve mitochondrial capacity (Tapia 2006; Ristow and Schmeisser 2014; Zhang and Hood 2016; Hood et al. 2018).

While some studies appear to counter the reproduction–longevity trade-off (Mowry et al. 2016; Hyatt et al. 2018), these studies have largely focused on the effects of reproduction on performance early in the reproductive life of animals. Under Williams's antagonistic pleiotropy hypothesis (Williams 1957), selection should favor senescence when genes that improve early life reproductive performance also reduce longevity. It is possible that the benefits of reproduction that we observe early in life are a function of pleiotropic genes, or gene networks, that also govern the decline in mitochondrial performance with increasing reproductive effort later in life. Thus, it is paramount to determine how both the accumulation of oxidative damage and mitochondrial performance vary with parity and across the reproductive life of organisms.

For this study, we evaluated the impact of parity on mitochondrial performance in laboratory mice. We used the outbred laboratory mice as a model for this investigation because they maintain the highest sustained metabolic scope recorded for any vertebrate species (Hammond and Diamond 1997). Under this high energetic demand, we expect that the impacts of reproduction on somatic maintenance would be high. We compared age-matched female mice that have not reproduced (nulliparous), females that have completed one reproductive event (primiparous), and females that have experienced four reproductive events (multiparous) in an attempt to capture the cost of reproduction in females with greater reproductive experience. Following the theories of mitochondrial hormesis and antagonistic pleiotropy, we predicted that primiparous females would display higher mitochondrial respiratory capacity and lower oxidative damage than nulliparous females. In contrast, multiparous females are predicted to have lower mitochondrial respiratory function and higher oxidative damage than both groups. Further, within the multiparous group, we evaluated the relationship between lifetime number of pups produced and the same mitochondrial measurements to further explore how differential allocation to reproduction impacts mitochondrial performance. Finally, we include body mass in our model to reveal how it interacts with condition to affect mitochondrial performance.

Materials and methods

Animal care

Adult outbred female ICR mice (Envigo, Inc., Prattville, AL) were used in this investigation. All husbandry and experimental procedures were approved by the Auburn University Institutional Animal Care and Use Committee (PRN 2017-3168). All animals had a body mass of at least 35 g when shipped and an estimated age of 4–7 weeks. Animals were maintained on a 12:12 light–dark cycle at a temperature of 24 °C. All mice were housed in pairs (two females for non-reproductive groups, male–female for reproductive)

in standard mouse boxes (11.5″ × 7.5″ × 5″). After one bout of reproduction for primiparous females (described below), they were housed with another female. Food and water were provided ad libitum.

Experimental design

Female mice were randomly assigned to one of the three groups: non-reproductive (nulliparous), one complete bout (primiparous) and four complete bouts (multiparous) of reproduction ($n = 12$/group). Since there were isolated incidents of stillborns and pup cannibalism within the multiparous group (one case of stillborn, one case of litter cannibalism), we designated a reproductive bout as one that included successful weaning. Males were added to the cages of reproductive group females and later removed just before the birth of their last expected litter. At 21 days postpartum, the litters were weaned, and the mass and sex of individual offspring recorded. All multiparous mice were euthanized 14 days after weaning their last successful fourth litter. To ensure that all animals were of the same age at the time of organ collection, each nulliparous and primiparous female were randomly assigned to be euthanized the same number of days post-arrival as a multiparous female (Fig. 1). The time since weaning in the primiparous group averaged 89.5 ± 3.4 days (± se). While this design meant that animals varied between groups in time since reproduction, we felt it was important to control for age in this study. There is strong selection pressure of prey species, such as house mice, to breed early. Thus, mice with limited reproductive performance are more likely to breed earlier, rather than later in their life. Thus, the data collected herein are designed to be comparable to age-matched cohort of animals collected from a wild population.

Mice were briefly anesthetized with isoflurane, then decapitated with a rodent guillotine. The left lateral and right medial lobes of the liver and about 0.5 g of the skeletal muscle of the left hind limb (including the rectus femoris, vastus lateralis, vastus medialis, biceps femoris, semitendinosus, gracilis, semimembranosus, and gastrocnemius; hereafter, "skeletal muscle") were dissected for immediate mitochondrial isolation. The rest of the liver, skeletal muscle, and heart were also harvested and flash-frozen in liquid nitrogen and stored at − 80 °C for future analyses.

We focused our mitochondrial respiratory performance measures on the liver because the liver plays a vital role in reproduction, making glucose available for the developing young in utero, as well as glucose and lipids available for milk synthesis by the mammary gland (Bell and Bauman 1997; Brett et al. 2014; Zhang et al. 2017). Further, the performance of liver mitochondria has been shown to decline with age (Yen et al. 1989). Additional tissues from the liver, skeletal muscle, and heart were also collected to evaluate markers of oxidative stress.

Mitochondrial isolation

Mitochondria were isolated following procedures outlined previously (Hyatt et al. 2018; Mowry et al. 2016). The liver was homogenized in a Potter–Elvehjem PTFE pestle and glass tube. The resulting homogenate was centrifuged at 500×*g* for 10 min at 4 °C, and the supernatant filtered through cheesecloth underwent another round of centrifugation at 3500×*g* for 10 min. The subsequent supernatant was discarded, and the mitochondria pellet was washed in liver isolation solution before it was put through another centrifugation. The final mitochondria pellet was suspended in a mannitol–sucrose solution.

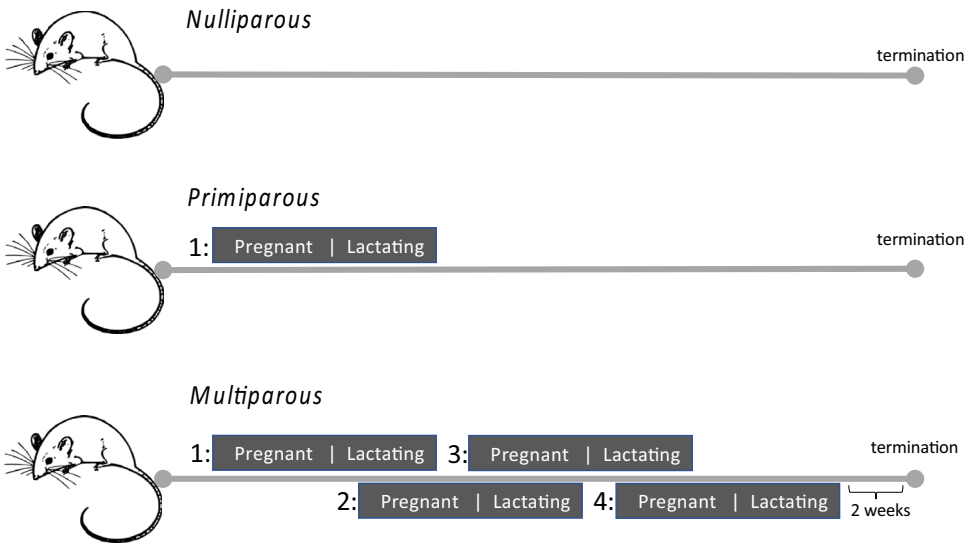

Fig. 1 Experimental design. Thirty-six age-matched female lab mice were randomly distributed into three groups of reproductive bouts: nulliparous, primiparous, and multiparous

Mitochondrial respiratory capacity

Mitochondrial respiration in liver was measured polarigraphically in a respiration chamber maintained at 37 °C (Oxytherm, Hansatech Instruments, UK) following Hyatt et al. (2016) and Mowry et al. (2016). Flux through complex I was measured using 2-mM pyruvate and 2-mM malate; whereas, flux through complex II was measured using 5-mM succinate. Rotenone (5 µM) was added to prevent electron backflow to complex I in the succinate-driven experiments. State 3, or maximal respiration, was measured as the rate of respiration in the presence of ADP. State 3 was initiated by adding 0.25-mM ADP to the chamber containing the mitochondria and respiratory substrates. State 4 respiration, or basal respiration, was measured after the phosphorylation of ADP was complete. The respiration states were normalized to mitochondrial protein concentration determined by Bradford assay (Bradford 1976). Respiratory control ratio (RCR) was calculated by dividing state 3 by state 4 respiration.

H_2O_2 (ROS) emission

H_2O_2 emission in isolated liver mitochondria was quantified with Amplex Red (Thermofisher, Waltham, MA) (Kavazis et al. 2009; Hyatt et al. 2017). 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 37 °C in a 96-well plate using succinate. Readings of resorufin formation were recorded every 5 min for 15 min. The obtained slope (rate of formation) was then converted into the rate of H_2O_2 production using a standard curve and was normalized to mitochondrial protein concentration.

Mitochondrial content

Citrate synthase is an enzyme present in all cells that catalyzes the first step of the Krebs cycle of the condensation of acetyl-CoA and oxaloacetate to form citrate, with the rate of citrate formation being widely used as a proxy for mitochondrial density (Larsen et al. 2012; Spinazzi et al. 2012). Whole tissue homogenate from the liver, skeletal muscle and heart was used for the citrate synthase enzyme activity assay. Each tissue was homogenized in a 1:10 (wt/vol) ratio of 5-mmol/l Tris HCl (pH 7.5), 5-mmol/l EDTA (pH 8.0), and protease inhibitor cocktail (14224-396, VWR, Radnor, PA) and was centrifuged at 1500×*g* for 10 min at 4 °C. Protein content of the supernatant was quantified by the Bradford assay (Bradford 1976). Citrate synthase was measured as a function of the increase in absorbance from 5,5′-dithiobis-2-nitrobenzoic acid reduction over time.

Antioxidant western blots

Western blots were used to quantify antioxidant enzymes in each tissue. Proteins from tissue homogenates were separated by electrophoresis via 4%–15% Criterion TGX precast gels (Bio-Rad, Hercules, CA). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes following separation. Nonspecific sites were blocked in phosphate-buffered saline (PBS) solution containing 0.1% Tween-20 and 5% nonfat milk. Membranes were then incubated overnight at 4 °C with primary antibodies for superoxide dismutase I, II, catalase and glutathione peroxidase (SOD1: GTX100554, SOD2: GTX116093, CAT: GTX110704, GPX: GTX116040; GeneTex, Irvine, CA). Membranes were washed (5 min × 3) with PBS-Tween and incubated with secondary antibodies for 1 h at room temperature. After another round of washing, a chemiluminescent system was used to detect labeled proteins (GE Healthcare, Buckinghamshire, UK). Each membrane was stained by Ponceau and was used as the loading and transfer control. Images of the membranes were captured and analyzed using the ChemiDocIt Imaging System (UVP, LLC, Upland, CA).

Lipid and protein oxidative damage western blots

Lipid peroxidation marker (4-hydroxynonenal; 4-HNE; ab46545; Abcam, Cambridge, MA) and protein oxidation marker (protein carbonyls; OxyBlot; s7150; EMD Millipore, Billerica, MA) were measured via Western blotting for each tissue as described by the manufacturer's instructions. Each membrane was stained by Ponceau and was used as the loading and transfer control. The resulting markers were visualized using a chemiluminescent system (GE Healthcare Life Sciences, Pittsburgh, PA) and analyzed with the ChemiDocIt Imaging System (UVP, LLC, Upland, CA).

DNA extraction

Genomic DNA (gDNA) was extracted from 25 mg of each of the frozen tissues using the Qiagen DNeasy kit (cat. 69,506). DNA extract purity and concentration were determined by measuring absorbance ratios A_{260}/A_{280} using NanoDrop Lite (Thermo Scientific, Waltham, MA). The DNA extracts were subsequently precipitated and hydrolyzed to deoxyribonucleosides using Nuclease P1 (Sigma N8630). Briefly, DNA pellets were resuspended in sodium acetate (20 mM, pH 5.0–5.4), then denatured by boiling for 10 min. Appropriate amounts of stock reagents zinc chloride (10 mM), nuclease P1 (5U/ml) were added to each DNA sample. The reaction mixtures were incubated for 30 min at 37 °C, and pH was

adjusted to 7.5–8.0 by addition of ~ 1/10 volume of Tris–HCl (1 M, pH 8.0). Alkaline phosphatase (0.15U) was added to each sample to dephosphorylate and prevent self-ligation and incubated at 37 °C again for 30 min. Samples were boiled again for 10 min to inactivate the alkaline phosphatase, and further diluted in ELISA buffer prior to being assayed.

DNA oxidative damage ELISA

We cumulatively quantified all three oxidized guanine species; 8-hydroxy-2′-deoxyguanosine from DNA, 8-hydroxyguanosine from RNA, and 8-hydroxyguanine in each sample (hereafter 8-OHdG) using an enzyme-linked immunofluorescent assay (ELISA, Cayman Chemical cat. 589,320). This assay is based on the competition between oxidatively damaged guanine species and an 8-OHdG-acetylcholinesterase conjugate (DNA/RNA Oxidative Damage Tracer) for a limited amount of DNA/RNA Oxidative Damage Monoclonal antibody. Because some RNA will be retained in the DNA extraction and the 8-OHdG kit recovers damage to guanine associated with both DNA and RNA, it is important to acknowledge that the values reported will include some damaged RNA, and thus, the ELISA values will be higher than other methods of oxidative DNA damage quantification such as LC/MS.

Statistical analysis

Grubb's outlier test was used to identify significant physiological outliers on our results using the GraphPad QuickCalcs (website: https://www.graphpad.com/quickcalcs/Grubbs1.cfm, accessed May 9, 2018). All the remaining comparisons were completed using Rstudio (2019). We used general linear models with maternal body mass included as a covariate to determine if parity (nulliparous, primiparous, or multiparous) impacted the dependent variables in the study. Each dependent variable was evaluated independently; for each, we tested for an interaction between reproductive groups and body mass. When the interactions were not significant, the interaction term was excluded from the model. When the interactions were significant, the p values for the interactions are reported, and then, independent linear models were run for each group, so that the regression line between body mass and the dependent variable could be characterized. All data presented include the outliers, as the results only changed for one variable (liver RCR with complex II substrate) when the outliers were removed.

In addition, given that the multiparous group bred multiple times and the other groups did not, we used this group to look for clues that differential allocation to reproduction was costly for females. To test this, we used a general linear model to determine if the cumulative mass of pups weaned across four reproductive events was associated with variation in any of the dependent variables evaluated herein.

Results

The age of the female mice at the termination of the study did not differ between groups ($p = 0.99$) and averaged 134 days post-arrival for all groups [NR (Non-reproductive/nulliparous): 134.4 ± 3.5 ($\pm$ se), 1-bout (primiparous): 134.3 ± 3.4, and 4-bouts (multiparous): 134.2 ± 3.4] or approximately 6 months of age. We found no significant difference in body mass ($p = 0.35$) at killing between NR, 1-bout, and 4-bout females (Table 1).

Liver

Liver mass increased with body mass ($p < 0.001$) and was significantly different between reproductive groups (NR vs 1-bout, $p = 0.012$; NR vs 4-bout, $p < 0.001$, Table 1). Citrate synthase activity, indicative of mitochondrial density, did not differ with body mass or parity (Table 1). In contrast, hydrogen peroxide production, indicative of ROS emission, varied between nulliparous and multiparous females ($p = 0.041$) and increased with body mass ($p = 0.027$).

When isolated liver mitochondria were provided substrates to support respiration via complex I, state 3 respiration did not differ with body mass or between groups ($p > 0.203$). However, state 4 respiration displayed significant interactions between body mass and reproductive group, such that the effect of body mass on state 4 respiration significantly differed between non-reproductive and 1-bout females ($p = 0.049$), and between non-reproductive and 4-bout females ($p = 0.003$). Results of independent linear models (lm) show that state 4 respiration displayed a trend suggestive of an increase with body mass in NR females [$Y = (0.53*\text{body mass}) - 10.09$, $p = 0.066$], was highly variable and did not vary with body mass in 1-bout females ($p = 0.81$), and decreased with body mass in 4-bout females [$Y = (-1.00*\text{body mass}) - 57.79$, $p = 0.011$] (Fig. 2a, Table 1).

For liver RCR with complex 1, the NR-group displayed a significantly different relationship with body mass compared to the 4-bout group ($p = 0.008$). RCR decreased with body mass in NR females [independent lm, $Y = (0.43*\text{body mass}) + 13.59$, $p = 0.011$] and displayed a trend suggestive of an increase in body mass in 4-bout females [independent lm, $Y = (-0.18*\text{body mass}) + 13.02$, $p = 0.068$] (Fig. 2b, Table 1, NR vs. 4 bout). Unlike state 4 respiration, RCR for 1-bout females did not display differences in the relationship with body mass when compared to NR females $p = 0.228$). When liver mitochondria were provided with substrate to support respiration via complex II, state 3 and

Table 1 Result of general linear models comparing body mass, organ mass, mitochondrial physiology and oxidative stress in the liver, skeletal muscle and heart of nulliparous (NR), primiparous (1-bout), and multiparous mice (4-bouts)

	NR	1-bout	4-bouts	Statistics, *p* value			
	$\bar{x} \pm$ se	$\bar{x} \pm$ se	$\bar{x} \pm$ se	NR vs. 1-bout	NR vs. 4-bout	BM	Interaction
Body mass (g)	44.5 ± 1.7	48.2 ± 2.7	46.4 ± 0.91	0.150	0.475	–	–
Liver mass (g)	1.69 ± 0.05	2.03 ± 0.08	2.51 ± 0.08	**0.012**	$2.40e^{-10}$	$2.49e^{-5}$	> 0.12
Leg skeletal mass (g)	1.07 ± 0.03	1.07 ± 0.03	0.993 ± 0.025	0.669	0.048	0.068	> 0.18
Heart mass (g)	0.155 ± 0.003	0.163 ± 0.005	0.206 ± 0.009	0.564	$3.21e^{-6}$	0.101	> 0.28
Liver							
Citrate synthase (nM/min/mg protein)	0.167 ± 0.08	0.159 ± 0.007	0.170 ± 0.007	0.474	0.770	0.491	> 0.34
H_2O_2 (pmols H_2O_2/mg protein/min)	47.9 ± 10.0	40.1 + 8.2	29.0 ± 5.02	0.203	**0.041**	**0.027**	> 0.49
Complex I substrates							
State 3 respiration (nmole O^2/mg/protein/min)	62.9 ± 3.8	71.7 ± 4.12	66.1 ± 6.86	0.203	0.589	0.570	> 0.11
State 4 respiration (nmole O^2/mg/protein/min)	13.2 ± 1.6	10.8 ± 1.4	11.2 ± 1.4	–	–	–	**NR vs. 1 = 0.049** **NR vs. 4 = 0.003**
Respiratory control ratio (state 3/state 4)	5.28 ± 0.53	7.38 ± 0.78	6.41 ± 0.58	–	–	–	NR vs. 1 = 0.228 **NR vs. 4 = 0.008**
Complex II substrates							
State 3 respiration (nmole O^2/mg/protein/min)	112.67 + 6.5	125.0 ± 8.6	121.1 ± 11.3	0.414	0.548	0.745	> 0.93
State 4 respiration (nmole O^2/mg/protein/min)	23.6 ± 1.7	22.6 ± 1.8	21.2 ± 2.0	0.721	0.375	0.943	> 0.46
Respiratory control ratio (state 3/state 4)	5.04 ± 0.51	5.68 ± 0.39	5.80 ± 0.41	0.359	0.254	0.922	> 0.40
Markers of oxidative stress							
4HNE adducts (arbitrary units)	1.00 ± 0.02	1.01 ± 0.01	0.998 ± 0.02	0.668	0.594	**0.006**	> 0.17
Protein carbonyls (arbitrary units)	1.00 ± 0.02	1.05 ± 0.02	1.06 ± 0.02	–	–	–	NR vs. 1 = 0.093 **NR vs. 4 = 0.044**
8-OHdG (pg/ml)	1303 ± 94	1386 ± 5	1337 ± 59	0.715	0.886	0.138	> 0.78
SOD1 (arbitrary units)	1.00 ± 0.02	0.994 ± 0.025	0.988 ± 0.03	0.764	0.677	0.605	> 0.36
SOD2 (arbitrary units)	1.00 ± 0.04	0.942 ± 0.052	0.913 ± 0.04	0.076	0.062	**0.002**	> 0.10
CAT (arbitrary units)	1.00 ± 0.06	1.06 ± 0.05	1.02 ± 0.04	0.754	0.970	**0.024**	> 0.07
GPX (arbitrary units)	1.00 ± 0.05	0.976 ± 0.043	0.992 ± 0.079	0.578	0.809	0.271	> 0.45
Skeletal muscle							
Citrate synthase (nM/min/mg protein)	0.305 ± 0.388	0.249 ± 0.029	0.280 ± 0.027	0.193	0.556	0.608	> 0.34
Markers of oxidative stress							
4HNE adducts (arbitrary units)	0.999 ± 0.016	1.01 ± 0.02	1.02 ± 0.02	0.658	0.536	0.618	> 0.41
Protein carbonyls (arbitrary units)	0.998 ± 0.012	0.981 + 0.014	0.989 ± 0.02	0.177	0.746	0.943	> 0.48
8-OHdG (pg/ml)	1386 ± 11	1644 ± 154	1367 ± 121	0.527	0.748	0.079	> 0.08
SOD1 (arbitrary units)	1.00 ± 0.040	0.996 ± 0.072	0.991 ± 0.050	–	–	–	NR vs. 1 = 0.064 **NR vs. 4 = 0.010**
SOD2 (arbitrary units)	1.00 ± 0.05	0.953 ± 0.100	1.01 ± 0.06	0.628	0.981	0.411	> 0.15
CAT (arbitrary units)	1.00 ± 0.07	1.04 ± 0.05	0.978 ± 0.08	0.995	0.636	0.094	> 0.31
GPX (arbitrary units)	1.00 ± 0.11	0.987 ± 0.124	1.15 ± 0.17	0.683	0.565	0.168	> 0.42
Heart							
Citrate synthase (nM/min/mg protein	0.873 ± 0.07	0.888 ± 0.054	0.936 ± 0.025	0.834	0.415	0.956	> 0.05
Markers of oxidative stress							
4HNE adducts (arbitrary units)	1.00 ± 0.03	1.02 ± 0.03	1.02 ± 0.03	0.903	0.748	0.192	> 0.96
Protein carbonyls (arbitrary units)	1.00 ± 0.04	1.00 ± 0.04	1.04 ± 0.05	0.719	0.710	0.112	> 0.39
8-OHdG (pg/ml)	1470 ± 117	1485 ± 49	1318 ± 50	0.649	0.086	**0.018**	> 0.06
SOD1 (arbitrary units)	0.934 ± 0.06	1.03 ± 0.03	0.981 ± 0.04	0.986	0.653	0.109	> 0.19
SOD2 (arbitrary units)	1.00 ± 0.07	0.923 ± 0.059	0.932 ± 0.04	0.216	0.209	**0.003**	> 0.12
CAT (arbitrary units)	1.00 ± 0.06	0.886 ± 0.060	1.06 ± 0.09	0.161	0.655	0.250	> 0.54
GPX (arbitrary units)	1.00 ± 0.09	1.43 ± 0.19	1.17 ± 0.15	0.216	0.167	0.839	> 0.78

Body mass were included as a covariate and interactions between body mass and group were evaluated. When the interaction terms were not significant, they were not removed from the model. The p values are given for all comparisons. When neither interaction (NR vs. 1 controlling for body mass or NR vs.4 controlling for body mass) was significant, the lowest value is reported. Significant findings are reported in bold

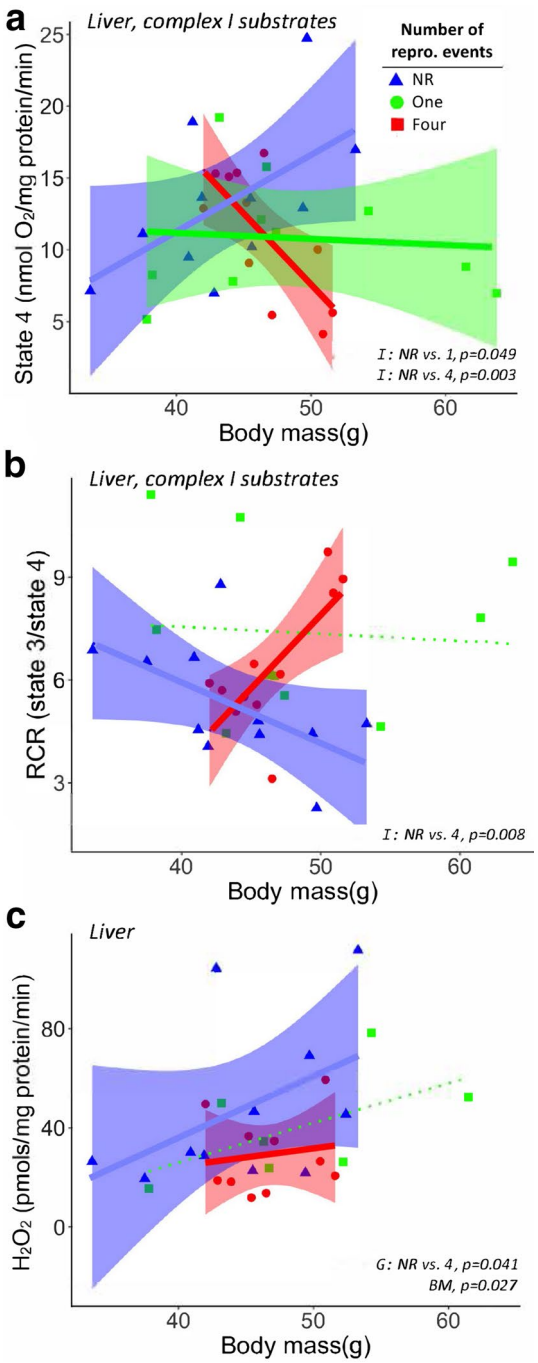

Fig. 2 Significant relationships between parity, body mass, and mitochondrial respiratory performance in liver mitochondria. Data include **a** state 4 respiration with complex I substrates, **b** RCR with complex I substrates and **c** reactive oxygen species production, as indicated by hydrogen peroxide production. Data for several groups covaried with body mass and displayed significant interactions. The groups with shaded 95% confidence intervals in **a** and **b** displayed significant interactions (Table 1). The groups with shaded confidence intervals in **c** were significantly different from one another and varied with body mass but did not interact (Table 1). Dashed regression lines are included for all groups to emphasize trends with parity. The statistics represent the significant effects of body mass (BM), pairwise differences between reproductive groups (G: NR vs. 1 or 4) or pairwise differences between reproductive groups and the slopes of body mass and mitochondrial respiration/capacity (I: NR vs. 1 or 4)

state 4 respiration, and RCR did not vary with body mass or parity (Table 1).

Several markers of oxidative stress in the liver were shown to vary as a function of body mass (Fig. 3a–d); while, only protein carbonyls displayed a significant interaction between body mass and the groups. Four-bout females displayed a significantly different relationship with body mass than NR females ($p=0.044$, Table 1, Fig. 3b). Protein carbonyls decreased with body mass in NR females [independent lm, $Y=(0.0077*\text{body mass})+1.34$, $p=0.074$] but showed a trend suggestive of increased protein carbonyls with body mass in 4-bout females [independent lm, $Y=(0.011*\text{body mass})+0.56$, $p=0.183$]. Protein carbonyls in the NR group did not differ from 1-bout females in the relationship between body mass ($p=0.093$, Table 1, Fig. 3b). 4HNE adducts [$Y=(0.0044*\text{body mass})+0.80$, $p=0.006$, Fig. 3a, Table 1], SOD2 [$Y=(0.011*\text{body mass})+0.46$, $p=0.008$, Fig. 3c, Table 1], and catalase [$Y=(0.011*\text{body mass})+0.50$, $p=0.025$, Fig. 3d, Table 1] in the liver all increased with body mass.

Finally, when we limited the analysis to the 4-bout multiparous females, which all produced 4 litters, we found that state 4 respiration with complex I substrate displayed a negative relationship with cumulative mass of the young weaned by each female [$Y=(-0.06*\text{cumulative mass of pups weaned})+45.79$, $p=0.021$, Fig. 4a, Table 2). This decline in state 4 respiration was associated with a positive trend in RCR relative to the cumulative mass of the pups weaned [$Y=(-0.02*\text{cumulative mass of pups weaned})-5.72$, $p=0.074$, Fig. 4b, Table 2]. Further, protein oxidation displayed significant positive relationship with the cumulative mass of the pups weaned [$Y=0.0009*\text{cumulative mass of the pups weaned})+0.4943$, $p=0.039$, Fig. 4d, Table 2]. Finally, citrate synthase was negatively correlated with cumulative mass of the pups weaned [$Y=-0.0003*\text{cumulative mass of the pups weaned})+0.3334$, $p=0.036$, Fig. 4c, Table 2].

Skeletal muscle

For the skeletal muscle, we found a significant reduction in muscle mass in 4-bout females (0.99 ± 0.03 g) relative to NR females (1.07 ± 0.03 g, $p=0.048$), and a trend suggesting that muscle mass increased with body mass ($p=0.068$). There was no difference in muscle mass between 1-bout and NR females (1.07 ± 0.03 g, $p=0.669$; Table 1).

Mitochondrial density and oxidative stress markers were compared between reproductive groups for the skeletal muscle. There was no impact of reproductive group on the citrate synthase activity or the oxidative damage markers (Table 1). The antioxidant SOD1 displayed a significant difference in the relationship with body mass between NR females and 4-bout females ($p=0.010$,

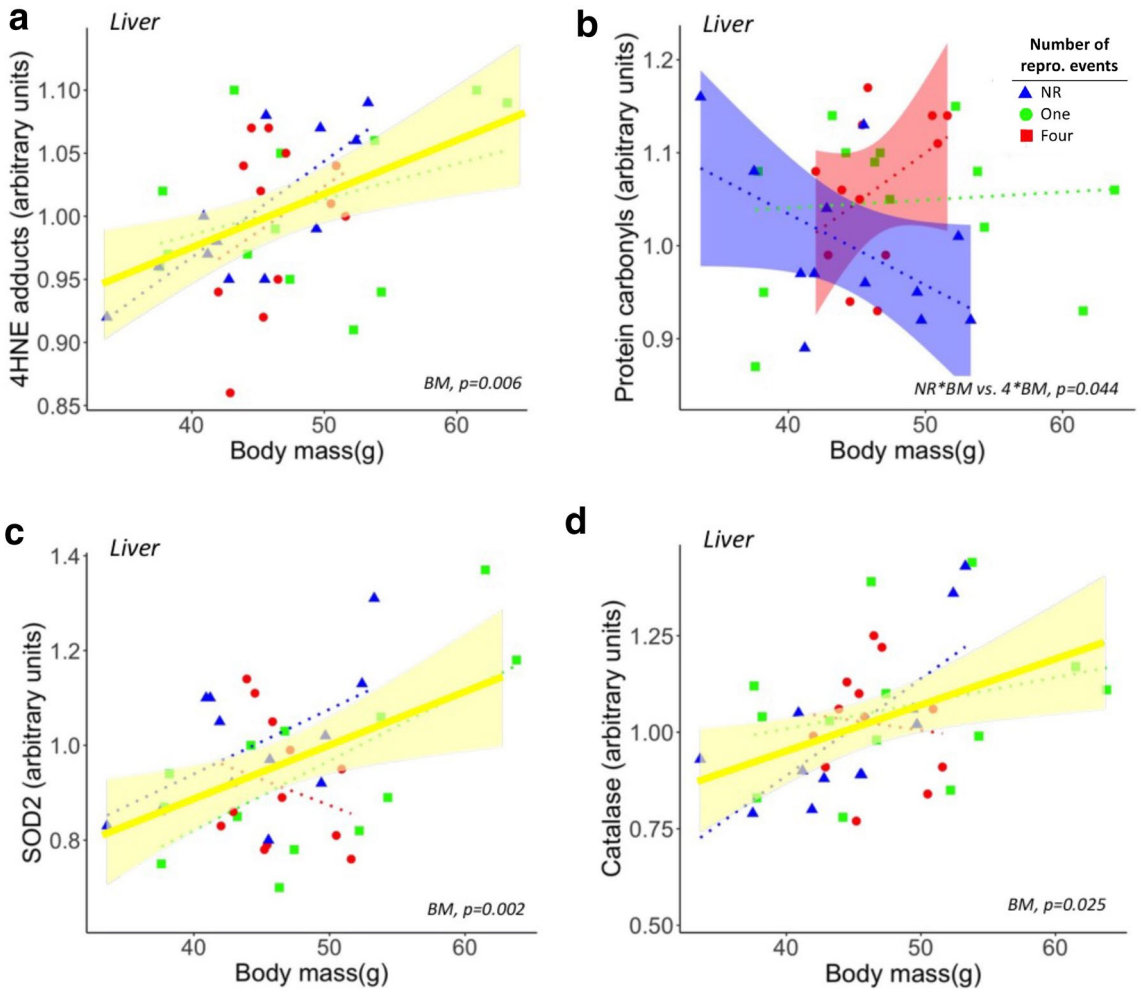

Fig. 3 Relationships between parity, body mass, and oxidative stress in the liver. Significant relationships included two measures of oxidative damage, **a** 4-hydroxynonenal (4HNE) adducts and **b** protein carbonyls, and two antioxidants, **c** superoxide dismutase 2 (SOD2) and **d** catalase. Protein carbonyls (B) displayed a significant differences between non-reproductive and the 4-bout group with respect to the relationship with body, as indicated by significant interaction terms for these groups and depicted in the 95% confidence intervals. There were no effects of parity on 4HNE, SOD2 or catalase. The effect of body mass on these variables is indicated by the yellow 95% confidence intervals. Dashed regression lines are included for all groups to emphasize trends with parity. The statistics represent the significant effects of body mass (BM), or pairwise differences between reproductive groups and the slopes of body mass and oxidative stress marker (I: NR vs. 4).

Table 1, Fig. 5a). In this case, SOD1 levels increased with body mass in NR females [$Y = (0.021*\text{body mass}) + 0.046$, $p < 0.001$] and decreased with body mass in 4-bout females [$Y = (-0.028*\text{body mass}) + 2.31$, $p = 0.078$]. SOD1 in NR females did not differ from 1-bout females with respect to its relationship with body mass ($p = 0.064$). No other antioxidants varied with reproductive group or body mass. No significant relationships between the cumulative mass of pups weaned by 4-bout females and oxidative stress markers were observed in skeletal muscle.

Heart

Heart mass increase with parity but was only significantly different between the NR and 4-bout females ($p = < 0.001$). Heart mass also increased with the mass of the female ($p < 0.001$). Most markers of oxidative damage (4HNE adducts and protein carbonyls) or antioxidants (SOD1, SOD2, CAT, and GPX) did not differ with body mass or parity (Table 1). However, the linear model revealed that heart 8-OHdG increased with body mass [$Y = (16.6*\text{body mass}) + 654.0$, $p = 0.018$, Fig. 5b, Table 1], as did SOD2

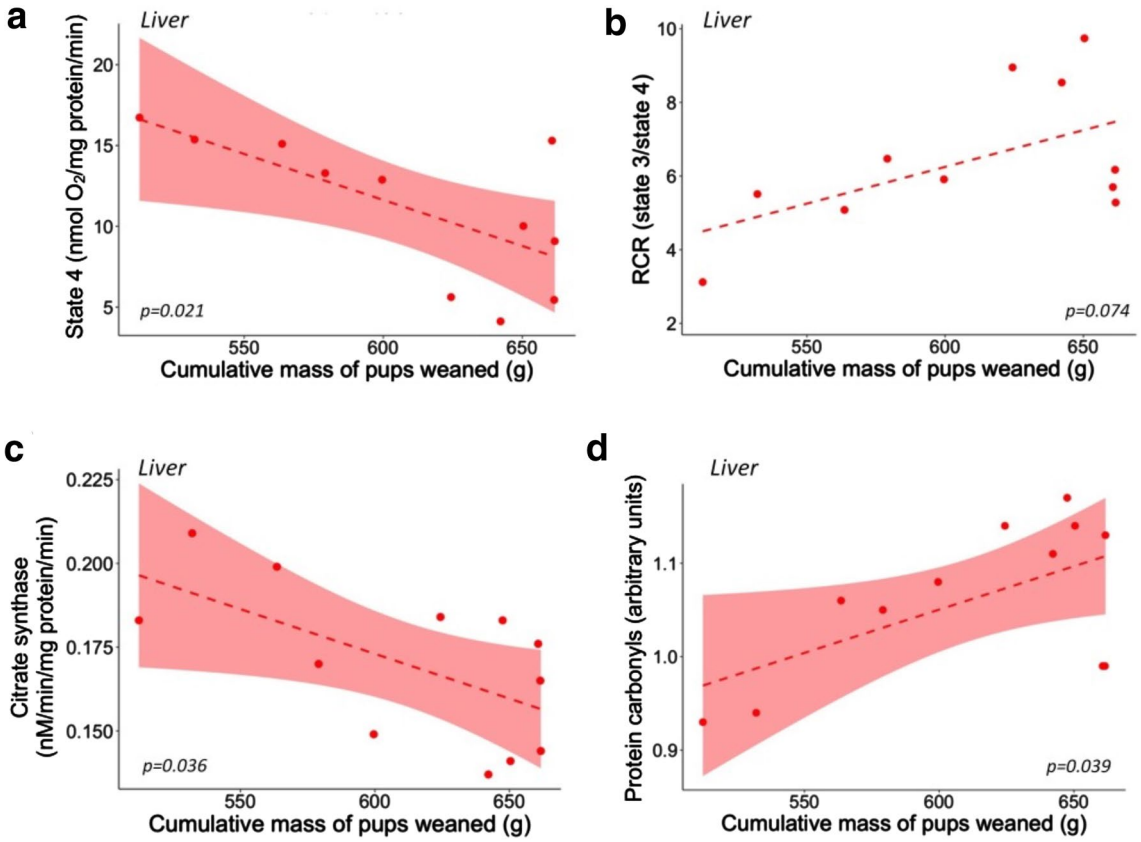

Fig. 4 Significant and near significant relationships between number of young produced and mitochondrial respiration and oxidative stress in mothers that had four litters. Data included **a** state 4 respiration and **b** RCR in the liver with complex I substrates, **c** citrate synthase, and **d** protein carbonyls

[$Y = (0.014$*body mass$) + 0.303$, $p = 0.003$, Fig. 5c, Table 1]. No significant relationships between the cumulative mass of pups weaned by multiparous females and oxidative stress markers were observed in the heart.

Discussion

We experimentally manipulated the number of reproductive bouts over the lifetime of female mice and measured the effect of parity on organ function. It is well established that the performance of both whole-animal metabolic rate and the mitochondria are impacted by body mass (Brand et al. 2003; Speakman et al. 2004a; Roussel et al. 2015; Mélanie et al. 2019). We also show that several markers of oxidative stress significantly increased with body mass, including a lipid peroxidation marker in the liver (4HNE), a DNA damage marker in the heart (8-OHdG), and antioxidant markers in the liver (SOD2 and catalase) and heart (SOD2). In contrast, several variables were also strongly impacted by parity, with nulliparous and multiparous females displaying opposite trends. Further, the RCR of the liver with complex I substrates increased with body mass in multiparous females but decreased with body mass in nulliparous females. For these groups, the performance of the mitochondria in adult female mice appears to be condition dependent.

We designed this experiment with the hopes of both capturing the beneficial effects that reproduction can have with mitochondrial performance (Hood et al. 2018, 2019) and the proposed decline that is predicted under antagonistic pleiotropy and the oxidative cost of reproduction hypothesis (Hood et al. 2018). Our results imply that the benefits of reproduction on mitochondrial performance are most robust in multiparous females that experienced four reproductive events. Comparisons between groups indicate that the liver of multiparous females in this study had a higher mitochondrial RCR associated with lower state 4 respiration when fueled via complex 1 substrate. Lower state 4 respiration reduces the carbohydrate demands of the liver mitochondria while idling; yet, this condition can also be associated with an increase in the production of ROS (Brand 2000; Speakman et al. 2004b), as observed in the multiparous females. We found a comparable trend in protein oxidation levels ($p = 0.064$), but this effect was limited in those with

Table 2 Results of general linear models evaluating the impact number of young weaned on measures morphology and mitochondrial physiology in 4-bout multiparous mice

Statistics						
	F	*P*	*F*	*P*	*F*	*P*
Body mass[b] (g)	0.827	0.385				
	Liver		Skeletal muscle		Heart	
Morphology						
Organ mass (g)	1.87	0.201	0.43	0.528	0.965	0.349
Mitochondrial physiology						
Citrate synthase (nM/min/mg protein)	**5.83**	**0.036**	3.73	0.082	0.07[a]	0.800
H_2O_2 (pmols H_2O_2/mg protein/min)	0.22[a]	0.649	---	---	---	---
Respiration with complex I substrates						
State 3 respiration (nmole O^2/mg/protein/min)	0.481[b]	0.506	---	---	---	---
State 4 respiration (nmole O^2/mg/protein/min)	**7.72**[b]	**0.021**	---	---	---	---
Respiratory Control Ratio (state 3/state 4)	4.10[b]	0.074	---	---	---	---
Respiration with complex II substrates						
State 3 respiration (nmole O^2/mg/protein/min)	1.51[a]	0.254	---	---	---	---
State 4 respiration (nmole O^2/mg/protein/min)	1.45[a]	0.263	---	---	---	---
Respiratory Control Ratio (state 3/state 4)	0.039[a]	0.848	---	---	---	---
Oxidative stress markers						
4HNE adducts (arbitrary units)	0.31	0.588	0.63	0.445	1.75	0.216
Protein carbonyls (arbitrary units)	**5.63**	**0.039**	1.46	0.255	1.76	0.217
8-OHdG (pg/ml)	1.69	0.222	0.31	0.590	1.35	0.273
SOD1 (arbitrary units)	0.12	0.738	1.05	0.329	0.29	0.602
SOD2 (arbitrary units)	1.04	0.332	0.61	0.451	0.30	0.596
CAT (arbitrary units)	0.77	0.400	0.05	0.827	0.18	0.683
GPX (arbitrary units)	0.41[b]	0.535	0.00	0.991	0.22	0.647

Significant findings are reported in bold

--- indicated values that were not quantified

Degrees of freedom = 11 unless otherwise indicated (see superscripts). Equations for significant and near significant trends given in the results section

[a]degrees of freedom = 9

[b]degrees of freedom = 10

the highest number of breeding events. Prior studies have shown inconsistent support for the idea that reproduction has an oxidative cost (Oldakowski et al. 2012; Plumel et al. 2014; Speakman and Garratt 2014; Blount et al. 2016). By waiting until after the liver and mammary tissue regressed, we targeted baseline conditions that could impact future reproduction and survival.

When considering the interaction between reproductive groups and body mass among mitochondrial performance variables, several interesting patterns emerged. For several variables (liver: state 4 respiration and RCR with complex I substrates, and protein carbonyls; skeletal muscle: SOD1), the direction of the best fit regressions for body mass in nulliparous and multiparous groups was in direct opposition with one another. These interactions suggest that body mass has very different effects on performance in each of these groups. Interestingly, the body mass of females was not statistically different between each of the reproductive groups (averaging 46 g), and the pivot point between the patterns displayed by nulliparous and multiparous groups was at or just below 46 g for all variables displaying interactions. The metabolic costs of reproduction are expected to be reduced in higher condition females (Reznick et al. 2000). This was exemplified by the tight positive linear relationship between body mass and liver RCR with complex substrates in multiparous females. RCR was lowest in the heaviest nulliparous females.

We suspect that body mass was not positively correlated with condition in nulliparous females because heavier nulliparous females may be experiencing the negative impacts of obesity. After breeding has ceased, multiparous females retain some of the increase in liver mass and blood volume that accompanies reproduction (Speakman and McQueenie 1996). Thus, increased liver mass and blood volume may have contributed to higher body mass and performance of multiparous, rather than body fat. Further, greater investment in reproduction has been shown to reduce non-alcoholic fatty liver disease and improve the condition of human subjects

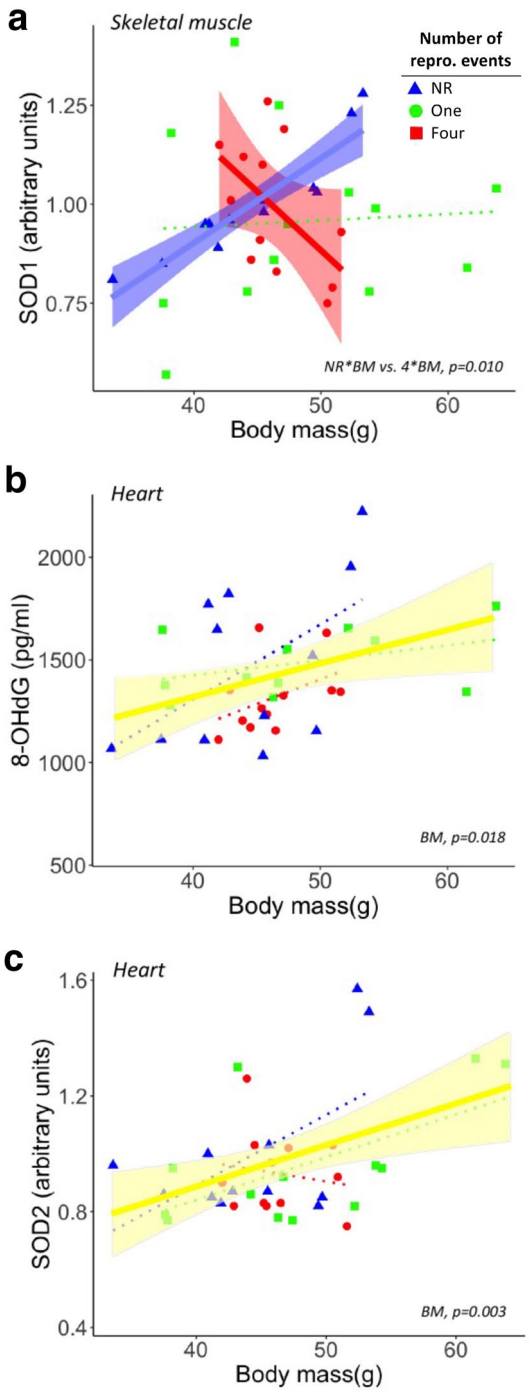

Fig. 5 Relationships between parity, body mass, and oxidative stress in skeletal muscle and the heart including **a** superoxide dismutase 1 (SOD1) in the skeletal muscle, **b** DNA damage in the heart, and **c** superoxide dismutase 2 (SOD2) in the heart. SOD1 displayed a significant differences between non-reproductive and 4-bout females with respect to the relationship with body mass, as indicated by significant interaction terms for these groups and depicted in the 95% confidence intervals. There was no effect of parity in **b** or **c**. The effect of body mass on these variables is indicated by the yellow 95% confidence intervals. Dashed regression lines are included for all groups to emphasize trends with parity. The statistics represent the significant effects of body mass (BM), or pairwise differences between reproductive groups and the slopes of body mass and oxidative stress marker (I: NR vs. 4)

(Ajmera et al. 2019). Obesity increases state 4 respiration, as observed in the nulliparous females; but high state 4 may also increase RCR associated with a greater increase in state 3 than state 4 respiration when obese animals remain otherwise healthy (Brady et al. 1985). Our findings suggest that the interaction between body mass and the condition of the individual is dependent on their reproductive history and is a vitally important covariate to consider in comparable studies.

Finally, we evaluated the relationship between the total mass of young produced by multiparous females and mitochondrial performance to determine if the costs of reproduction are revealed within this group. The consequences of reproduction were most apparent on the bioenergetic performance of the liver. In the liver, we found that state 4 respiration of mitochondria displayed a negative relationship with the cumulative mass of all the pups a mother weaned. Further, a decline in state 4 respiration was also associated with a near significant trend suggesting that females that allocate more to reproduction may also have higher RCR. While this condition suggests that the most productive females would have the greatest mitochondrial capacity, the more productive females also have relatively fewer mitochondria than females with lower maternal investment, as indicated by reduced citrate synthase activity. Reduced state 4 respiration is also associated with increased oxidative damage in females with the highest levels of investment. Under the uncoupling to survive hypothesis (Brand 2000; Speakman et al. 2004b), these females with lower state 4 respiration would be predicted to have higher oxidative damage because of tighter coupling, and as a consequence, they would have reduced longevity. While support for the hypothesis that oxidative damage to DNA contributes to aging has waned (Hood et al. 2019), it is possible that protein damage is more critical in the liver (Reeg and Grune 2015). Further, an accumulation of oxidative damage to DNA in the heart in the most productive females was also observed.

Conclusions

With this investigation, we found that multiple reproductive events and high reproductive output appear to be associated with both improved respiratory performance and an increase in protein oxidation. Given the high performance of mitochondria in these animals, we expect that the detected oxidative damage has had minimal impact on performance of mitochondria. This response is noteworthy given the intense role that the liver plays in supporting reproductive demand. Animals that experience oxidative stress prior to reproduction have shown both a delay in the onset of reproduction and reduced future reproductive performance (Stier et al. 2012; Costantini et al. 2016; Hood et al. 2019). Thus, it is

possible that the consequences of increased oxidative damage could be revealed in future reproductive events (Zhang and Hood 2016).

In laboratory mice, females can produce 5–10 litters per year, with an average lifespan of 2–3 years (Wolf and Austad 2010). Newman et al (1985) documented changes in litter mass at weaning (a strong indication of energy allocated to reproduction) across the life of females belonging to three different strains of laboratory mice. They found that litter mass at weaning does not drop below that of the first reproductive event until at least an average of 8 reproductive events was completed. It is probable that in the optimal conditions of a laboratory environment, the cellular consequences of reproduction may not be apparent until the later stage of a female's reproductive life when female performance begins to decline.

This study revealed that the cost of reproduction in laboratory mice is limited early in their reproductive life and highlights the importance of including maternal body mass as a covariate in life-history studies. Further evaluation of mice experiencing 8 or more reproductive bouts is warranted to determine if the physiological trade-offs responsible for the reproduction and longevity trade-off are revealed.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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