Mitochondrial function and oxidative stress in response to induced reactive oxygen species and reproduction

by

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A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
August 4th, 2018

Keywords: mitochondria, reactive oxygen species, oxidative stress, cost of reproduction, longevity, life history tradeoffs

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Abstract

Mitochondria are pivotal in the survival of complex organisms, generating metabolic energy in all eukaryotic cells by breaking down carbohydrates, fatty acids, and protein from diet and converting them to ATP (adenosine triphosphate) through oxidative phosphorylation. As a byproduct of this process, reactive oxygen species (ROS) can form, a form of free radical that have the capacity to damage macromolecules. When there is an imbalance between relative levels of ROS and antioxidants, damage can accumulate within the cell; this condition is referred to as "oxidative stress" and results in macromolecular damage such as lipid peroxidation, protein oxidation, and DNA mutations that are associated with various diseases and aging.

Paradoxically, however, the notion that oxidative stress is always harmful is controversial. Besides generating damage, ROS have also been shown to act as signaling molecules stimulating processes that promote increased mitochondrial biogenesis, antioxidant production and repair of damaged macromolecules. Thus, mitochondria are hypothesized to display a biphasic response to ROS exposure referred to as mitochondrial hormesis. For my thesis, I investigated the mitochondrial function and oxidative stress under two scenarios in mice: induced ROS production and natural life-history event of reproduction.

The majority of studies evaluating the impact of oxidative stress on animals have largely focused on quantifying damage to proteins and lipids. Therefore, in chapter one, I examined the temporal response to induced ROS via radiation on DNA in wild-derived mice, extrapolating upon a previous study from our lab (Zhang et al., 2017) looking at the effect of radiation-induced

ROS on mitochondrial function and physiological parameters. I measured a biomarker of oxidative DNA damage 8-oxo-7,8-dihydroguanine (8-OHdG), and its primary repair protein 8-oxoguanine glycosylate (OGG1), using the frozen tissues from the past study. I found there to be a mitohormetic response at the DNA level, consistent with past study's findings of the same pattern on other macromolecules.

In chapter two, I asked if mitohormesis can be seen in a natural life-history setting. Reproduction is an energetically demanding activity, with individuals that allocate more to reproduction typically having reduced longevity. But despite numerous studies and reviews, the mechanisms behind the tradeoff between reproduction, bioenergetics and longevity are still poorly understood. I asked if oxidative stress could be the mediator of this tradeoff, if and when the cost of reproduction is seen with varying parity in laboratory mice. I hypothesized that females that have undergone one bout of reproduction will display improved mitochondrial function relative to non-reproductive mice, while multiparous females display negative effects of continuous reproduction. The results revealed no negative effects of reproductive effort on the bioenergetic capacity of female lab mice. Instead, females with the highest reproductive performance had a heavier liver and heart, which would equate to more liver and heart mitochondria, and their skeletal muscle mitochondria displayed higher respiratory performance when oxidizing lipid.

Together, my results suggest that there is evidence of a mitohormetic response at the DNA level under induced ROS, and that the assumed negatively linear tradeoff between reproduction and longevity does not hold in the context of oxidative stress.

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ROS	Reactive oxygen species
8-OHdG	8-oxo-7,8-dihydroguanine
OGG1	8-oxoguanine glycosylate
NFE2L2	nuclear factor (erythroid-derived 2)-like 2

Chapter 1

Temporal response to ROS exposure: oxidative DNA damage and repair in wild-derived mice

Abstract

Reactive oxygen species (ROS) can induce lipid peroxidation, protein oxidation, and DNA mutations that can accumulate and contribute to reduced performance, disease, and senescence. In this study, I quantified DNA damage and repair induced with radiation to model the response to an acute oxidative stressor. Thirty-two wild-derived mice were equally divided among four groups (non-irradiated control and three experimental groups that varied in time between radiation exposure and euthanasia (1, 4, or 10 days after x-irradiation). I measured a biomarker of oxidative damage to DNA, 8-oxo-7,8-dihydroguanine (8-OHdG), and its primary repair enzyme 8-oxoguanine glycosylate (OGG1) in liver, skeletal muscle, and heart at each time point. At day 10, 8-OHdG levels were significantly lower in the liver, skeletal muscle and heart. There was no statistical change in levels of the repair enzyme OGG1 in the liver. OGG1 in the skeletal muscle significantly increased on day 10 from day 1. In the heart, OGG1 level on day 10 was significantly higher than control, day 1 and day 4. A decrease in the levels of DNA damage and upregulation of its primary repair enzyme for these organs suggest a benefit from ROS exposure, and that DNA displays a hormetic response to a brief, modest exposure to ROS.

Introduction

Oxidative stress has been proposed to underlie variation in the performance of individuals under numerous conditions including, but not limited to: stress, disease, and reproduction (Monaghan et al., 2008; Speakman and Garratt, 2014; Zhang and Hood, 2016). While there is little doubt that oxidative stress has the potential to contribute to individual differences in performance, the results of empirical studies have often yielded equivocal support for proposed hypotheses. One possible reason for this inconsistency is because the production of reactive oxidative species (ROS) and resulting oxidative damage are not consistently harmful (Mowry et al., 2016; Ristow and Zarse, 2010; Zhang et al., 2018, 2017).

Methods that allow for the experimental induction of ROS production are crucial for understanding the role of ROS in biological processes. In a recent study, Zhang et al. (2017) used x-radiation as an acute pro-oxidant to understand the response of cells and mitochondria of several organs to an oxidative event. While several exogenous stressors can be used to induce ROS, radiation is particularly valuable because it can uniformly increase ROS production via the direct ionization of water found in cells while producing few additional side effects (Koch and Hill, 2017; Riley, 1994; Szumiel, 2015).

Zhang et al. (2017) found that following x-radiation on mice, lipid and protein damage increased and mitochondrial respiratory function declined over the first 24 hours, but then both returned to control levels by 4-7 days post-exposure. Even more impressively, mitochondrial complex activity exceeded that of the controls, and ROS and oxidative damage levels dropped below control levels by day 10. While a few mechanisms for this effect were proposed, including the increased induction of antioxidants via NFE2L2 (nuclear factor (erythroid-derived 2)-like 2)

and increased signaling of mitochondrial biogenesis via PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha), it is clear that these processes only partially describe the observed response. Oxidative damage repair processes were not evaluated and almost surely contribute to the observed response, as well.

Studies that have evaluated the impact of oxidative stress on individuals and isolated cells have often focused on the impact of oxidative stress on proteins and lipids due to the ease and reliability with which these variables can be measured (Powers et al., 2010). DNA damage and repair were missing components of Zhang et al.'s study. Guanine is the main target of ROS in nucleic acids, with the base lesion of 8-hydroxy-2'-deoxyguanosine (8-OHdG) being the most prominent indication of oxidative damage to DNA (Cheng et al., 1992; Kasai, 1997; Kong and Lin, 2010; Valavanidis et al., 2009). To counteract DNA damage, base excision repair involving glycosylase subsequently removes 8-OHdG lesions in DNA (Maynard et al., 2009), with 8-oxoguanine glycolylase 1 (OGG1) being one of the three mammalian glycosylase enzymes that most efficiently removes these oxidative lesions (David-Cordonnier et al., 2001). Thus, the goal of this study was to describe the temporal change in DNA damage and repair in response to ROS exposure using the same tissues from Zhang et al's.

Furthermore, the allocation of resources to protect organ systems against damage and to repair damage is thought to follow priority rules, where the protection and repair of damage to organs required for instantaneous survival are prioritized over those with less immediate demand (Zera and Harshman, 2001). Therefore, the last goal of this project was to qualitatively assess if the patterns observed in organs from damage and repair in response to ROS exposure follow these priority rules. I predicted damage to nucleic acids would follow the pattern described for lipids and protein with repair processes being upregulated and damage returning to baseline

levels. And following priority rules, I predicted that damage would be lower and repair would occur most rapidly in the heart, followed by the liver, and finally the skeletal muscle.

Materials and Methods

Experimental Design and Sample Collection

All husbandry and experimental procedures were approved by the Auburn University Institutional Animal Care and Use Committee (PRN 2016-2903). Adult (4-6 months old) female wild-derived house mice (*Mus musculus*) were used for this study. I chose wild-derived house mice for a variety of reasons: 1) their laboratory counterparts have been selected for a variety of traits, and thus 2) display differences from wild mice such as robustness in size and fecundity but about 20% shorter life span (Miller et al., 2002). Most laboratory species are also inbred and carry homozygosity at all genetic loci, and are less sensitive to stressors and subject to inbreeding depression in a variety of traits (Phelan and Austad, 1994). Therefore, I deemed it most apposite to carry our study in wild-derived mice, who are expected display responses most similar to animals in their natural life history settings. Animals were maintained on a natural light-dark cycle and temperature (average 30˚C/17˚C). Food and water were provided ad libitum, and all animals were provided with a running wheel as enrichment.

Animals were maintained, ROS was induced, and samples were collected as described previously (Zhang et al., 2017). Briefly, mice were randomly assigned to one of four groups (n=8/group). All mice within each group were selected from different parental lineages to ensure genetic diversity. Group was defined by the time euthanized relative to x-irradiation. A non-irradiated control group was also included in the study. Mice were exposed to x-irradiation dosage of 5 Gy at 2 Gy/min. At 1 day, 4 days, or 10 days after irradiation, mice were

anesthetized with an overdose of isoflurane vapors and then decapitated with a rodent guillotine. Liver, both hind limb muscles, and heart were harvested and flash frozen in liquid nitrogen and moved to -80˚C freezer. These frozen tissues were used in this current investigation.

DNA Damage and Repair

Genomic DNA (gDNA) was extracted from 25 mg of each of the frozen tissues using the Qiagen DNeasy kit (cat. 69506). DNA extract purity and concentration were assessed by measuring absorbance ratios A_{260}/A_{280} using the NanoDrop Lite instrument (Thermo Scientific, Waltham, MA). The DNA extracts were subsequently precipitated and hydrolyzed to deoxyribonucleosides using Nuclease P1 (Sigma N8630) following manufacturer's instructions. Briefly, DNA pellets were re-suspended in 20 mM sodium acetate, pH 5.0-5.4, then denatured by boiling for ten minutes. Appropriate amount of stock reagents zinc chloride (10 mM), nuclease P1 (5U/ml) were added to each DNA sample. The reaction mixtures were incubated for 30 minutes at 37 C, and pH was adjusted to 7.5-8.0 by addition of ~1/10 volume of 1 M Tris-HCl, pH 8.0. Alkaline phosphatase (0.15U) was added to each sample to dephosphorylate and prevent self-ligation, and incubated at 37ºC again for 30 minutes. Samples were boiled again for ten minutes to inactivate the alkaline phosphatase, and further diluted in ELISA buffer prior to being assayed.

I 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. 589320). This assay is based on the competition between oxidatively damaged guanine species and an 8-OH-dG-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. The ELISA values will be higher than other methods of oxidative DNA damage quantification such as LC/MS.

Oxidative repair enzyme OGG1 (GTX20204, GeneTex**)** protein levels were quantified through western blot in the liver, muscle and heart tissues. I followed the western blot described by Mowry et al (2016). Briefly, tissue was homogenized 1:10 (wt/vol) in 5 mM Tris HCl (pH 7.5), 5 mM EDTA (pH 8.0), and protease inhibitor cocktail and was centrifuged at 1500 *g* for 10 minutes at 4 °C. Proteins were loaded onto a 4-20% SDS-polyacrylamide gels (Bio-Rad) and were subjected to electrophoresis (200V at 60 min). Proteins were then subsequently transferred to polyvinylidene difluride membranes (Bio-Rad). All samples from each tissue were run on the same gel, along with the ladder and control sample to account for variance between different gels. Each western membrane was stained by ponceau and protein content of these blots was normalized to ponceau stain levels. A chemiluminescent system was used to visualize OGG1 protein (GE Healthcare Life Sciences, Pittsburgh, PA). Images were taken and analyzed with the ChemiDocIt Imaging System (UVP, LLC, Upland, CA).

Statistics

Grubb's outlier test was used to identify and remove significant physiological outliers using the GraphPad QuickCalcs (website: https://www.graphpad.com/quickcalcs/Grubbs1.cfm, accessed May 9, 2018). All remaining comparisons were completed with GraphPad Prism version 7.02 for Windows (Graphpad Software, La Jolla California, USA), and RStudio 1.1.453

(RStudio, Boston, MA, USA). One-way ANOVA was used to compare relative concentrations of 8-OHdG and OGG1 within tissue, followed by Tukey's post-hoc tests when appropriate, for all other organs. Because liver is expected to have higher levels of RNA than the other tissues, I did not compare the tissues directly. Instead, I discuss the qualitative differences between organs relative to the control.

Results

8-OHdG, a marker of oxidative damage to DNA, was measured in the liver, muscle and heart. In liver, DNA oxidative damage levels were similar between control, day 1 and day 4 animals, but day 10 level was lower than all other groups ($F_{3,28}$= 3.30, P=0.023; Fig. 1A). In skeletal muscle, average 8-OHdG levels were greater than control levels on day 1 and 4 post-exposure but the observed pattern was not significant (P=0.06; Fig. 1B). On day 10, 8-OHdG levels were lower than on day 1 and 4 (day 1 vs. day 10: P<0.001; day 1 vs. day 4: P =0.014). 8-OHdG levels in the heart were substantially lower than the controls on day 4 and 10 post-exposure (control vs. day 4: P<0.001; control vs. day 10: P < 0.001; Fig. 1C).

The DNA damage repair protein OGG1 was measured in the liver, muscle and heart. OGG1 levels stayed consistent in all tissues measured across all time points ($F_{3,27}$= 1.36, p=0.278; Fig. 2A). There was a significant increase in OGG1 level on day 10 from day 1 in the skeletal muscle (overall: $F_{3,27}$= 5.82, P=0.003; day 1 vs. day 10: P=0.002; Fig. 2B). In the heart, day 10 level was significantly higher than control, day 1 and day 4 (overall: $F_{3,28}$=7.09, P=0.001; control vs. day 10: P=0.022, day 1 vs. day 10: P=0.003, day 4 vs. day 10: P=0.002; Fig. 2C).

Discussion

In this study, I characterized the temporal response to induced-ROS at the DNA level in mice. While a prior study suggested that dramatic changes in lipid peroxidation and protein oxidation occurred in response to the same conditions, I found no increase in oxidative damage to DNA following the pro-oxidative insult of radiation. Indeed, all changes in DNA damage was associated with a reduction in levels of damage relative to the control individuals. Conversely, proteins that repair DNA damage significantly by day 10, confirming this response. Thus, DNA appears to be highly protected from oxidative damage.

Zhang et al. (2017) evaluated the damage to lipids and proteins following the same conditions described herein and found that ROS production increased by nearly 50%. Oxidative damage biomarkers for lipid peroxidation increased from nearly 1 to 3-fold in response to the oxidant across organs while protein oxidation displayed a similar but dampened response relative to lipid damage. ROS rapidly dropped within the first day and then damage to lipids and protein dropped to near or below control levels within 4-10 days of exposure in all tissues measured (Zhang et al., 2017). However, according to this current study's results, DNA does not display the same pattern of damage and recovery displayed by lipids and proteins. Instead, DNA oxidative damage remained unchanged from control levels until a significant drop from control values was detected on day 10 in liver and between day 4 and 10 in skeletal muscle. This drop is more rapid and more pronounced in the heart, where damage levels drop by nearly half by day 4 post-radiation. While it is feasible that oxidative damage to DNA occurred and was repaired within the 24-hour period, it is more likely that DNA is more highly protected from oxidative damage than lipid and proteins. Change in the redox condition of cells stimulates a number of signaling cascades that allow cells to adapt their intracellular environment (Dröge, 2002; Ray et

al., 2012). These processes undoubtedly contributed to the recovery from x-radiation, drop in baseline ROS levels, and improved mitochondrial complex performance described by Zhang et al (2017) and the improvement in baseline levels of oxidative damage carried by DNA.

Given that an increase in oxidative damage to DNA was not detected in the irradiated mice, it is surprising that a change in the base excision repair processes occurred and most likely aided in the drop of the damage level. There was no change in the liver OGG1 level across the timepoints, consistent with its oxidative DNA damage pattern. However, not only was there a significant difference in level of OGG1 by day 10 in skeletal muscle and heart, there was a trend in skeletal muscle suggesting that the repair enzyme may have actually become reduced in response to oxidant exposure on one day post radiation ($P=0.067$). But the level in both tissues were elevated by day 10, concurrently with the drop in damage. It is notable that the increase in the heart OGG1 level on day 10 is even more dramatic, matching its pronounced decrease in the damage level.

The energy and maintenance demands of organ systems that are vital for survival, like the brain and the heart, are expected to be prioritized over others that are not essential for immediate survival, such as those that contribute to reproduction and the immune response (Segerstrom, 2007; Zera and Harshman, 2001). As a result, it was no surprise to see such drastic difference in the levels of DNA damage and repair in the heart. The heart derives essentially all of its energy from aerobic metabolism, and cardiac myocytes have the highest mitochondrial volume density in mammals (Else and Hulbert, 1985; Izzi et al., 1991). It has also been shown that skeletal muscle and liver display a substantial age-related decrease of mitochondrial DNA copy number and oxidative DNA but not in the heart, presumably due to its constant aerobic activity (Barazzoni et al., 2000). These facts suggest that the heart may be more highly protected from

these studies and following the priority rule, and the results of our current investigation support this notion.

A caveat for studies utilizing radiation to induce oxidative stress like our study is that radiation can directly produce double strand break. They are the most threatening type of DNA damage, since a single unrepaired double strand break is sufficient for the induction of cell death (Jackson, 2002). To prevent this, cells respond rapidly to locate double stranded breaks in the chromatin and repair the damage as quickly and accurately as possible through activation of large numbers of proteins and induction of genes associated with cell cycle and growth control (Schmidt-Ullrich et al., 2000). Thus, putting in a measure of potential radiation damage besides oxidative stress and its repair mechanisms should be considered in studies using radiation.

Furthermore, even though almost all extracted gDNA will be of nuclear content (Gould et al., 2015), it is vital to note that genetic information occurs in two locations: nucleus and mitochondria in mammalian cells. Mitochondrial genome furthermore lacks histones, which suggests the reported high rate of mitochondrial DNA mutagenesis (ten times greater than nuclear DNA; Ballard and Whitlock, 2004; Brown et al., 1979; Tatarenkov and Avise, 2007) lack of protection from histones. Differentiating damage to mitochondrial and nuclear DNA will be another pivotal question to address in the future.

Conclusions

In conclusion, this study showed that DNA is highly resistant to oxidative damage from an acute pro-oxidant. I found that a mild dose of radiation of 5 Gy at 2 Gy/min that was shown previously to induce oxidative stress response on physiological parameters had no adverse effect at the DNA level. Rather, the 8-OHdG levels decreased on day 10 in liver and skeletal muscle,

and by day 4 in the heart. The significant drop in the oxidative DNA damage level seems to be driven by its respective repair protein, OGG1, in the skeletal muscle. But in the heart, the increase in OGG1 was delayed relative to the drop in DNA damage, suggesting that other processes may play a more important role in recovery. The heart also showed the most drastic response in the markers for oxidative damage and repair, suggesting the priority rules do appear to govern patterns of protection from acute oxidant. These findings countered previous work that suggest that DNA is the more sensitive to oxidative stress than other intracellular macromolecules (Martinez and Kolter, 1997).

Figures

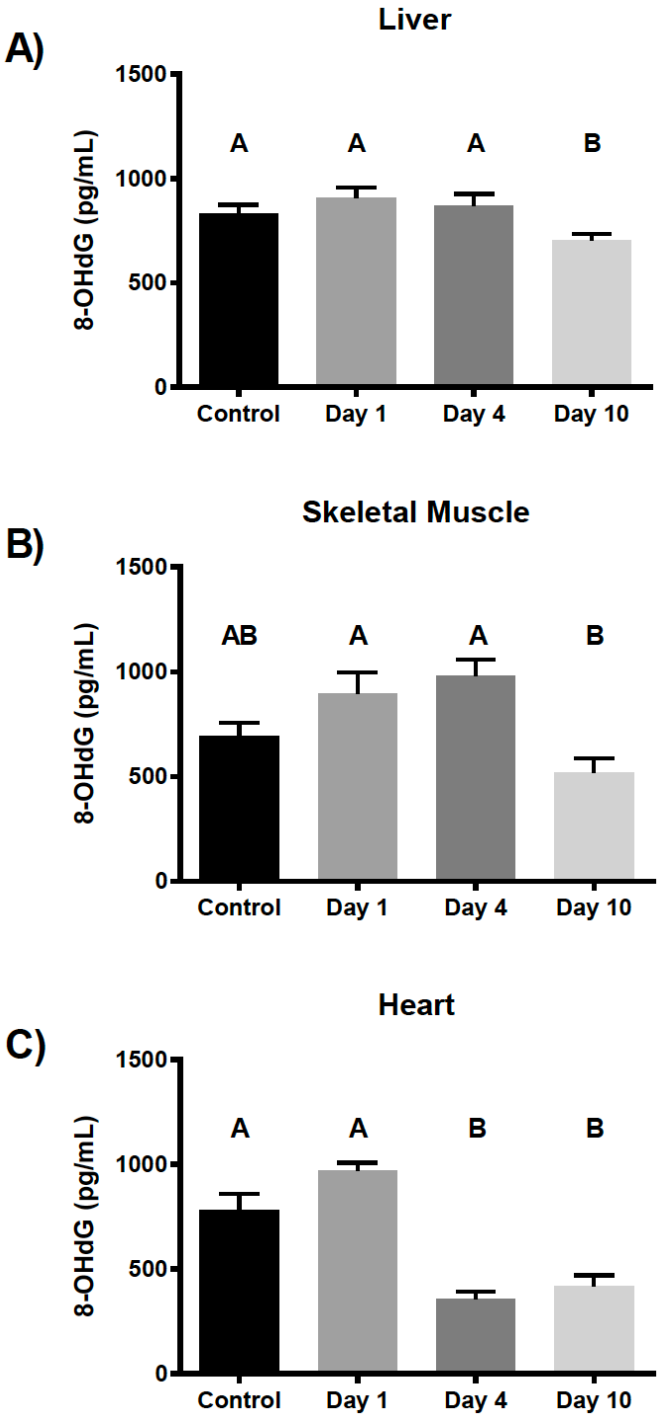

Figure 1. 8-OHdG levels in (**A**) liver, (**B**) skeletal muscle and (**C**) heart. Tissues were collected from non-irradiated control mice and mice 1 day, 4 day and 10 day post-irradiation. Standard error bars are given (n=8/group). Bars with different superscripts are significantly different.

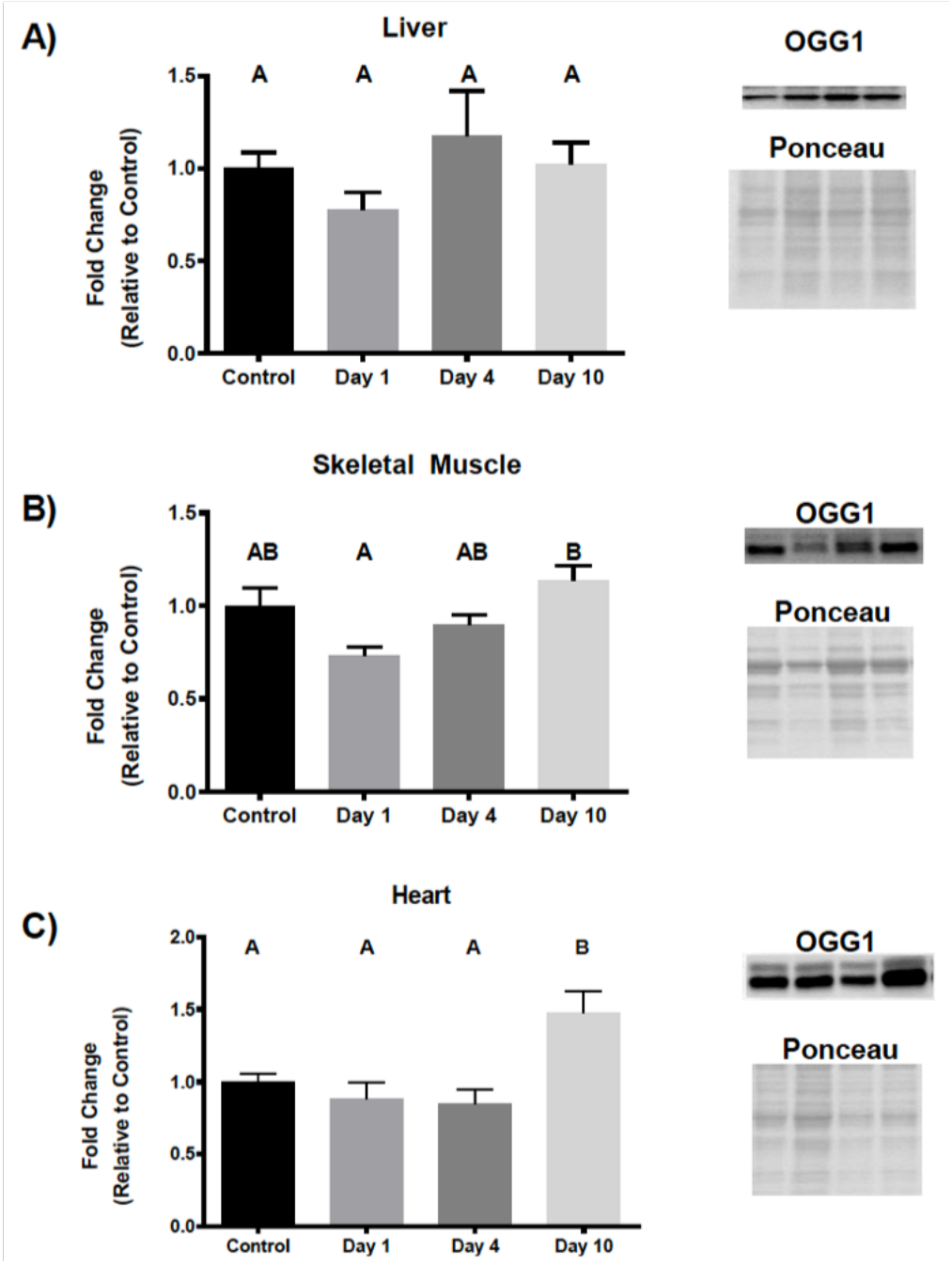

Figure 2. Relative expression of OGG1 in (**A**) liver, (**B**) skeletal muscle and (**C**) heart. Tissues were collected from non-irradiated control mice and mice 1 day, 4 day and 10 day post-irradiation. Standard error bars are given (n=8/group). Bars with different superscripts are significantly different. Representative western blot images are to the right of graphs. Representative images for the protein at each timepoint and th respective ponceau stains are on the right.

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Chapter 2

Variation in reproductive effort has few differential effects on the bioenergetic capacity of laboratory mice

Abstract

Oxidative stress has been hypothesized to underlie the tradeoff that exists between reproduction and longevity. Yet, empirical tests of this hypothesis have yielded equivocal results. I propose at least one reason for oxidative stress are not consistently harmful. Instead, consistent with the concepts of mitochondrial hormesis and antagonistic pleiotrophy, I propose that modest ROS production early in an animal's reproductive life may benefit maternal bioenergetic capacity while negatively affecting processes that are revealed later in a female's reproductive life. Thus, the aim of this study was to examine the impact of parity on mitochondrial parameters in laboratory mice under the theory of mitohormesis. I hypothesized that females that have undergone one bout of reproduction will display improved mitochondrial function relative to non-reproductive mice, while multiparous females display negative effects of continuous reproduction. The results of this study provide limited support for a cost or benefit of reproduction on mitochondrial bioenergetic capacity. Females that produced four litters had the highest liver and heart mass and consistent mitochondrial density, suggesting that there are more mitochondria in each of these organs. Female that had four litters displayed higher state 3 and state 4 respiration in the skeletal muscle when oxidizing carbohydrate substrates, and females

that bred once displayed reduced respiratory function (RCR) than nulliparous mice when oxidizing lipid substrate in the same tissue. Parity did not impact oxidative stress in these mice. The results of this investigation suggest that reproduce effort has limited physiological ramifications for these mice. It is possible that four reproductive bouts are insufficient in invoking energetic tradeoffs with processes that impact longevity in these mice.

Introduction

Reproduction is an energetically demanding activity, particularly in female mammals (Kirkwood & Rose, 1991). This high demand drives energetic tradeoffs, where the allocation of energy to a trait that benefits fitness comes at the expense of energy allocation to another potentially beneficial trait (Cody, 1966; Reznick, 1985; Stearns, 1989). Indeed, when the demands for reproduction are high, it is thought to reduce energy allocation to somatic maintenance and repair (Skogland, 1989; Zera & Harshman, 2001). This tradeoff could reduce an individual's subsequent reproductive performance and longevity (Williams, 1957, 1966). But despite numerous empirical studies and reviews delving into this phenomenon, the mechanisms behind these tradeoffs are still poorly understood (Blount, Vitikainen, Stott, & Cant, 2016; Costantini, 2014; Metcalfe & Monaghan, 2013; Monaghan, Metcalfe, & Torres, 2009a; John R. Speakman & Garratt, 2014).

One putative mechanism responsible for this tradeoff is oxidative damage that results from production of reactive oxygen species (ROS) (Dowling & Simmons, 2009). Following the

assumption that ATP production enhances the generation of ROS by the electron transport chain, this hypothesis suggests that higher ROS emission during reproduction would increase oxidative stress. In turn, the resulting oxidative damage to macromolecules would subsequently reduce a breeder's longevity (Monaghan et al., 2009a). Empirical tests of this hypothesis have yielded equivocal results, with some studies showing an increase in oxidative damage, while others show a reduction in damage or no change during reproduction (Speakman and Garratt 2014, Blount et al., 2016).

There are at least two potential problems with this approach. First, oxidative damage is measured during reproduction, a period of high metabolic plasticity, where changes associated with the reproductive event could mask the impact of reproduction on maintenance. Thus, it is preferable to measure the impact on reproduction on the female after the reproductive event has ended and the reproductive tissues have regressed (Mowry, Kavazis, Sirman, Potts, & Hood, 2016; Zhang & Hood, 2016). Second, oxidative damage is potentially ephemeral, as damaged molecules can be removed or damage mitochondria recycled (Zhang and Hood 2016). Thus, it is ideal to include a measure of mitochondrial or cellular performance after the reproduction bout has ended (Zhang and Hood 2016; Hood et al in press).

Hyatt et al (2018) showed that mitochondrial respiratory performance in the liver (RCR with pyruvate and malate as substrates) was greater in age-matched laboratory rats a week after a single reproductive event, while Mowry et al (2016) showed a similar trend for multiparous females relative to nulliparous females approximately one month after weaning in wild-derived

house mice. The mechanism that is likely responsible for mitigating the effects of oxidative damage is the benefical signaling that occurs with modest levels of ROS within the mitochondria. Under normal physiological conditions, 1-5% of the electrons transported to the mitochondria to support oxidative phosphorylation are converted to ROS (Wei, Lu, Wei, Ma, & Lee, 2001). But besides generating damage, ROS have also been shown to contribute to the change in the cellular redox environment, which stimulates downstream processes that promote increased mitochondrial biogenesis, antioxidant production and repair of damaged to protein, lipid and DNA (Croteau & Bohr, 1997; Davies, 2001; Finkel, 1998; Friguet, 2006; Handy & Loscalzo, 2012; Jornayvaz & Shulman, 2010; Juránek & Bezek, 2005; Pham-Huy, He, & Pham-Huy, 2008; Wang & Yi, 2008; Yan, 2014).

As a consequence of this ROS signaling, mitochondria are hypothesized to display a biphasic response to ROS exposure referred to as mitochondrial hormesis. While high levels of ROS are generally accepted to cause cellular damage and to promote aging, low levels such as from caloric restriction and exercise may rather improve systemic defense mechanisms by inducing an adaptive response that benefits mitochondrial and cellular performance, and may ultimately contribute to increased longevity (Ristow & Schmeisser, 2014; Ristow & Zarse, 2010; Sena & Chandel, 2012; Tapia, 2006; Zhang et al., 2018; Zhang & Hood, 2016). If ROS production is modest during a reproductive event, one should anticipate the mitochondrial function would be enhanced since adaptive responses, such as mitochondrial biogenesis and antioxidant production, would be upregulated. It is unknown how long the beneficial effects of

ROS signaling will last and when they arise during reproduction. Nor is it known if these benefits will persist across many reproductive events. Under Williams's antagonistic pleiotropy hypothesis (1957), selection should favor early reproduction when genes that improve early life reproductive performance are indelibly tied to reduce longevity. Thus, if pleiotropic genes, or gene networks, underlie improved mitochondrial performance following reproduction early in life, it is also feasible that they underlie a decline in mitochondrial performance later in life, particularly if it is associated with increasing parity.

Much of the early work lacks concrete evidence for the oxidative stress theory because they put too much emphasis on antioxidant defenses without the concurrent measurements of oxidative damage and repair (Metcalfe & Monaghan, 2013; Monaghan, Metcalfe, & Torres, 2009b). Furthermore, there is a dirth of evaluation of the impact of the stressor on cellular mitochondrial performance, which is necessary to identify a hormetic response (Hood et al, in press). Thus, the aim of this investigation is to evaluate the ramifications of reproductive parity on oxidative stress and mitochondrial performance.

I have selected the outbred laboratory mouse 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, the impact of reproduction on somatic maintenance is more likely to be revealed than in other species whose demand is substantially lower. For this investigation, I 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). Following the theories of mitochondrial hormesis and antagonistic pleiotropy, I predict that primiparous females will display higher mitochondrial respiratory function, and potentially lower oxidative damage, than nulliparous females, while multiparous females are predicted to have lower mitochondrial respiratory function and high oxidative damage than both groups. DNA appears to be more protected from oxidative damage than lipids or proteins (Park, chapter 1), but oxidative DNA damage may ultimately play a more insidious role in the decline of mitochondrial performance with repeated stressors (Yakes & Van Houten, 1997). Thus, I predict that DNA damage will be highest in the multiparous group.

Materials and Methods

Animal Care

Adult outbred female ICR mice were used in this investigation (Envigo, Inc.). All animals had body mass of at least 35 g when shipped and an estimate age of 4-7 weeks. Animals were maintained on a 12:12 light-dark cycle at a temperature of 24C. All mice were housed in pairs (two females for non-reproductive groups, male-female for reproductive) in standard mouse boxes (11.5" x 7.5" x 5"). After one bout of reproduction for 1-bout designated females (described below), they were housed with another female. Food and water were provided *ad libitum*. All husbandry and experimental procedures were approved by the Auburn University Institutional Animal Care and Use Committee (PRN 2017-3168).

Experimental Design

Female mice were randomly assigned to one of 3 groups: nonreproductive (NR, nulliparous), 1 complete bout (primiparous) and 4 complete bouts (multiparous) of reproduction (n=12/group). Since there were isolated incidents of stillborn and cannibalism of the pups within the multiparous group (1 case of stillborn, 1 case of litter cannibalism), I designated parturition to successful weaning of the pups as a complete bout. Males were added to the cages of females in the 1-bout and 4-bout groups and later removed just before the birth of their last expected litter. Litters were weaned 21 days postpartum, with individual mass weighed and sex recorded. All 4-bout mice were euthanized 14 days after weaning their last successful fourth litter. To ensure that all animal were the same age at the time of organ collection, each NR and 1 bout female was randomly assigned to be euthanized the same number of days post-arrival as a 4-bout female (Fig. 3).

Mice were anesthetized with isoflurane then decapitated with a rodent guillotine to conserve mitochondrial integrity. The left lateral and right medial lobe of the liver and the skeletal muscle of the left hind limb (hereafter, "skeletal muscle") were dissected for immediate mitochondria isolation. The rest of the liver, left skeletal muscle, heart and brain were also harvested and flash frozen in liquid nitrogen and stored in -80 C for future analyses.

Mitochondria isolation

Mitochondria were isolated following procedures outlined previously (Hyatt et al, 2018; Mowry et al., 2016). The liver was homogenized in a Potter-Elvhjem PTFE pestle and glass tube. The resulting homogenate was centrifuged at 4°C at 500xg for 10 minutes, and the supernatant filtered through cheesecloth underwent another round of centrifugation at 3500xg for 10 minutes. The subsequent supernatant was discarded, and the mitochondria pellet was washed in liver isolation solution, and was put through another centrifugation. The final mitochondria pellet was suspended in a mannitol-sucrose solution. The skeletal muscle was minced and then homogenized with a VITRUS polytron. Trypsin (protease) was added for 7 minutes to release the mitochondria from myofibrils. The digested mince was centrifuged at 500xg for 10 minutes at 4C, and the rest of the steps for skeletal muscle followed the procedure for liver mitochondria isolation. The final mitochondrial pellets from liver and skeletal muscle were suspended in a mannitol sucrose solution.

Mitochondrial function

Mitochondrial respiration 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. Maximal respiration (state 3), or the rate of respiration in the presence of ADP, 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. 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 mitochondria was accomplished with Amplex Red (Thermofisher, Waltham, MA) (Kavazis et al., 2009b; 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 37ºC in a 96-well plate using succinate. Readings of resorufin formation were recorded every 5 minutes for 15 minutes. 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-CoaA 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, Casarin, Pertegato, Salviati, & Angelini, 2012). Whole tissue homogenate 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 1,500g for 10 minutes 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.

Antioxidant Western Blots

Western blots were used to quantify antioxidant enzymes in each tissue. Proteins from tissue homogenates were separated by electrophoresis via 4% to 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 (five minutes x3) with PBS-Tween and incubated with secondary antibodies for 1 hour at room temperature. After another round of washing, a chemiluminescent system was used to detect labeled proteins (GE Health- care, 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 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. 69506). 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 (20mM, pH 5.0-5.4), then denatured by boiling for ten minutes. Appropriate amount of stock reagents zinc chloride (10 mM), nuclease P1 (5U/ml) were added to each DNA sample. The reaction mixtures were incubated for 30 minutes at 37 C, and pH was adjusted to 7.5-8.0 by addition of ~1/10 volume of Tris-HCl (1M, pH 8.0). Alkaline phosphatase (0.15U) was added to each sample to dephosphorylate and prevent self-ligation, and incubated at 37^{o}C again for 30 minutes. Samples were boiled again for ten minutes to inactivate the alkaline phosphatase, and further diluted in ELISA buffer prior to being assayed.

DNA Oxidative Damage ELISA

I 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. 589320). This assay is based on the competition between oxidatively damaged guanine species and an 8-OH-dG-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 and remove significant physiological outliers using the GraphPad QuickCalcs (website: https://www.graphpad.com/quickcalcs/Grubbs1.cfm, accessed May 9, 2018). All remaining comparisons were completed with GraphPad Prism version 7.02 for Windows (Graphpad Software, La Jolla California, USA), and RStudio 1.1.453 (RStudio, Boston, MA, USA). For all analyses, significance was established at $\alpha = 0.05$. ANOVA was used to compare the impact of parity on maternal body mass, organ mass, and mitochondria function measurements. Tukey's post hoc test was employed to test significant differences between groups.

Given that the multiparous group bred multiple times and the other groups did not, I used this group to look for clues that high allocation to reproduction was costly for females. First, I

employed ANOVA to determine if either litter size or the cumulative mass of the litter changed over the course of the 4 reproductive bouts. Then, I compared the amount of time between reproductive events to determine if time between bouts changed over time. Finally, I evaluated the impact of time at our animal facility (arrival to termination) as an indication of time it took females to complete four bout of reproduction, the impact of the total number of pups produced across the 4 bouts, and the total number of young produced on measurements of maternal body and organ mass and mitochondrial function mesures using Pearson's correlation. Regression was used to describe the pattern for significant relationships. Because there was limited evidence for offspring size versus number tradeoff, I did not test for correlations between total offspring mass over four bouts relative to maternal body and organ mass and mitochondrial function.

Results

Effect of parity on maternal body mass, organ mass, and mitochondria parameters

When comparing the three experimental groups, maternal body mass was not statistically different between groups ($F_{2, 34}$=1.08, P=0.350; Fig. 4A). Liver mass increased in a stepwise fashion from nulliparous to multiparous (overall: $F_{2,34}$=26.1, P<0.001; nulliparous vs. primiparous: P=0.021, nulliparous vs. multiparous: P<0.001, primiparous vs. multiparous: P<0.001; Fig. 4B). Heart mass was also significantly larger in multiparous females than in primiparous and nulliparous females (overall: $F_{2, 34}$=20.2, P<0.001, multiparous vs. nulliparous P<0.001, multiparous vs. primiparous: P<0.001s; Fig. 4C). But, there was no change in the mass of the calf muscle ($F_{2, 34}$=2.16, P=0.131; Fig. 4D).

There were no statistical differences detected in the relative mitochondrial content of the liver, skeletal muscle and heart between the reproductive bouts, as indicated by citrate synthase activity (liver: $F_{2,33}$=1.72, P=0.196; skeletal muscle: $F_{2,33}$=1.15, P=0.330; heart: $F_{2,26}$=0.393, P=0.679; Fig. 5), although differences in organ mass indicate that the mitochondrial content of each organ should vary with its mass.

For isolated isolate mitochondria, there were no statistical differences in liver state 3, state 4 and RCR between groups in the liver while complex I substrates (state 3: $F_{2,31}$=0.423, P=0.659; state 4: $F_{2,31}$=0.503, P=0.609; RCR: $F_{2,31}$=0.853, P=0.436; Fig. 6A-C). There were also no statistical differences in liver state 3, state 4 and RCR using complex II substrate, although RCR showed a trend towards multiparous female having significantly higher RCR than nulliparous females (state 3: $F_{2,30}$=0.465, P=0.633; state 4: $F_{2,30}$=0.425, P=0.658; RCR: $F_{2,29}$=2.85, P=0.074; Fig. 6D-F).

In skeletal muscle using complex I substrates, state 3 and state 4 in multiparous females were significantly higher than primiparous females (state 3: $F_{2,27}$=3.84, P=0.027; state 4: $F_{2,27}$=4.70, P=0.018; Fig. 7A-B), while RCR was not significant ($F_{2,27}$=1.58, P=0.225; Fig.7C). There were also no differences in skeletal muscle state 3 and state 4 using complex II substrate (state 3: $F_{2,28}$=2.07, P=0.146; state 4: $F_{2,28}$=0.355, P=0.705; Fig. 7D-E). However, skeletal muscle RCR using complex II substate was significantly lower in primiparous females than nulliparous ($F_{2,25}$=4.85, P=0.016; Fig. 7F).

There were no statistical differences detected in oxidant emission in isolated liver and skeletal muscle with changes in parity (liver: $F_{2,27}$=2.31, P=0.118; skeletal muscle: $F_{2,27}$=0.182, P=0.834, respectively; Fig. 8). There were also no statistical differences detected in 4-HNE

adducts (liver: $F_{2,34}$=0.096, P=0.909; skeletal muscle: $F_{2,33}$=0.779, P=0.467; heart: $F_{2,34}$=0.148, P=0.863). Nor were there differences in protein carbonyls (liver: $F_{2,34}$=1.76, P=0.188; skeletal muscle: $F_{2,33}$=0.387, P=0.682; heart: $F_{2,34}$=0.203, P=0.817). Oxidized guanine species 8-OHdG were measured in the liver, skeletal muscle and heart of the animals, and no differences were detected (liver: $F_{2,32}$=0.326, P=0.724; skeletal muscle: $F_{2,32}$=0.935, P=0.403; heart: $F_{2,32}$=1.34, P=0.275). Relative expressions of the endogenous antioxidants, superoxide dismutase 1, superoxide dismutase 2, glutathione peroxidase, and catalase, were also consistent across groups and tissues as presented in Table 1.

Change in reproductive performance across four bouts of reproduction

In the multiparous females, the litter size difference between reproductive events was significantly different ($F_{3,44}$=3.04, P=0.039, Fig. 9A), with the size of the second litter being significantly greater than the first (P=0.035). The cumulative mass of the litter also differed between groups ($F_{3,44}$=6.30, P=0.004, P=0.002; Fig. 9B), with mass of the second litter being greater than the third (P=0.004) and fourth litters (P=0.002). The time inbetween weaning and each subsequent litter was not statistically different ($F_{2,33}$=1.27, P=0.295).

Evidence for individual variation in the cost of reproduction

For the multiparous females producing four litters, the number of young that females produced had a significant impact of the function of their mitochondria. First, state 4 respiration of isolated live mitochondria using complex I substrate was negatively correlated with number of pups produced (Y=-0.4174*X+32.17, P=0.040; Fig. 10A, Table 2), suggesting that the liver mitochondria in animals that gave birth to more young had lower basal respiration. Associated with this effect, RCR with the same substrates displayed a trend toward increasing performance

with number of pups (Y=0.3495*X-10.69, P=0.055; Fig. 10B, Table 2). Liver mitochondria also displayed a negative relationship between RCR using complex II substrate (-0.2127*X+19.59, P=0.050; Table 2).

The time it took females to complete four reproductive bouts varied by approximately 50 days (120 to 170 days from arrival to termination). RCR in skelelal muscle mitochondria with complex II substrates was positively correlated with time (Y=0.064*X-1.902, P=0.055; Table 2). This relationship was clearly driven by the results of a signal female that took longer to breed that the other females. When this outlier was removed, RCR was no longer significant (P = 0.664).

There were no effects of number of pups produced or time to complete four reproductive bouts on liver or skeletal muscle ROS production (Table 2). There was also no effect of number of pups produced or time to complete four reproductive bouts on mitochondrial density in the liver, skeletal muscle, or heart (Table 2). There was no effect of number of pups produced or time to complete four reproductive bouts on oxidative damage or antioxidant levels in the liver or skeletal muscle (Table 1). However, the amount of catalase expressed in the heart decreased with number of pups produced (P = 0.019, Table 2) and the level of lipid peroxidation (4HNE) in ,the heart increased (P = 0.038, Table 2). and the antioxidant glutathione peroxidase in the heart increased (P = 0.007, Table 2) with the time it took to complete four reproductive bouts.

Discussion

This experiment was designed to evaluate the relative costs of reproduction and specifically evaluate the persistent effects of prior reproduction on the function of mitochondria, as mitochondrial performance plays a pivotal role in aging (Bratic and Larsson, 2013). When

comparing age-matched animals (~6 months old), I hypothesized that relative to nulliparous females, primiparous females would display improved mitochondrial function and reduced oxidative damage while multiparous females would display reduced mitochondrial function and higher oxidative damage. The data do not support these predictions, but instead suggest that multiparous females may have the highest bioenergetic potential among the three groups examined. They are shown to have the highest liver and heart mass and consistent mitochondrial density, which suggests that there are more mitochondria per organ. In addition, skeletal muscle mitochondria displayed higher respiratory performance in multiparous females when oxidizing carbohydrates. Within females that completed four bouts of reproduction, breeding quickly in succession may be costly with those females displaying increased lipid peroxidation in their heart after their bodies returned to nonreproductive state.

In females of comparable age, the number of times a female bred impacted liver and heart but not maternal body mass. Pregnancy involves enlargement of the liver, with nutrient supply and GI hormones such as gastrin exerting a trophic effect (Svennersten-Sjaunja & Olsson, 2005). Milk synthesis is an energetically demanding process to the mothers of which the liver is highly involved, and it has been shown that the resting metabolic rate during peak lactation and the liver size could be as high as 2-fold than controls (J R Speakman & McQueenie, 1996). Contrary to studies showing post-weaning involution of the liver as fast to 8-10 days in rodents (Goddard et al., 2017; Hollister, Okubara, Watson, & Chaykin, 1987; Widdowson, 1976), we found a stepwise increase in liver and heart mass with prior allocation to reproduction. The impact of prior reproduction was persistent despite the fact that primiparous females would have ended their first reproductive event approximately 3 months earlier and all multiparous females would have ended their fourth reproductive event 2 weeks earlier.

Despite these persistent impacts on liver mass, parity had no impact on mitochondrial respiratory function (RCR, state 3, and state 4 respiration) in the liver when using both complex I substrate (pyruvate and malate) and complex II substrate (succinate). Yet, RCR with complex II substrate did show a trend of increasing in the multiparous females when compared to the nulliparous females. Indeed, liver has been shown to increase lipid beta-oxidation to facilitate production of glucose to be used for milk production in mammary glands (Hyatt, Zhang, Hood, & Kavazis, 2017; Rawson et al., 2012). This trend could be reflective of an increase in fat metabolism in females that could reduce stored adipose and improve maternal body condition in multiparous females.

Because the multiparous females also had heavier livers and consistent mitochondrial content relative to liver mass, their livers are predicted to have a greater number of mitochondria, and thus greater capasity to produce ATP than primiparous and nulliparous females. These effects are suggestive of mitochondrial hormesis, and have the potential to improve immediate energetic performance of multiparous females. Further work is needed to determine if these benefits are ultimately tied to an increase or reduced longevity.

In skeletal muscle, parity did not impact hind limb mass; but it did impact mitochondrial respiration. Both state 3 and state 4 respiration using glucose-derived complex I substrates were significantly higher in multiparous than primiparous mice. However, there was no significant difference between RCR values, since state 3 and state 4 values changed proportionately to each other. It is likely that the skeletal muscle mitochondria in this study's females is displaying an adaptive response to the energetically demanding activity of reproduction without solely altering respiratory capacity. In fact, Montero et al. (2015) (Montero et al., 2015) showed that the skeletal

muscle hematologically induced adaptive increase in oxygen uptake during endurance training rather than mitochondrial respiratory performance.

Conversely, state 3 and state 4 respiration using complex II substrate did not differ with parity, but RCR was significantly lower in primiparous than nulliparous females. This finding is consistent with previous study which also showed decreased RCR with succinate and decreased PPARδ protein levels in skeletal muscle 3 months after lactation in rats; PPARδ mediates lipid metabolism in skeletal muscle (Hyatt et al., 2017; Lee et al., 2006). A logical hypothesis for this trend is that the females are mobilizing adipose reserves to prepare for future lactations. Indeed, the most rapid rates of lipolysis and lipogenesis observed both in vitro or in vivo systems were in lactating animals (J.P. McNamara, 1991; J P McNamara & Huber, 2018; John P McNamara, 1994). Thus, the decrease in fat oxidation in skeletal muscle in primarous females may be a consequence of this shift in energy metabolism when facing demands of reproduction for the first time to supply large amounts of nutrients to the offspring.

For liver, skeletal muscle, and heart, there were no effects of parity on antioxidant levels nor oxidative damage levels across all macromolecules tested, suggesting that the change in these variables that many have occurred during reproduction is not persistent. Oxidative stress has been hypothesized to be the mediator of the somatic cost of reproduction and longevity. Our data counter this theory and suggest that despite the high reproductive output of our strain of the laboratory mouse used (Choi, Seng, & Toyoda, 2000; Shin et al., 2017), oxidative damage does not accumulate across approximately four months of breeding.

Because the demand for reproduction was highest in the multiparous females, we focused on this group to further explore any fitness costs of breeding. I evaluated change in litter size and litter mass of multiparous females. Litter size and mass were greatest in the second reproductive

bout, but were then maintained at the level of the first bout after the second reproductive event. This increase during bout two could be a consequence of maternal experience, or an increase in energetic capacity following the first reproductive event (Hood et al, in press). There were no significant differences in the duration of the delay between reproductive events, suggesting that there was no effect of prior reproduction on capacity to initiate the next reproductive event.

I also quantified the costs of producing more young and breeding more rapidly by assessing the impact of number of young produced and time to complete four bouts of reproduction in the multiparous females. Liver mitochondria for females that produced more young displayed lower state 4 respiration, which contributed to a trend toward higher RCR when oxidizing carbohydrate (complex I substate). In contrast, liver mitochondria displayed reduced RCR in females that produced more young when oxidizing lipid substrate (complex II substrate). This shift in efficiency of oxidizing different substate seen in this investigation is supported by other studies preferential use of carbohydrates during pregnancy and lactation (Burnol, Leturque, Ferré, & Girard, 1983; Butte, Hopkinson, Mehta, Moon, & Smith, 1999; Jones, Ilic, & Williamson, 1984). In addition, females with more young produced less catalase in the heart, which could contribute to future oxidative stress- and yet, the heart also displayed reduced lipid peroxidation and increased glutathione peroxidase activity in females that completed four bouts to reproduction more quickly. An increase in oxidative stress has been shown to delay reproduction in canaries (Costantini, Casasole, AbdElgawad, Asard, & Eens, 2016). Thus, if oxidative stress levels at termination were consistent across the females' reproductive lives, it is possible that lower oxidative stress allowed some females to breed more rapidly.

There are at least two potential interpretations of relatively consistent oxidative stress with variation in parity, and the trend we see within the multiparous females. First, despite the

high demand placed on these females, it is possible that the lab mice were able to adequately support maintenance and repair processes that sustain maternal condition under the optimal environment of a laboratory animal facility. In many cases, tradeoffs are not revealed unless females face energetic stress (Kaplan & Gangestad, 2004), this may also be true for the lab mice. Second, oxidative stress may not underlie the relationship between reproduction and longevity. An underlying premise of the oxidative cost of reproduction hypothesis is that oxidative damage is responsible for cellular senescence, yet an increasing number of studies are providing evidence against this supposition (Greaves & Turnbull, 2009; Itsara et al., 2014; Lagouge & Larsson, 2013; Park & Larsson, 2011; Ross et al., 2013).

Conclusions

This study revealed no negative effects of reproductive effort on the bioenergetic capacity of female lab mice. Instead, females with the highest reproductive performance had a heavier liver and heart, which would equate to more liver and heart mitochondria and their skeletal muscle mitochondria displayed higher respiratory performance when oxidizing lipid. It is probable that the negative ramifications of reproduction do not come into play until much later in a female's reproductive life, in concurrence to antagonistic pleiotropy. Female mice can produce 5 to 10 litters per year, with the average lifespan of 2 to 3 years (Wolf & Austad, 2010). Even our investigation's designated 4-bout effort may ultimately equate to an early reproductive effort. Further study with an increased number of reproductive bouts is warranted to determine if the physiological tradeoffs responsible for the reproduction and longevity tradeoff are revealed.

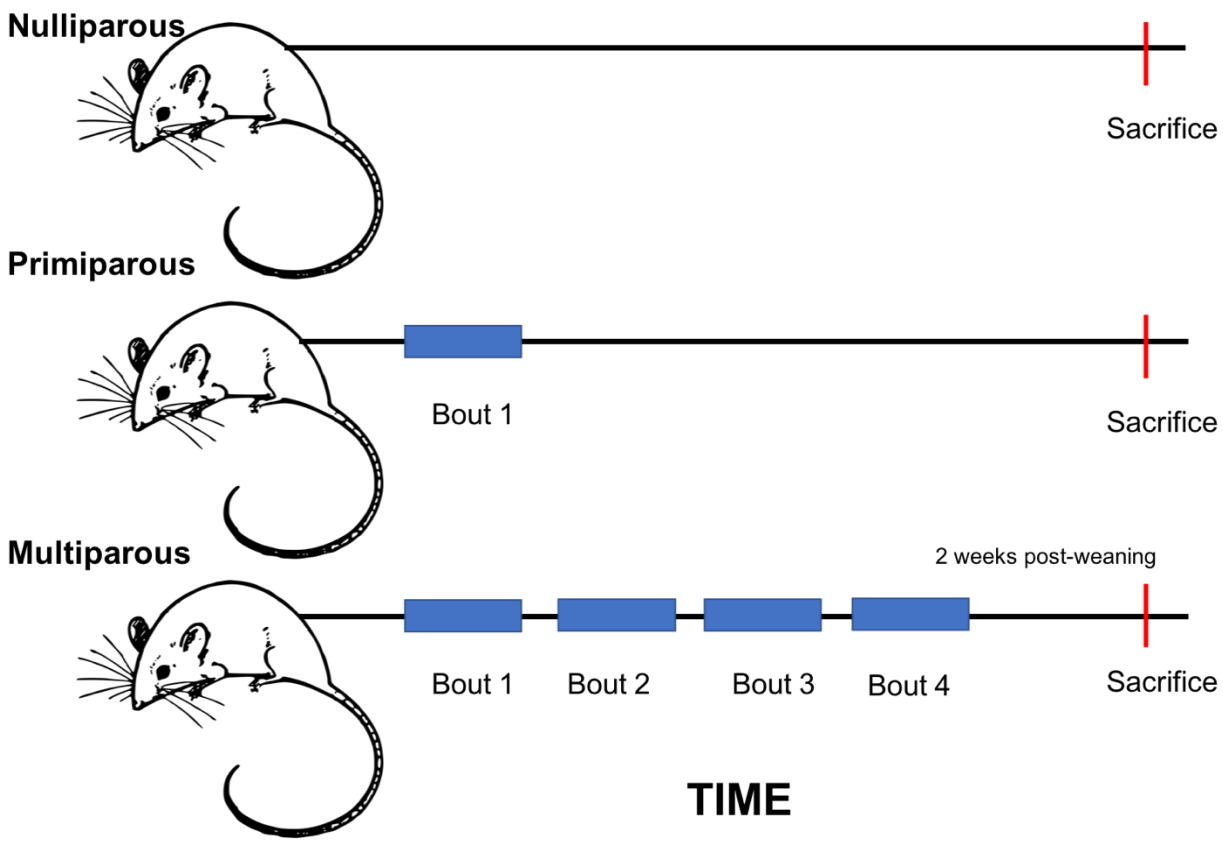

Figure 3. Experimental design. Thirty six age-matched female mice are randomly distributed into three groups of reproductive bouts: nulliparous, primiparous and multiparous.

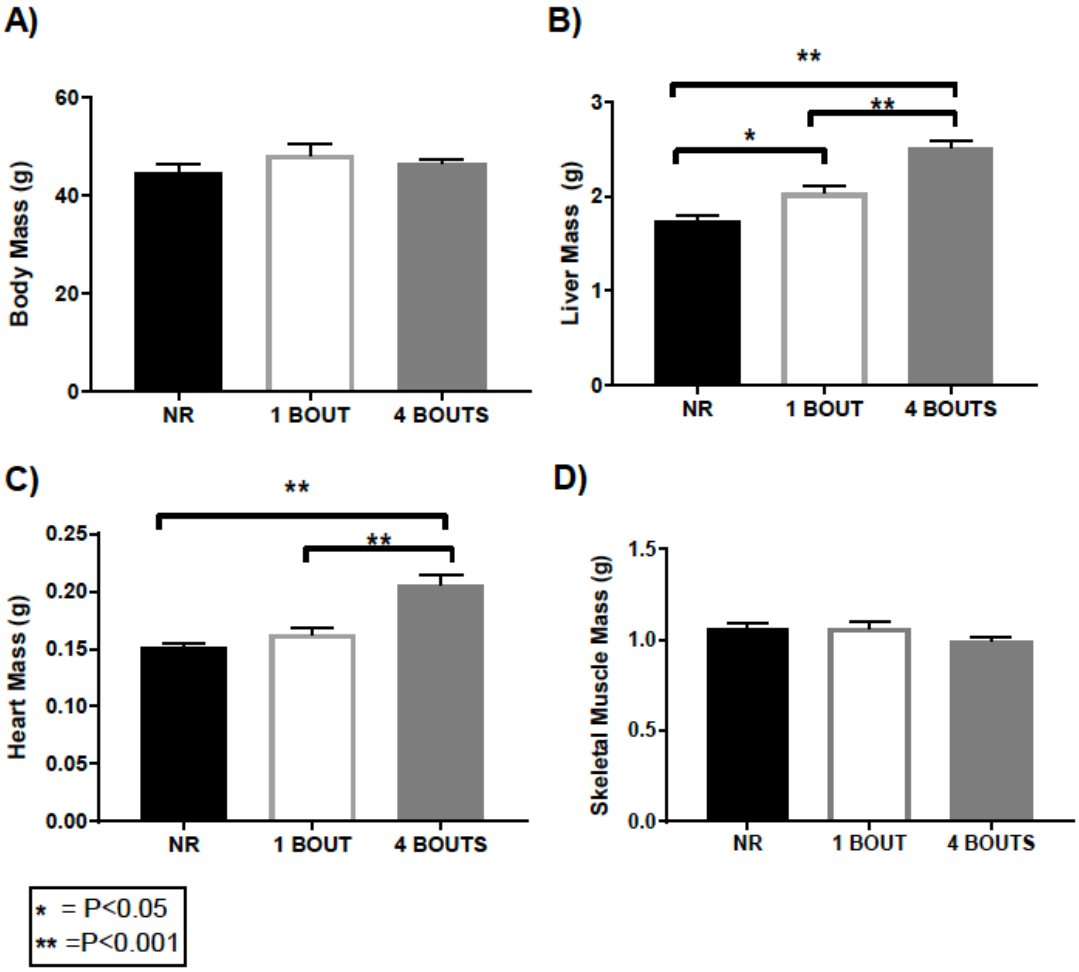

Figure 4. Effect of parity on maternal A) body mass, B) liver mass, C) heart mass, D) skeletal muscle mass in female mice. Standard error bars are given.

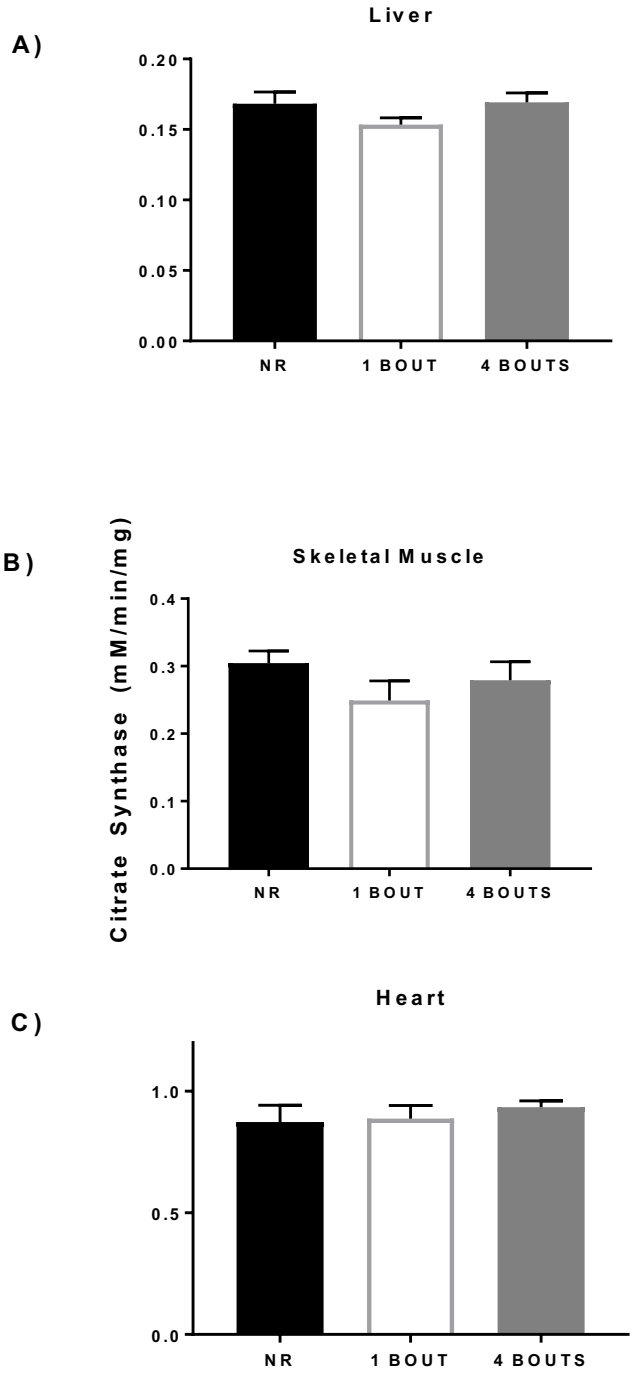

Figure 5. Effect of parity on mitochondrial content in the A) liver, B) skeletal muscle, C) heart between the reproductive bouts. Standard error bars are given.

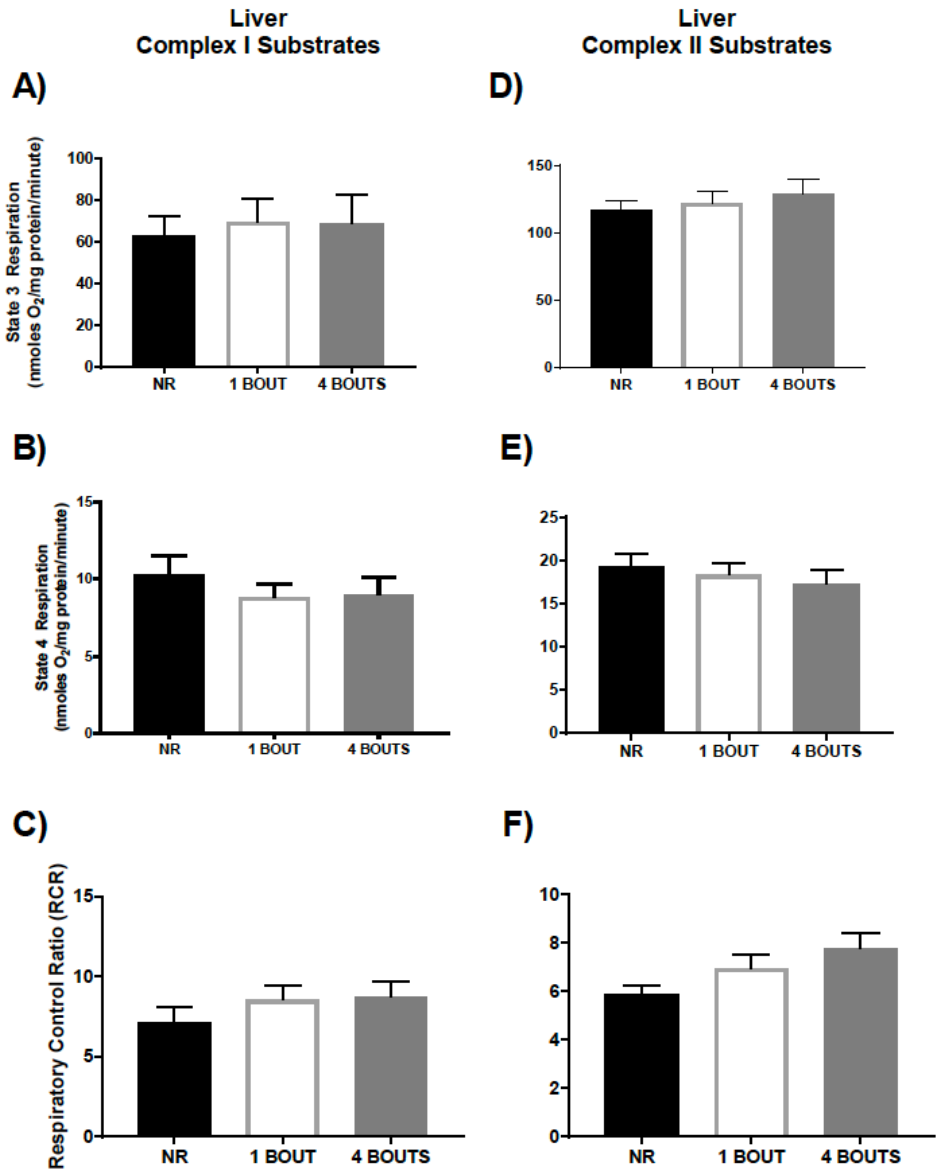

Figure 6. Effect of parity on the mitochondrial respiratory function of the liver using complex I and complex II substrates. Pyruvate and malate were used as complex I substrates to obtain A) state 3 respiration, B) state 4 respiration, C) RCR. Succinate was used as complex II substrate to obtain D) state 3 respiration, E) state 4 respiration and F) RCR. Standard error bars are given.

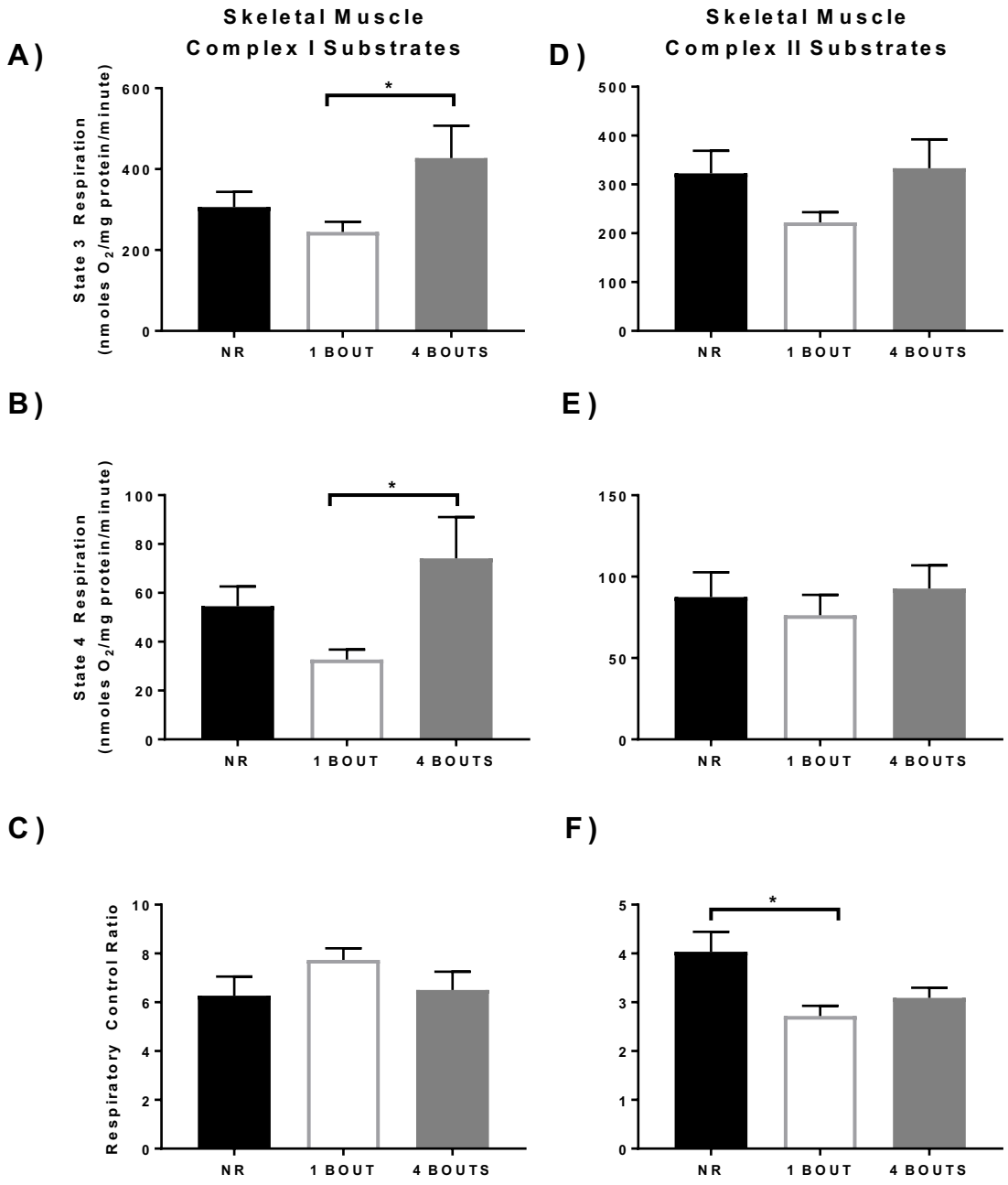

Figure 7. Effect of parity on the mitochondrial respiratory function of the skeletal muscle using complex I and complex II substrates. Pyruvate and malate were used as complex I substrates to obtain A) state 3 respiration, B) state 4 respiration, C) RCR. Succinate was used as complex II substrate to obtain D) state 3 respiration, E) state 4 respiration and F) RCR. Standard error bars are given.

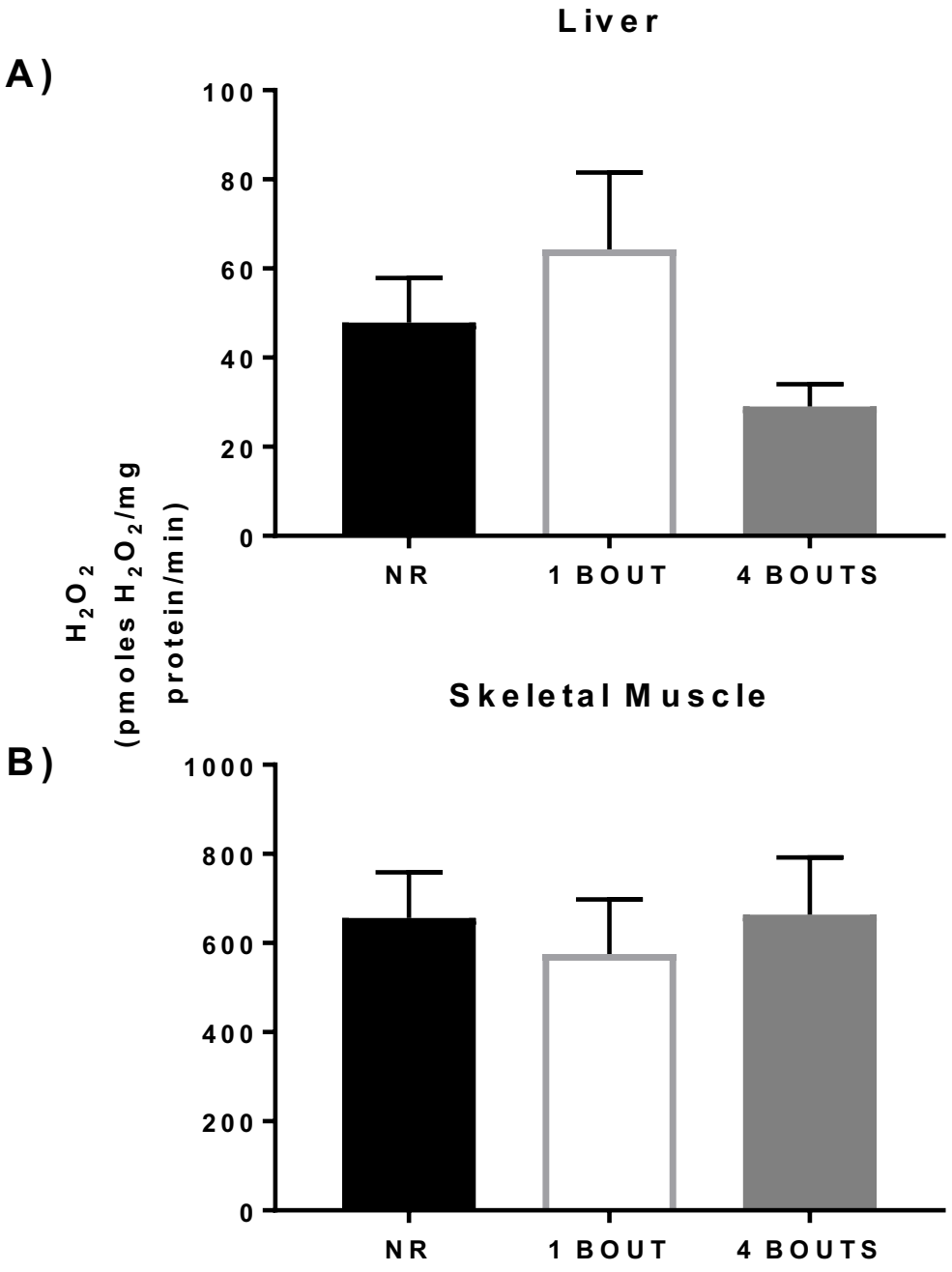

Figure 8. Effect on parity on oxidant (ROS) emission in isolated A) liver mitochondria and B) skeletal muscle mitochondria. Standard error bars are given.

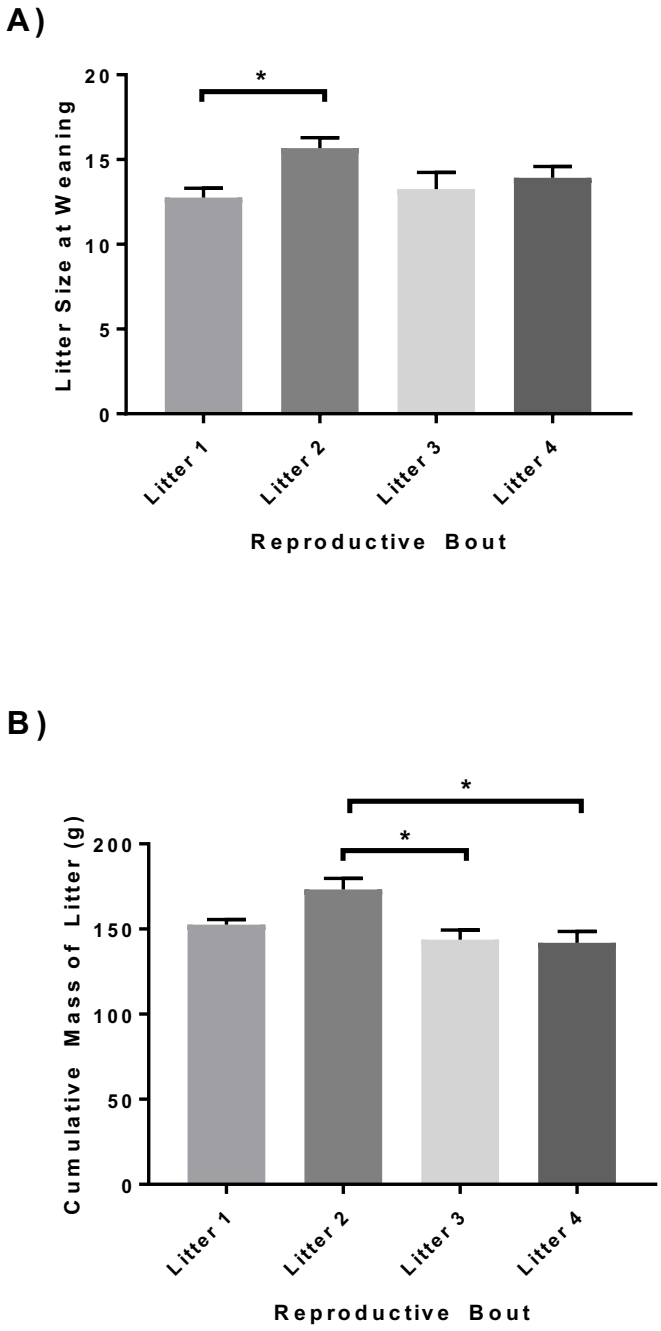

Figure 9. A) Number of pups weaned and B) total mass of each litter at weaning for female mice across four reproductive bouts. Standard error bars are given.

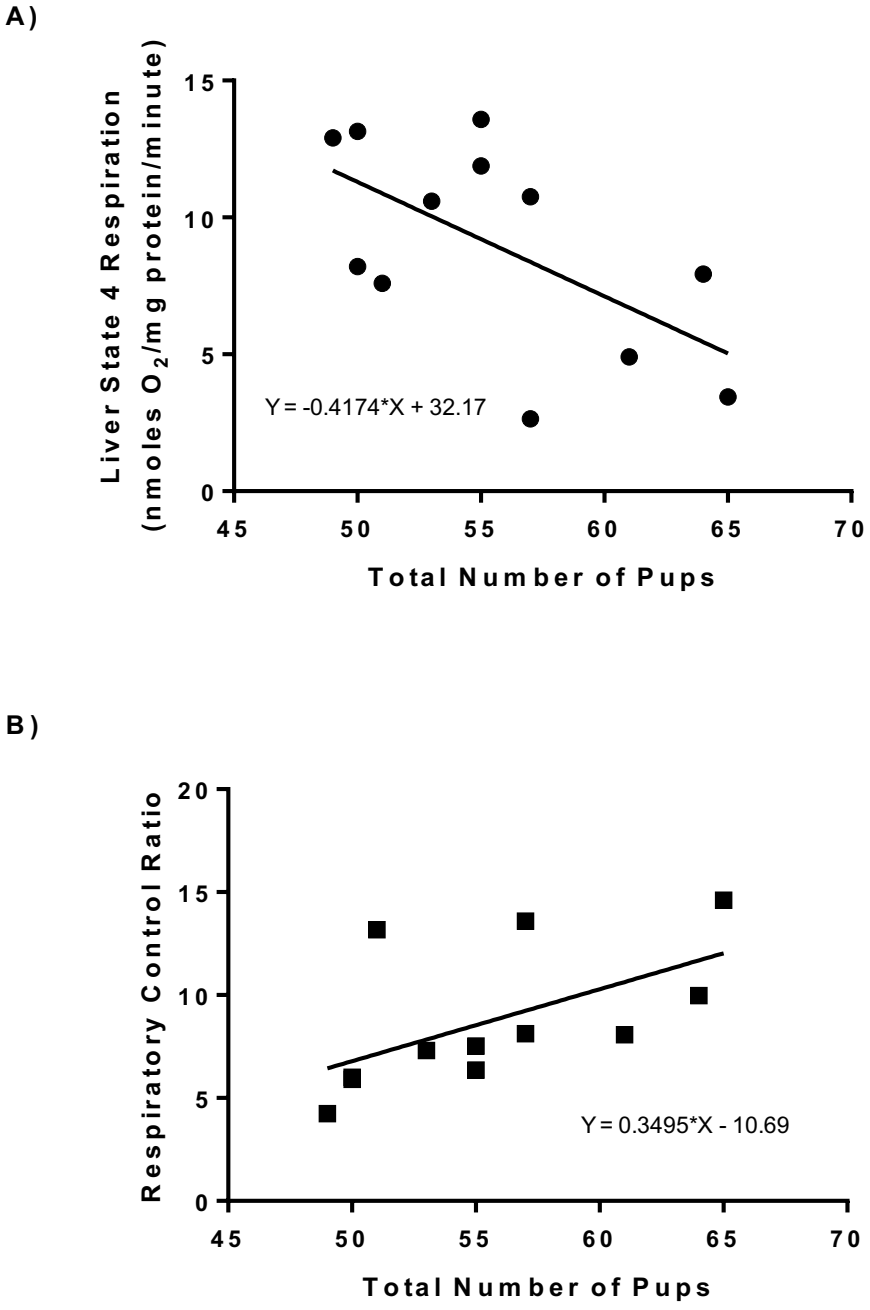

Figure 10. Relationship between total number of pups produced across four reproductive bouts and A) state 4 respiration in liver mitochondria, and B) the respiratory control ratio in the liver mitochondria. All measurements completed with Compelx I substrates. Regression lines are given.

Table 1. Results of ANOVA comparing the relative expression levels of endogenous antioxidants in liver, skeletal muscle and heart between nulliparous, primiparous, multiparous mice.

Antioxidant	**Liver**			**Skeletal Muscle**			**Heart**		
	F	**df**	**P**	**F**	**df**	**P**	**F**	**df**	**P**
SOD1	0.0659	2,34	0.936	0.190	2,34	0.828	1.291	2,33	0.289
SOD2	0.960	2,34	0.393	0.096	2,34	0.909	0.322	2,34	0.727
CAT	0.386	2,34	0.683	0.248	2,34	0.782	1.66	2,34	0.205
GPX	0.045	2,33	0.956	0.488	2,31	0.619	2.244	2,33	0.122

Table 2. Impact of total number of pups produced and time to complete four reproductive bouts on maternal mass, organ mass, and mitochondrial function measurements for females that bred four times. Results of Pearson's correlations given. Significant values in bold.

	Total Number of Pups		**Days to complete 4 reproductive bouts** (days, arrival to termination)	
	r	P-value	r	P-value
Maternal body and organ mass:				
Body mass (g)	0.36	0.257	-0.21	0.507
Liver mass (g)	0.36	0.256	-0.27	0.397
Skeletal muscle mass (g)	-0.23	0.478	0.03	0.918
Heart mass (g)	0.46	0.136	0.44	0.153
Liver				
RCR – complex I substrate	0.57	0.055	-0.20	0.541
- state 3 respiration	-0.23	0.468	0.08	0.816
- state 4 respiration	-0.60	0.040	0.17	0.598
RCR – complex II substate	**-0.63**	**0.050**	-0.22	0.540
- state 3 respiration	-0.30	0.407	0.18	0.615

- state 4 respiration	0.22	0.547	0.33	0.357
Mitochondrial content	0.12	0.700	0.46	0.130
H_2O_2 emission	0.30	0.396	-0.31	0.389
DNA oxidative damage	0.34	0.275	-0.02	0.940
Lipid peroxidation	0.42	0.179	0.23	0.473
Protein oxidation	0.31	0.330	-0.16	0.629
Superoxide dismutase 1	0.12	0.710	-0.07	0.828
Superoxide dismutase 2	0.02	0.949	0.48	0.114
Glutathione peroxidase	0.05	0.882	0.26	0.448
Catalase	-0.66	0.629	0.35	0.132
Skeletal muscle				
RCR – complex I substrate	-0.10	0.809	0.55	0.156
- state 3 respiration	0.18	0.669	0.03	0.942
- state 4 respiration	0.36	0.387	-0.30	0.466
RCR – complex II substrate	0.48	0.193	**0.78**	**0.013**
- state 3 respiration	0.39	0.302	0.33	0.388
- state 4 respiration	0.02	0.969	-0.34	0.376
Mitochondrial Content	0.36	0.250	-0.05	0.881
H_2O_2 emission	-0.02	0.962	-0.20	0.633
DNA oxidative damage	0.21	0.502	-0.12	0.712
Lipid peroxidation	0.41	0.180	0.28	0.378

Protein oxidation	0.55	0.063	0.24	0.444
Superoxide dismutase 1	0.28	0 .377	0.32	0.311
Superoxide dismutase 2	0.18	0.578	0.43	0.167
Glutathione peroxidase	-0.51	0.089	-0.36	0.257
Catalase	0.12	0.706	-0.14	0.657
Heart				
Mitochondrial content	-0.26	0.472	0.36	0.308
Heart DNA Damage	0.12	0.700	-0.36	0.250
Lipid peroxidation	0.13	0.690	**0.60**	**0.038**
Protein oxidation	-0.15	0.642	0.18	0.585
Superoxide dismutase 1	0.46	0.136	0.28	0.370
Superoxide dismutase 2	-0.23	0.463	-0.03	0.934
Glutathione peroxidase	0.41	0.188	**0.73**	**0.007**
Catalase	**-0.66**	**0.019**	-0.46	0.132

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