Energetic tradeoffs between reproduction and longevity in the house mouse (*Mus musculus)*

by

Annelise Valerie Rossetti Mowry

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 1, 2015

Keywords: life history, cost of reproduction, longevity, self-maintenance, mitochondrial efficiency, oxidative damage

Approved by

Wendy Hood, Chair, Assistant Professor of Biological Sciences
Andreas Kavazis, Assistant Professor of School of Kinesiology
Geoffrey Hill, Professor of Biological Sciences

Abstract

Energy investment in reproduction comes at the cost of other life history traits, including future reproductive output and ultimately, lifespan. The disposable soma theory suggests that there is a negative relationship between reproduction and longevity, proposing that allocation of energy to reproduction compromises the availability of energy for tissue repair and maintenance and, therefore, reduces longevity. However, the mechanism behind this tradeoff is fully not understood. Recently, investigation has focused on the negative byproducts of respiration, free radicals, and mitochondria as the source of the cost that reproduction exacts on self-maintenance and longevity. Herein, I investigate the changes to mitochondrial efficiency and cell redox state of skeletal muscle and liver of house mice at peak-lactation, as well as measure the effects of reproduction on mitochondrial function in a post-reproductive, maintenance state in the house mouse.

Life history theory predicts that during reproduction, energy will be allocated more heavily to tissues that support reproduction than to those that primarily support maintenance efforts. In chapter 1, I examined the plasticity of mitochondrial function in the liver and skeletal muscle during reproduction to determine if organs adopt different strategies for sparing substrate for the high-energy demand of supporting offspring growth. In addition, I asked if these changes were likely to expose the organ to damage that could impact the ability of the organ to maintain itself. Reproductive female house mice were collected at peak-lactation of their third successful litter and mitochondrial function of their tissues was compared to non-reproductive individuals. Skeletal muscle maintained mitochondrial functionality during lactation with a more coupled

basal metabolism. These changes were coupled with lower antioxidant production and lower mitochondrial subunit expression, both of which are substrate-sparing changes, potentially sparing nutrients to support milk synthesis. However, at peak-lactation there is evidence of both a substrate-sparing strategy and higher damage in the liver. A higher respiratory efficiency during reproduction suggests the liver maximizes ATP production from the substrate it takes in, a strategy that could facilitate greater substrate use by the mammary gland. Although lactating females displayed a higher basal respiratory rate that limits ROS production, lactating females also displayed higher oxidative damage than non-reproductive females, which could have long-term negative effects on longevity.

The disposable soma theory predicts that the increased energetic demand of reproduction will decrease allocation to self-maintenance, allowing for the accumulation of harmful damage to mitochondrial DNA. This damage results in the replication and propagation of inefficient mitochondria post-reproduction. To evaluate the impact of reproduction on maintenance, and thus longevity, in chapter 2 I completed measurements of mitochondrial efficiency, oxidative damage, and antioxidant capacity at least one month following reproduction to exclude the immediate metabolic changes associated with pregnancy and lactation. While oxidative damage is higher in liver mitochondria post-reproduction, I found no evidence that this damage adversely affects mitochondrial function and, thus, would not be a major contributor to senescence. Combined, my results suggest that while there is a momentary cost to self-maintenance during reproduction and a lasting increase in free radical production, there is no long-term adverse affect on mitochondrial function, calling into question the role that mitochondria play in the tradeoff between reproduction and longevity.

Acknowledgments

I owe countless thanks to my advisor, Dr. Wendy Hood, for the all the work, advice, and direction she provided, which has been invaluable to the success of my thesis work. You are an inspiring mentor and have set a fine example of work ethic, standards, and teamwork in your lab that all of the multitudes of your students feel and admire. Even with your enormous responsibilities and many projects, you always find time to discuss and offer suggestions on the littlest things. You always treat your students' projects, grants, and classes with the same weight as if they were your own and I cannot express how appreciative I am of the attention. I am grateful that I have had the opportunity to work with you.

Additionally, I owe my gratitude to Dr. Andreas Kavazis for the months of help with my lab work, sample analysis, and the invaluable input he contributed to the evolution of my project. Your contribution has been invaluable and I greatly appreciate the time you committed to teaching me.

I also owe a large thanks to the Hood lab and the Hood-Hill-Wada lab group, including my final committee member, Dr. Geoff Hill, for their input and comments on grants and project work. In the Hood lab, I must thank Aubrey Sirman for the work she set-up and passed to me at the mouse house, as well as the hours of training she put in to help me start my thesis work. Further, this project would not have been possible without the dedication and care of the undergraduate team in the Hood lab that has been invaluable to the success of our mouse facility.

And finally, I must thank my family for their support and encouragement. Thank you for listening to my problems, helping me work through the stress, and always being there when I needed a loving hand. Your constant belief in my abilities and potential have gone far to get me this far successfully. I need to thank my hero and mother, my hard-working father, my fabulous sister, my unique brother, and my adopted family, Pepper, Rex, Thena, and Merlin for they are everything. And, I would especially like to thank Brendan Roy for his praise and encouragement. You have made my experiences at Auburn better than I ever imagined, and I am so thankful that I got to share this time with you. I am blessed to have such a supportive family around me. Thank you!

Funding for my research was provided by the Auburn University College of Science and Mathematics (W.R. Hood), the Auburn University School of Kinesiology (A.N. Kavazis), an American Society of Mammalogists Grant-in-Aid of Research awarded to A.V. Mowry, and a Alabama Academy of Science Grant-in-Aid of Research awarded to A.V. Mowry.

Table of Contents

List of Figures

CHAPTER ONE

Energetic efficiency and metabolic measurements at peak-lactation in *Mus musculus*: A tradeoff between reproduction and self-maintenance

ABSTRACT

Reproduction exacts a cost on future reproductive potential and longevity by damaging the ability of the body to maintain itself. I hypothesized that energetic tradeoffs are likely to occur between tissues during reproduction that would play an important role in shaping future performance. Thus, I predicted that mitochondrial bioenergetics, redox state, and substrate demand plasticity in individual organs plays a crucial role in setting relative energy allocation to reproduction and the accumulated damage to tissue maintenance capabilities during reproduction. I evaluated changes in liver and muscle mitochondria function and redox state between non-reproductive and lactating house mice to determine how the mitochondria respond to the demands of reproduction and lactation. I found that skeletal muscle of lactating house mice living under semi-natural conditions display substrate sparing strategies, including a more efficient resting respiration rate, reduced antioxidant production, and an expression of fewer mitochondrial subunits. The liver, however, showed a mix of costly and substrate-sparing changes, with costs such as tissue growth, increased basal respiratory rate, and greater lipid peroxidation, and substrate conserving strategies, including reduced antioxidant production and a higher maximum respiratory rate at peak-lactation. While this organ-specific plasticity to

mitochondrial function suggests a preferential allocation of nutrients to a reproductive effort, there is a potential long-term negative impact of the costly changes seen during peak-lactation.

INTRODUCTION

Availability of energy both directly impacts relative energy allocation to reproduction and indirectly impacts future fecundity and longevity (Speakman 2008). Factors such as food availably, gut capacity, capacity of mammary glands to produce milk, and capacity of the body to dissipate heat have been evaluated as potential constraints on the amount of energy that is allocated to individual reproductive effort (Hammond and Diamond 1994; Hammond et al. 1996; Speakman and Krol 2005). Reproduction is thought exact a cost on future reproductive potential and longevity by damaging the ability of the body to maintain itself. As a result of limiting the body's maintenance ability, the future capacity to allocate energy to reproductive tasks or the maintenance of tissues and cellular function that support longevity is reduced (Promislow & Harvey, 1990; Zera & Harshman, 2001; Roff 1992; Stearns 1992; Flatt & Heyland, 2011; Kleiber, 1975). Although this relationship is often described as being responsible for the negative interactions between life-history events, such as growth and reproduction, current and future reproduction, and reproduction and lifespan (Roff 1992; Stearns 1992), energetic tradeoffs also occur within the body (Crescenzo et al 2012) and it is this effect that may play an important role in future reproductive performance.

Lactation is typically the most energetically demanding activity in the life of a female mammal (Speakman 2008). To maximize reproductive performance, selection would act to maximize the total amount of energy partitioned both to developing offspring and the maternal organs that support offspring development. To support the high energetic demand, the timing of

reproduction in most mammals coincides with the time of year when food abundance is increasing or at its peak (Bronson, 1989; Lane, 2011; Speakman, 2008). As a result, physiological constraints on reproduction are likely more important than food availability in dictating maximum reproductive output in most species (Speakman 2008). Whether limited by food availability or constraints on physiological capacity, partitioning of available energy between organs likely plays an important role in determining how much energy is allocated to the reproductive effort and how much is allocated toward maintaining the integrity and functionality of cells and tissues. More specifically, I predict that the number of mitochondria in a tissue and efficiency by which the mitochondria use substrate for oxidative phosphorylation's production of ATP determines demand and thus how much ingested nutrient is available for alternate organ systems.

Numerous physiological changes occur throughout the body to support the initial demands of pregnancy and later demands of lactation (Akers 2002). I posit that changes to the function of mitochondria in the most demanding tissues, specifically the liver and skeletal muscle (Rolfe and Brown 1997), could have a substantial impact on substrate allocated to and energy produced for the reproductive effort. I anticipate that this effect is greatest, and thus easiest to detect, in lactating mice due to the high demands of lactation in these species. Indeed, lactating laboratory mice display the highest maximum sustained metabolic rate measured in any mammal (Hammond and Diamond 1997). The mammary gland of a lab mouse produces a volume of lipid and protein rich milk that is roughly equivalent to 25% of its body mass each day (Oftedal 1984) and thus supporting the mammary gland is undoubtedly the most energetically demanding process in a lactating female.

In the non-reproductive animal, the liver and muscle are the most demanding tissues in the body, totaling approximately 50% of the body's daily resource use combined (Rolfe and Brown 1997). The liver and muscle both go through functional changes during reproduction. For example, the mitochondria in the liver of lactating house mice are reported to have reduced respiratory efficiency and oxidative damage, but increased antioxidants capacity and mitochondrial density compared to non-reproductive mice (Pichaud et al 2013). Skeletal muscles show an almost complete down-regulation of proteins related to the citric acid cycle in favor of an increased expression of glycolytic protein, as well as decreases in ATP production, amino acid use, and lipid metabolism (Xiao et al 2004a). Furthermore, there is evidence that there is down-regulation of proteins related to respiratory efficiency, such as uncoupling proteins, in skeletal muscle during the period of lactation (Xiao et al 2004b). There is also evidence that a reduction in the energy demands of one organ may benefit another. Specifically, Crescenzo et al (2012) showed that in response to food restriction, despite mitochondrial respiratory function remained unchanged, the density of mitochondria was higher in the liver while it was lower in muscle, suggesting that the energetic demand of these tissues changed in a manner that would be energy sparing in muscle and help support the energy requirements of gluconeogenesis in the liver.

As mitochondria are the main source of ATP that supports the energetic demand of cells and organs, organ specific adjustments to mitochondrial efficiency determine the relative amount of substrate required to produce that ATP (Rolfe and Brown 1997). A very representative measure of the functional and coupled state of mitochondria is the respiratory control ratio (RCR). Brand & Nicholls (2011) suggest that RCR is the most valuable measure of mitochondrial function because it responds to almost any change in variables of the electron

transport chain. RCR is a ratio of state 3 (maximum respiratory rate) and state 4 (basal respiratory rate) respiration, representing the mitochondria's increase in respiratory rate in response to newly available ADP (Brand & Nicholls, 2011). Tissue-specific alterations to any contributing variable will alter the respiratory capacity and demand of the mitochondria. State 3 respiration is diminished when there is a decrease in ATP turnover, decreased substrate acquisition, smaller concentrations of necessary proteins, fewer available electrons, or less oxygen. State 4 respiration is controlled by the amount of proton leak and functional ATP synthases (Brand & Nicholls, 2011). It is hypothesized that organisms expend energy to maintain proton leak back across the inner mitochondrial membrane. While some organisms and tissues use proton leak as a means of producing heat for thermogenesis (Ricquier and Bouillard, 2000), it is also a relatively inexpensive way to regulate energy production and maintain control over the membrane and reactive oxygen species (ROS) production (Brand, 2000). The expression of uncoupling proteins (UCPs) is one way to increase proton leak of the inner mitochondrial membrane, uncoupling electron movement and ATP synthesis. ROS are created by the escape of electrons from the electron carriers in the inner mitochondrial membrane and mitochondrial matrix (Brand 2000; Speakman, 2004; Murphy 2009; Brand *et al* 2004; Brand & Nicholls 2011; Ricquer & Bouillard 2000). Organ specific adjustments in mitochondrial function also influence free-radical production (Ricquer & Bouillard 2000; Jastroch *et al* 2010), which have been posited to contribute to the decline in mitochondria and organ function and lifespan (Harman 1956). The slower the electrons move through the electron transport chain (ETC), the greater the chance is that some will escape from electron carriers (creating free radicals) or come into contact with molecular oxygen (creating superoxides) either before entering the ETC, while associated with an ETC complex, or while being shuttled between complexes by electron carriers (Murphy 2009;

Brand 2000; Ricquer & Bouillard 2000; Jastroch *et al* 2010). To neutralize the damaging effects of free radicals, mitochondria and cells produce antioxidants. The balance or ratio between tissues or cells free radical production and antioxidant capacity is termed the redox state of the cells, which is an important variable related to energy demand of a tissue for maintenance. Production of antioxidants is energetically expensive, as is protein synthesis related to mitochondrial biogenesis and maintenance of ETC complexes. Protein synthesis makes up almost 75% of a cells energy expenditure (Lane and Martin 2010), and as each mitochondria requires multiple expression of almost 600 genes (Mootha et al 2003) and the entire intracellular population of mitochondria is turned-over every 2-4 days (Lipsky and Pedersen 1981), tissue specific changes to mitochondrial biogenesis, complex density, and antioxidant production is an energetically expensive endeavor.

Thus, I predict that organ specific plasticity in the demand for substrate by the mitochondria and plasticity in oxidative damage and mitochondrial function plays a formative role in determining relative energy allocation to reproduction and the relative damage to tissue maintenance capabilities that is exerted during a reproductive event. The first step in testing this hypothesis is to evaluate mitochondrial processes in the liver and muscle of lactating and non-reproductive mice to determine if tradeoffs between organs exist. While, similar measures in mammary tissue would be informative, mammary tissue is virtually non-existent in non-reproductive mice. Organ hypertrophy is common in periods of high-energy demand and changes in the mass of these organs gives us a cursory evaluation of allocation to the liver and muscle during lactation. The mass of the liver increases along with the other physiological changes that occur during reproduction, but skeletal muscle mass remains unchanged (Williamson 1980). Thus, I predict that lactating females will display reduced muscle

mitochondrial respiratory efficiency, mitochondrial biogenesis, and antioxidant production during lactation in order to support the high demands of mammary function. I predict that in the liver there will be a shift in how available substrates are used to maintain high levels of glycolysis (Williamson 1980) and spare fatty acids for milk synthesis and glucose for lipid synthesis in the mammary glands, following the findings of Pichaud et al (2013; Williamson 1980). I selected wild-derived house mice maintained in semi-natural enclosures for this study in an effort to mimic the natural variation in activity and stressors that would be experienced by wild mice, as limited activity and environmental stimulation may result in very different substrate tradeoff profiles than those experienced by free-ranging individuals.

METHODS

Animals

I evaluated mitochondrial function, antioxidant potential, and oxidative damage in house mice, *Mus musculus*. The mice used in this study are descended from individuals that were obtained from Dr. Wayne Potts and the University of Utah. These outbred lines of house mice were originally collected in Gainesville, FL. Individuals included in this study were 16 generations removed from the wild and were maintained in semi-natural enclosures designed to mimic the natural environment, home range size, and social structure of wild mouse populations living in a barn-like location. The Institutional Animal Care and Use Committee of Auburn University approved the experimental set-up and research parameters (PRN 2014-2440; SOP 2012-2106).

Experimental design

In the wild, mice live in social groups, referred to as demes, which usually include 7-12 adults. Each adult maintains a home range size of approximately a few meters (Klein 1975). For this study, mice were maintained in enclosures designed to simulate these natural conditions. Four $5m^2$ enclosures were divided between 2 secure buildings, each with a roof and hardware-cloth windows to excluded predators while exposing the mice to ambient temperatures, noises and seasonal weather conditions. At sexual maturity (~2 months of age), each population was created with 3 male and 5 female mice. Within each population's enclosure, mice were allowed to maintain a natural social structure and breed at a natural pace. Mice were offered eight terracotta pots (4 in tall, 4 in diameter) with an opening added to the side and saucers on top for lids for nesting. The small size of these pots limited communal nesting and suckling that could reduce the energetic output of reproduction. Pups were removed at peak lactation (~day 14) to maintain population densities and encourage rapid reproductive turnover. In mice and many other species, primiparous mothers often allocate less to reproduction than experienced females (Fuchs 1982), which may be a byproduct of lower relative care and reduced mammary development. Thus to limit my data to females expected to maximize their allocation to reproduction, I allowed all reproductive females to breed and produce 3 successful litters before tissues were collected. Litters were deemed successful when at least 2 pups survived to peak-lactation (day 14). All non-reproductive females were maintained in a single enclosure with 7 female mice age-matched to the reproductive females. All animals were offered *ad libitum* access to rodent chow diet (Tekland Global Diet 2020).

All adult mice were pit tagged (model number HPT12, Biomark, Inc., Boise, ID) and ear punched for identification purposes. Litters and putative mothers were carefully monitored by

daily census. On day 14 of their third successful litter, females and their litter were sacrificed. Non-reproductive controls were sacrificed in an age-matched manner to control for aging differences.

Sample Collection

At peak-lactation of their third successful litter, females were euthanized humanely. All necessary adult euthanasia's were performed using isoflurane vapors and decapitation. Euthanasia of litters at peak lactation was performed by overdose of isoflurane followed by cervical dislocation. Immediately upon adult euthanasia, a sample of the liver (approximately 0.5g) and hind limb skeletal muscle (tibialis anterior, extensor digitorum longus, soleus, plantaris, quadriceps femoris, and gastrocnemius; approximately 0.3g) was removed and placed in a buffer made of sucrose, HEPES, and EGTA (described below) to isolate mitochondria to be used for mitochondrial respiratory measurements and Western blot analysis. Once isolated, mitochondria were stored at -80°C for future use. Additionally, a second sample of liver (approximately 0.75g) and skeletal muscle (hind limb, approximately 0.3g) were collected and flash frozen in liquid nitrogen and stored at -80°C for mRNA analysis.

Mitochondrial isolation

Mitochondrial isolation was performed according to the methods of Makinen & Lee at 4°C (1968) with minor adjustments for different tissues. Livers were weighed and put into 10 volumes of a solution made up of 250mM sucrose, 5mM HEPES, and 1mM EGTA and minced with scissors. This minced tissue was further homogenized with a Potter-Elvhjem PTFE pestle and glass tube. The resulting homogenate was centrifuged for 10minutes at 500g at 4°C,

pelleting the cellular debris. The supernatant was then decanted through cheesecloth and then centrifuged for 10 minutes at 3,500g at 4°C, pelleting the mitochondrial fraction. The supernatant was removed and the pellet resuspended in the sucrose solution. This solution was centrifuged for 10 minutes at 3,500g at 4°C, the supernatant discarded and the final mitochondrial pellet suspended in 250µl of a solution made up of 220mM mannitol, 70mM sucrose, 10mM Tris+HCl, and 1mM EGTA, at a pH of 7.4.

Excised skeletal muscles was trimmed of fat and connective tissue, weighed, and put in 10 volumes of BSA solution (100mM KCl, 40mM Tris HCl, 10mM Tris base, 1mM MgSO4, 0.1mM EDTA, 0.2mM ATP, and 2% (wt/vol) free fatty acid bovine serum albumin, pH 7.40). Muscles were minced with scissors and then homogenized for 5 sec with a VITRUS polytron. Trypsin (5mg/g of wet muscle) was added and mixed continually for 7 minutes to digest the minced muscle. This reaction was terminated with the addition of another 10 volumes of BSA solution. The homogenate was centrifuged at 500g for 10 minutes at 4ºC pelleting down cellular debris. The supernatant was decanted through cheesecloth and centrifuged at 3,500g for 10 minutes to pellet the mitochondrial fraction. The supernatant was discarded and the remaining mitochondrial pellet resuspended in BSA solution. This was then centrifuged at 3,500g for 10 minutes. The supernatant was discarded and the pellet was resuspended in 10 volumes of a no-BSA solution (similar to BSA solution, but without BSA). This resuspended pellet was centrifuged at 3,500g for 10 minutes and the final mitochondrial pellet suspended in 250µl of a solution made up of 220mM mannitol, 70mM sucrose, 10mM Tris+HCl, and 1mM EGTA, at a pH of 7.4.

Mitochondrial efficiency

Liver and skeletal muscle respiration was measured by mitochondrial oxygen consumption, quantified by respiratory control ratio (RCR). RCR is a ratio between state 3 and state 4 respiratory rates. RCR is the ratio of state 3 and state 4 respiration, representing the mitochondria's increase in respiratory rate in response to newly available ADP (Brand & Nicholls, 2011). State 3 respiration represents the maximum metabolic capacity of the mitochondria. State 4 respiration, the basally maintained respiration rate, is controlled by the amount of proton leak and functional ATP synthases (Brand & Nicholls, 2011; Brand 2000; Brand et al 2004). Respiration was measured using a technique described by Messer *et al* (2004), utilizing a respiration chamber maintained at 37°C to measure respiration polarographically (Hansatech Instruments, UK). Isolated mitochondria were placed in 1ml of respiration buffer adjusted from the solution described by Wanders *et al* (1984) (100mM KCL, 50mM MOPS, 10mM KH_2PO_4, 20mM glucose, 10mM MgCl2, 1mM EGTA, and 0.2% fatty acid free BSA; pH =7.0) in the respiratory chamber and spun constantly at 37°C. 2mM pyruvate and 2mM malate as complex I substrates and 0.25mM ADP were added to determine State 3 respiration. State 4 respiration follows the phosphorylation of all the ADP to ATP, described in Estabrook (1967). These measurements were recorded the electrode respiration chamber connected to an Oxylab control system and O_2View software (Hansatech Instruments, UK). Respiratory control ratio (RCR), relating the maximum respiration rate (State 3) and the basal respiration rate (State 4), were calculated by taking "State 3 per State 4" oxygen consumption in nm Oxygen per mg protein per minute.

RNA isolation, reverse transcription, and RT-PCR

Whole liver and skeletal muscle tissue were homogenized with a Polytron homogenizer in Ribozol. The sample were vortexed and centrifuged at 13,000g for 10 minutes at 4°C. The supernate was transferred to a new tube and mixed with chloroform before being centrifuged at 13,000g for 15min at 4°C. The aqueous phase was transferred to a new tube and RNA was precipitated with isopropanol and pelleted out using centrifugation and then washed twice with two volumes of 75% ethanol. The pellet was resuspended in RNase-free water. The concentration was measured using NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Total RNA was stored at -80°C.

cDNA was synthesized with 1µg RNA, 4µL qScript, and DEPC water. The mixture was incubated at 25°C for 5 minutes, 42°C for 30 minutes, 85°C for 5 minutes, and then held at 4°C. The cDNA was diluted for a final concentration of 5ng/µL. Quantitative real-time PCR was performed by SYBR green chemistry (Quanta BioSciences, Gaithersburg, MD) using CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Primers and probes for uncoupling protein 2 (UCP2), uncoupling protein 3 (UCP3), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α), and beta-actin (β-actin) as a standard were obtained from Integrated DNA Technologies, Inc. Each 20-µl PCR reaction was performed in duplicate with a 25.0 ng cDNA template. Primer accuracy was confirmed by melting curve.

Bradford protein quantification and protein electrophoresis

Protein concentration of isolated mitochondrial pellet and homogenized whole tissue was measured following the technique of Bradford (1976a, 1976b). Copper-zinc superoxide dismutase (CuZnSOD), manganese superoxide dismutase (MnSOD), glutathione peroxidase 1 (Gpx-1), Cytochrome c, 4-hydroxynonenal (4-HNE), and catalase levels were quantified from isolated mitochondrial samples to measure antioxidant ability, oxidative stress, mitochondrial uncoupling, and prevalence of cytochrome c electron carrier in the mitochondria of liver and skeletal muscle. Ponceau staining standardized sample loading. The amount of each individual protein in tissue and isolated mitochondria samples were analyzed by Western blot. Proteins (20ug) were separated in 0.1% sodium dodecyl sulfate polyacrylamide gels by polyacrylamide gel electrophoresis. Proteins were then transferred to PVDF membranes. Non-specific proteins were blocked using a buffer of phosphate-buffered saline (PBS) with 0.05% Tween and 5% non-fat milk. Membranes were incubated with primary antibodies (GeneTex, Irvine, CA) for 1 hour at room temperature. Membranes were then be washed thoroughly with PBS containing 0.05% Tween and incubated again with secondary antibodies (GeneTex, Irvine, CA) and then washed again. A chemiluminescent system was used to visualize the marked proteins (GE Healthcare). Luminescent membrane images were taken and visualized with the ChemiDocIt^2 Imaging System (UVP, LLC, Upland, CA).

Statistical analyses

All statistical analyses were done using R version 3.1.2 (R Core Team 2012). Comparisons between reproductive and non-reproductive groups were analyzed by two-sample t-test with consideration for unequal variance. Statistical significance was established at $p < 0.05$.

RESULTS

To identify potential energetic tradeoffs that may exist between reproduction and self-maintenance, indicators of mitochondrial efficiency, tissue redox state, mitochondrial uncoupling, and mitochondrial turnover were measured in the liver and skeletal muscle of non-reproductive females and females at peak-lactation period of their third litter.

Organ Weights

Organ weights were compared between treatments to evaluate costly changes of tissue accretion during lactation. There was no difference in the combined weight of the tibialis anterior, extensor digitorum longus, soleus, plantaris, quadriceps femoris, and gastrocnemius at peak-lactation compared to non-reproductive controls ($t_{12.15}$=0.102, p=0.920). Livers were significantly heavier in female mice at peak-lactation than in non-reproductive females ($t_{11.8}$=5.4, p=0.00017).

Mitochondrial oxidative phosphorylation

RCR was calculated from isolated mitochondria in the liver and skeletal muscle as a general measure of mitochondrial efficiency at peak-lactation. The RCR of skeletal muscle mitochondria showed a slightly higher RCR in reproductive individuals at peak lactation ($t_{12.1}$=1.91, p=0.08; Fig. 1a) compared to non-reproductive controls. This difference is driven by a significantly lower state 4, or basal respiration, in the skeletal muscle mitochondria of females at peak lactation ($t_{8.3}$=2.94, p=0.017; Fig. 1c). There is no significant difference to the state 3 respiration at peak-lactation compared to non-reproductive individuals ($t_{12.0}$= 1.19; p= 0.26; Fig. 1b).

The RCR of liver mitochondria was also only slightly higher efficiency at peak-lactation ($t_{11.9}$=1.67, p=0.12; Fig. 1d) compared to non-reproductive individuals. A significantly higher State 3 ($t_{14.0}$=4.54, p=0.0004; Fig. 1e), or maximal respiratory capacity, drives this difference, though there is also a significantly higher state 4 at peak-lactation compared to non-reproductive controls ($t_{12.6}$=2.18; p=0.048; Fig. 1f).

Antioxidants and oxidative damage

To evaluate how peak-lactation affects the redox state of the tissues of interest, measures of the antioxidant capacity and oxidative damage were taken, including copper-zinc superoxide dismutase (CuZnSOD), manganese superoxide dismutase (MnSOD), glutathione peroxidase-1 (Gpx-1), Cytochrome c, catalase, and 4-hydroxynonenal (4-HNE).

At peak lactation, the isolated skeletal muscle mitochondria have significantly lower catalase levels ($t_{7.8}$=2.89, p=0.02; Fig. 2a) than non-reproductive controls. Similarly, at peak-lactation, isolated muscle mitochondria have a significantly lower level of MnSOD ($t_{10.3}$=5.25, p=0.0003; Fig. 2b) and Gpx-1 ($t_{14.7}$=2.58, p=0.021; Fig. 2c) than the skeletal muscle mitochondria of non-reproductive controls. The skeletal muscle mitochondria also exhibit lower, though not significant, levels of 4-HNE, or lipid peroxidation ($t_{9.2}$=1.29, p=0.23; Fig. 2d).

In the liver at peak lactation, isolated mitochondria have significantly less catalase ($t_{15.0}$=2.96, p=0.009; Fig. 3a) and CuZnSOD ($t_{6.5}$=3.32, p=0.014; Fig. 3c). There is a no significant difference in levels MnSOD ($t_{12.5}$=1.023, p=0.33; Fig. 3b). However, there are significantly higher levels of 4-HNE, or lipid peroxidation ($t_{8.8}$=2.34, p=0.044; Fig. 3d) in the liver mitochondria of females at peak-lactation than in non-reproductive individuals.

Uncoupling proteins

The efficiency of energy production through oxidative phosphorylation is affected by several regulated variables, including the uncoupling of the electron transport chain and ATP synthesis. To evaluate the how the physiological demand of reproduction alters the degree of uncoupling, and thus the efficiency of energy production of a tissue, the mRNA expression of uncoupling protein 2 (UCP2) was evaluated.

In skeletal muscle, there was no difference in the expression of UCP2 ($t_{12.8}$=0.08, p=0.93; Fig. 4a). However, at peak-lactation, the liver showed a 2.16 fold higher increase expression of UCP2 than expressed by non-reproductive mice ($t_{12.6}$=2.48, p=0.027; Fig. 4b).

Biogenesis and cytochrome c

To measure how the energetic demand of reproduction alters the turnover of and creation of new mitochondria and mitochondrial subunits, the expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α) was measured, as well as the prevalence of the cytochrome c protein.

At peak lactation, mitochondria from skeletal muscle have significantly fewer cytochrome c carriers ($t_{8.3}$=3.46, p=0.008; Fig. 5) than the skeletal muscle mitochondria of non-reproductive individuals. However, the skeletal muscle of individuals at peak-lactation showed no difference in the expression of the mitochondrial biogenesis signaler PGC1-α ($t_{10.0}$=0.0162, p=0.98; Fig. 4c). In the liver, the expression of the PGC1-α was 1.41 fold greater in lactating than in non-reproductive mice ($t_{11.9}$=1.13, p=0.28; Fig. 4d).

DISCUSSION

With this investigation, I evaluated differences in liver and muscle mitochondria bioenergetics and redox state between non-reproductive and lactating house mice to determine if and how mitochondria in the liver and muscle respond to the energy and substrate demands of reproduction, and milk synthesis in particular. I found that skeletal muscle of lactating house mice living under semi-natural conditions display substrate sparing strategies, including a more energy efficient basal respiration (state 4), reduced antioxidant production, and reduced mitochondrial subunit production. In contrast, the livers of these animals displayed a mix of costly and substrate sparing changes. Costly changes included tissue accretion, increased basal respiratory rate (state 4), and greater oxidative damage, all of which could have long-term impacts on tissue maintenance. Substrate sparing changes included reduced antioxidant production and a higher maximum respiratory rate (state 3) at peak-lactation.

Liver mitochondria

Efficient mitochondria idle at a low state 4, consuming little O_2 to maintain their membrane potential, or proton motive force (pmf), while still having the ability to achieve a high maximal substrate oxidation and ATP turnover, or state 3, when necessary (Brand and Nicholls 2011; Ricquier and Bouillard 2000; Speakman et al 2004; Brand 2000; Brand et al 2004). The increased ATP turnover is associated with a decrease in pmf. However, this process is complicated by the fact that less proton leak through the inner mitochondrial membrane increases the pmf resulting in a slower electron flux and increased possibility of ROS production (Erlanson-Albertsson 2003; Jastroch et al 2000; Ricquier and Bouillard 2000; Speakman et al 2004; Brand 2000; Brand et al 2004).

Our results show that at peak-lactation in the liver there is a trend toward increased RCR, driven by a significantly increased state 3, and slightly increased state 4 (Fig. 1). This result contradicts Pichaud et al's (2013) findings that ETC activity is lower in the liver at peak-lactation. The differences between Pichaud et al's (2013) findings and our results highlight formative role that the environment may play in RCR. Although Pichaud et al's (2013) mice were also derived from wild populations, their lactating females were housed singly in standard laboratory 48cm x 11.5cm x 12cm rodent boxes. The mice described herein had the opportunity to travel greater distances, experienced greater social interaction with other members of their deme, male and female, and contended with natural weather conditions and exposure to the sounds of potential predators. These interactions and conditions likely play an important role in determining the bioenergetics profile of animals during reproduction. Garratt et al (2011) found that while singly housed, reproductive females displayed lower levels of oxidative damage in liver tissue compared to non-reproductive individuals, this decrease disappeared when reproductive females were in forced to contend with territorial intrusion. Our findings indicate that our mice displayed an adaptive increase in liver mitochondrial efficiency during lactation, as an energy sparing strategy in a highly demanding periods. I predict that this effect is more typical of wild mice than the responses of mice maintained in isolation. The increase in state 4 respiration in the liver at peak-lactation is a predicted response to the increase expression of UCP2 observed in this group. Increased uncoupling acts to limit ROS production (Ricquier and Bouillard 2000). While increased basal respiratory rate is more energetically costly, with increased proton leak, it spares energy required to mitigate damaging effects of ROS. Establishing and maintaining higher respiratory efficiency in the liver during reproduction and

lactation is suggestive of a reproductively skewed energy allocation toward reproductive activities.

While previous studies report that at peak-lactation the mitochondrial density of the liver increases (Pichaud et al 2013), I found no difference in mRNA coding for PGC1-α, a key regulator of mitochondrial biogenesis, in the liver of lactating and non-reproductive females (Fig. 4d). However, I did find a significant increase in liver size of lactating mice. This mass and vasculature of the liver increases steeply during reproduction and lactation to support the nutrient delivery to and from the liver and mammary glands. During lactation, the liver increases lipogenesis and lipid esterification to support milk synthesis (Vernon 1989). While no increase in PGC1-α RNA expression was detected, the energy allocation to liver growth and function is clearly still increased during lactation, indicating an energetically costly strategy to support milk synthesis.

All of the antioxidants measured were lower in the liver of reproductive females compared to non-reproductive females (Fig 2; Fig 3). Garratt et al (2011; 2013) reported an increase in SOD and a change in the proportion of glutathione: oxidized glutathione in lactating females. Cells rely in multiple antioxidant networks to protect them from free radical damage (Powers et al 2010). Our results cover multiple antioxidants expressed in the isolated mitochondria, the biomolecules closest to newly formed ROSs. As a reduction in antioxidants has the potential to allow a greater percent of existing free radicals to interact with and damage intracellular proteins, lipids, and mitochondrial DNA, the cost of down-regulating antioxidants production must improve reproductive performance and, in turn, represent a potentially long-term cost to reproduction. Indeed, the livers of lactating females displayed higher oxidative damage than the livers of non-reproductive mice.

Although numerous measures of oxidative damage exist in the literature, I chose to measure lipid peroxidation (4-HNE), as one of the more stable measures of oxidative damage (Powers et al 2010). 4-HNE is an aldehyde formed from lipid peroxidation and our analysis has the benefit of identifying all aldehyde-protein conjugates, which allows for quantification of bound aldehydes that are often missed in assays of free aldehydes. Furthermore, unlike other oxidative damage measurements, such as TBA, I can be more confident that our results are related to increased ROS production (Powers et al 2010). Our findings show a significant increase in oxidative damage in the liver at peak-lactation (Fig. 3d). Increased oxidative damage has been found in livers of female breeders that were required to engage in territorial defense compared to those that were not (Garratt et al 2011). Thus, it is possible that the lactating females in our study displayed higher oxidative damage compared to non-reproductive counterparts both as a function of reduced antioxidant production and the semi-natural environment that the mice experienced in this study.

Muscle mitochondria

Unlike the liver, which displays a mix of costly and substrate sparing changes during reproduction, the mitochondria of the skeletal muscle appear to adjust all major function to spare substrate for milk synthesis. The mitochondria of the skeletal muscle of lactating females in this study displayed a trend toward greater RCR relative to non-reproductive females. This pattern was driven by significantly lower state 4 respiration (Fig. 1). This would allow the muscle to maintain its mitochondrial functionality without expending as much energy, though increases the possibility of ROS production when it switches to state 3 respiration. While I found no change in mRNA levels of UCP2 (Fig. 4a), there is strong evidence in the literature that UCP3 is down

regulated in skeletal muscle at peak-lactation (Xiao et al 2004a). However, these measurements were done with actual expressed protein levels rather than mRNA levels, which could potentially explain the difference in results between studies. UCP2 and UCP3 are two of several homologs of uncoupling proteins. These are the two uncoupling proteins that are hypothesized to be involved in modulating superoxide production (Vidal-Puig et al, 2000). Their presence and abundance varies across tissues (Ricquier and Bouillard, 2000). Lower expression of uncoupling proteins in the skeletal muscle could explain the significantly lower state 4 at peak-lactation (Fig. 1e). Cytochrome c subunit expression was also lower in mitochondria skeletal muscle of lactating mice than in non-reproductive controls (Fig. 5). This effect is indicative of fewer maintained subunit and complex structures in mitochondria and perhaps a reduced rate of mitochondrial turnover (Barazzoni et al 2000). This interpretation is inconsistent with my results for PGC1-α, which did not differ between muscle of lactation and non-reproductive females (Fig. 4c). However, PGC1- α was a measure of RNA expression while Cytochrome c was measured in relative protein levels. Our results do not directly measure the post-transcriptional levels of PGC1-α. However, increased RCR in the muscle during lactation could serve as a mechanism to compensate for fewer mitochondria in muscle tissues, though further work is required. Regardless, significantly fewer Cytochrome c subunits and increased RCR suggests a substrate sparing strategy in skeletal muscle during lactation.

Antioxidant concentrations were lower at peak-lactation, but lipid peroxidation was unchanged in the mitochondria of skeletal muscle of mice at peak-lactation compared to non-reproductive mice. Again, this suggests that the substrate requirements for antioxidant production are spared during lactation, while in this case the drop in antioxidants is not

associated with an increase in oxidative damage. Reduced oxidative damage in lactating females is consistent with lower aerobic respiration and possibly mitochondrial density overall.

Conclusions

One of the central tenets of life history theory is that reproduction exacts a cost on maintenance and longevity. As a result of limiting the body's maintenance ability, the future capacity to allocate energy to reproductive tasks or the maintenance of tissues and cellular function that support longevity is reduced. Herein I show that there is organ specific plasticity in the demand for substrate, mitochondrial bioenergetics, and redox state during lactation in house mice. I posit that this plasticity plays an important role in determining allocation to reproduction and the damage accrued in tissue maintenance capabilities during a reproductive event. While skeletal muscle mitochondria displayed substrate-sparing changes, including more energy efficient state 4 respiration, reduced antioxidant production, and fewer Cytochrome c subunits, the livers of lactating mice showed a mix of costly and substrate sparing changes. Liver mass was significantly higher, as was state 4 respirations and oxidative damage. However, the complete down regulation of antioxidants and higher RCR suggest an effort to conserve substrate for milk production. While many of these substrate-sparing changes in both liver and skeletal muscle suggest a strategy to allocate nutrients preferentially to mammary tissue, there is a potential long-term negative impact of the costly changes seen during peak-lactation. Theory suggests that the long-term ramifications of this tradeoff will eventually result in a cost to lifespan. But that is beyond the scope this study. These results do not extend past the period of lactation, but suggest that multiple bouts of breeding could take a toll on the condition of tissues

that could ultimately lead to decreased lifespan. More research is needed to determine the mechanism and nature of this tradeoff and how it applies to life history of over time.

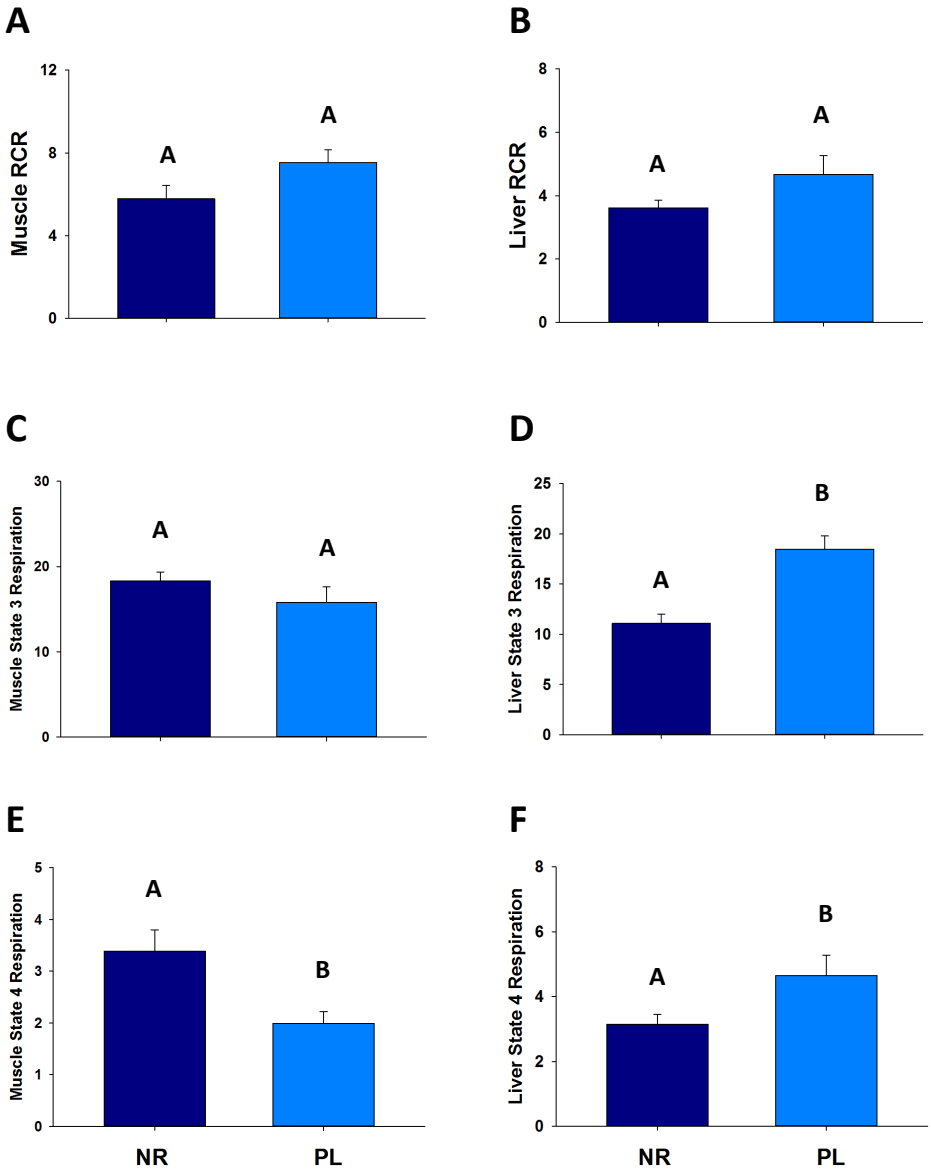

Figure 1. Skeletal muscle RCR (A), liver RCR (B), skeletal muscle state 3 respiration (C), liver state 3 respiration (D), skeletal muscle state 4 respiration (E), and liver state 4 respiration (F) isolated mitochondria of non-reproductive (NR) and peak-lactation (PL) female mice. Bar graphs show means and standard error bars. Letters above bars indicate results of t-test with significant differences represented by different letters. Significance established at $P<0.05$.

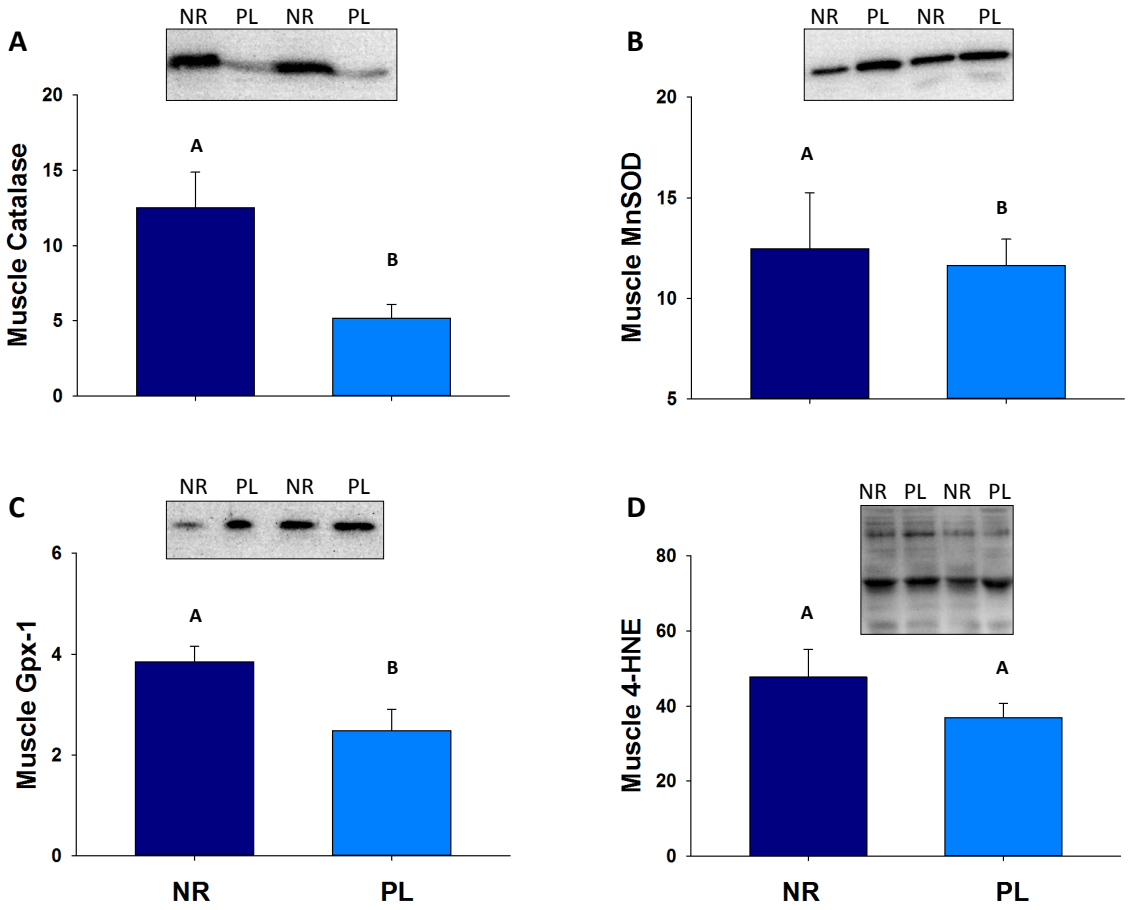

Figure 2. Skeletal muscle catalase (A), manganese superoxide dismutase (B), glutathione peroxidase 1 (C), and 4-hydroxynonenal (D) levels in arbitrary units from isolated skeletal muscle mitochondria of non-reproductive (NR) and peak-lactation (PL) female mice. Bar graphs show means and standard error bars. Letters above bars indicate results of t-test with significant differences represented by different letters. Significance established at $P<0.05$. Representative Western blots shown above graphs.

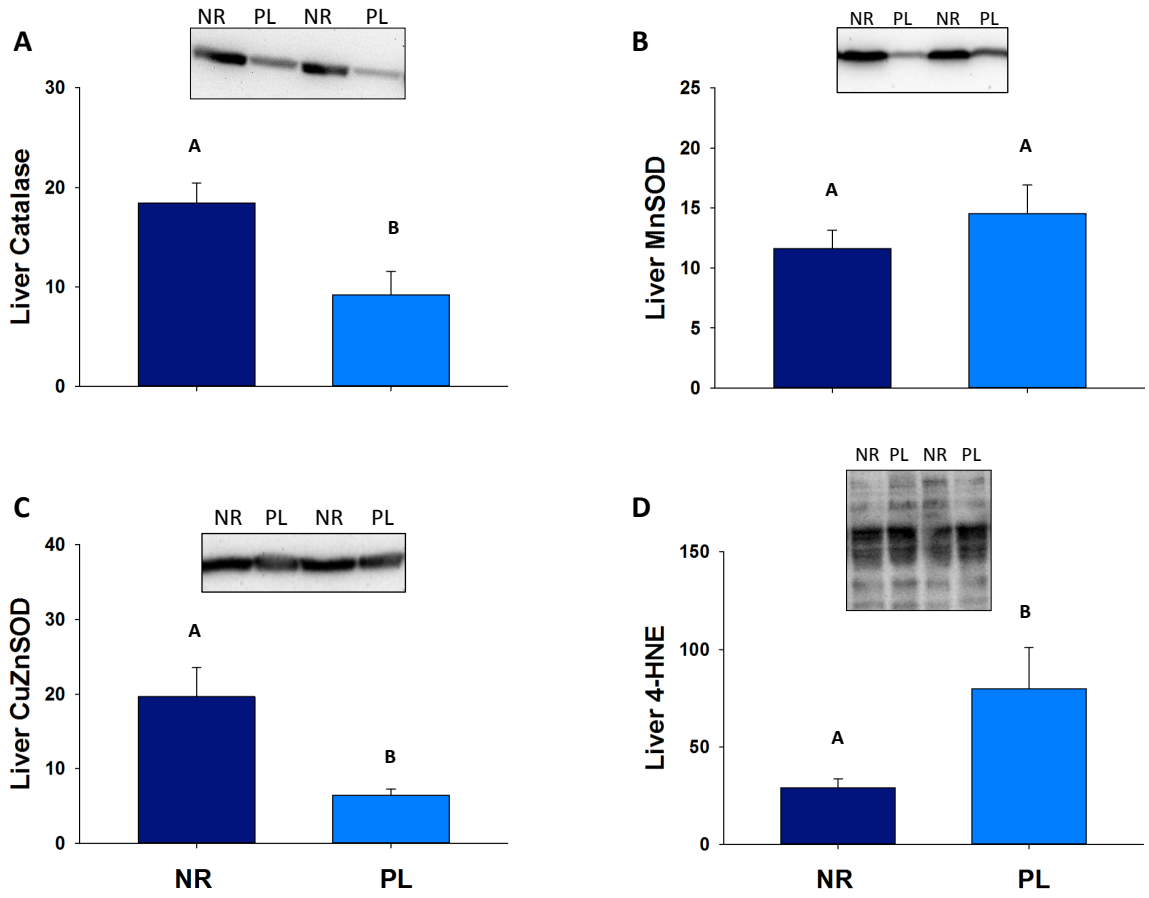

Figure 3. Liver catalase (A), manganese superoxide dismutase (B), copper-zinc superoxide dismutase (C), and 4-hydroxynonenal (D) levels in arbitrary units from isolated liver mitochondria of non-reproductive (NR) and peak-lactation (PL) female mice. Bar graphs show means and standard error bars. Letters above bars indicate results of t-test with significant differences represented by different letters. Significance established at $P<0.05$. Representative Western blots shown above graphs.

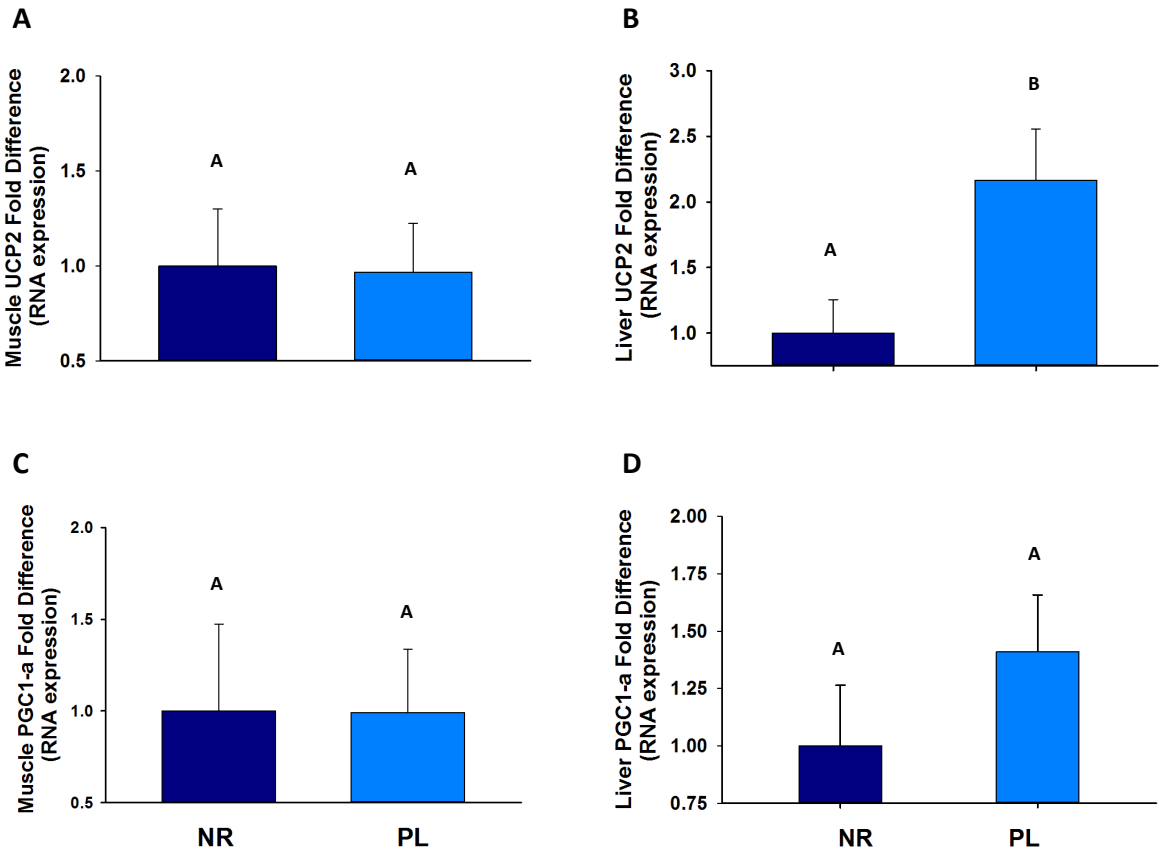

Figure 4. Fold-change in skeletal muscle (A) and liver (B) uncoupling protein 2 RNA expression and fold-change in skeletal muscle (C) and liver (D) peroxisome proliferator-activated receptor gamma coactivator 1-alpha RNA expression levels from non-reproductive (NR) and peak-lactation (PL) female mice. Bar graphs show means and standard error bars. Letters above bars indicate results of t-test with significant differences represented by different letters. Significance established at $P<0.05$.

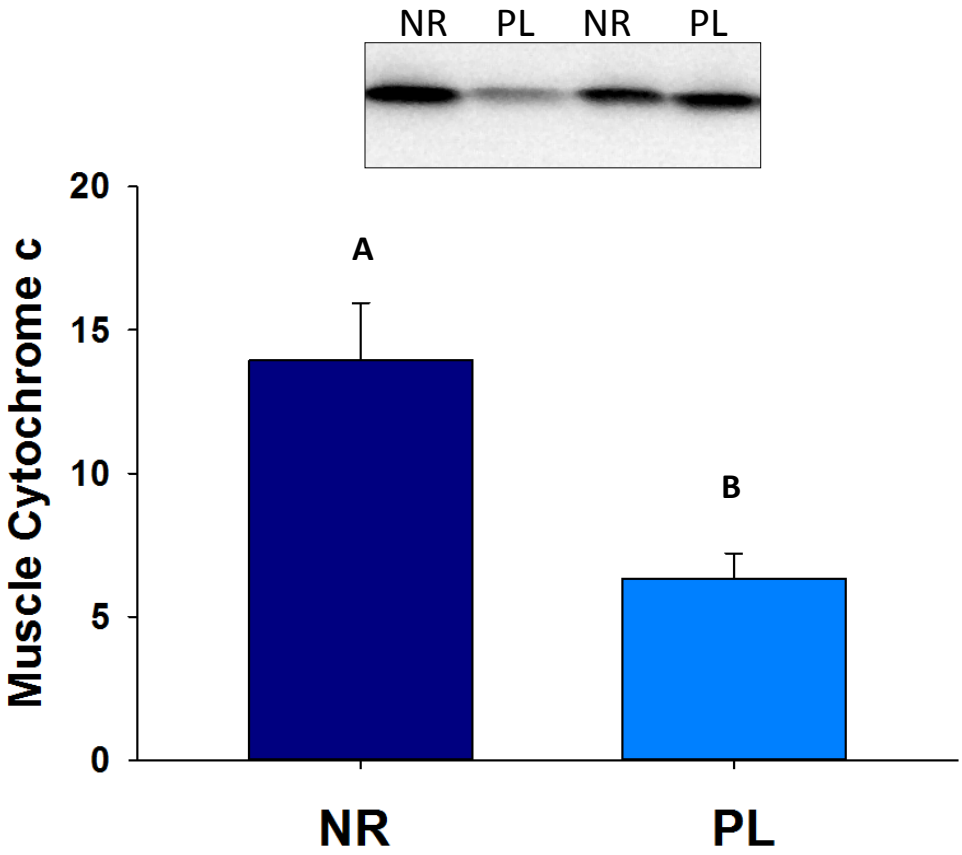

Figure 5. Skeletal muscle Cytochrome c protein levels in arbitrary units from isolated skeletal muscle mitochondria of non-reproductive (NR) and peak-lactation (PL) female mice. Bar graph show means and standard error bars. Letters above bars indicate results of t-test with significant differences represented by different letters. Significance established at $P<0.05$. Representative Western blot shown above graph.

LITERATURE CITED

Akers,RM. 2002. Lactation and the mammary gland. Iowa State University Press, Ames, Iowa, 2002.

Barazzoni R et al. 2000. Effects of aging on mitochondrial DNA copy number and cytochrome c oxidase gene expression in rat skeletal muscle, liver, and heart. The Journal of Biological Chemistry 5: 3343-3347.

Bottje W. *et al*. 2002. Association of mitochondrial function with feed efficiency within a single genetic line of male broilers. Poultry Science 81: 546-555.

Bradford M.M. 1976a. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Analytical Biochemistry 72: 248-254.

Bradford M. 1976b. Differential color change of a dye in response to various concentrations of protein. Analytical Biochemistry 72: 248-253.

Brand M.D. 2000. Uncoupling to survive? The role of mitochondrial inefficiency in ageing. Experimental Gerontology 35: 811-820.

Brand M.D. 2005. The efficiency and plasticity of mitochondrial energy transduction. Biochemical Society Transactions 33: 897-904.

Brand M.D. *et al*. 2004. Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. Free Radical Biology and Medicine 37: 755-767.

Brand M.D. and Esteves T.C. 2005. Physiological functions of the mitochondrial uncoupling proteins UCP2 and UCP3. Cell Metabolism 2: 85-93.

Brand, M.D. and Nicholls D.G. 2011. Assessing mitochondrial dysfunction in cells. Biochemical Journal 435: 297-312.

Bronson F.H. 1989. Mammalian Reproductive Biology. University of Chicago, Chicago.

Burton T. *et al*. 2011. What causes intraspecific variation in resting metabolic rate and what are its ecological consequences? Proceedings of the Royal Society B- Biological Sciences 278: 3465-3473.

Crescenzo, R., F. Bianco, I. Falcone, P. Coppola, A.G. Dulloo, G. Liverini and S. Iossa. 2012. Mitochondrial energetics in liver and skeletal muscle after energy restriction in young rats. British Journal of Nutrition 108: 655-665.

Erlanson-Albertsson C. 2003. The role of uncoupling proteins in the regulation of metabolism. Acta Physiol Scand. 178: 405-412.

Estabrook R.W. 1967. in *Methods in enzymology,* Ronald, M.E.P. and Estabrook, W., Eds. Academic Press. 10: 41-47.

Eya J.C. *et al*. 2012. Genetic variation in feed consumption, growth, nutrient utilization efficiency and mitochondrial function within a farmed population of channel catfish (Ictalurus punctatus). Comparative Biochemistry and Physiology Biochemistry & Molecular Biology 163: 211-220.

Fisher, R.A. 1930. *The Genetical Theory of Natural Selection*, Clarendon Press, Oxford.

Flatt, T. and Heyland, A. 2001. *Mechanisms of Life History Evolution, The Genetics and Physiology of Life History Traits and Trade-offs.* Oxford University Press, Oxford.

Fuchs S. 1982. Optimality of parental investment: The influence of nursing on reproductive success of mother and female young house mice. Behav Ecol Sociobiol 10: 39-51.

Garratt, M. *et al*. 2011. Is oxidative stress a physiological cost of reproduction? An experimental test in house mice. Proceedings of the Royal Society B-Biological Sciences 278: 1098-1106.

Garratt M. *et al*. 2013. Physiological adaptations to reproduction. I. Experimentally increasing litter size enhances aspects of antioxidant defence but does not cause oxidative damage in mice. The Journal of Experimental Biology 216: 2879-2888.

Hammond, K.A., K.C.K. Lloyd and J. Diamond. 1996. Is mammary output capacity limiting to lactational performance in mice? Journal of Experimental Biology 199: 337-349.

Hammond, K., and J. Diamond. 1994. Limits to dietary nutrient intake and intestinal nutrient-uptake in lactating mice. Physiological Zoology 67: 282-303.

Hammond, K.A., and J. Diamond. 1997. Maximal sustained energy budgets in humans and animals. Nature 386: 457-462.

Harman, D. 1956. Aging: a theory based on free radical and radiation chemistry. Journal of Gerontology 11: 298-300.

Jastroch M. *et al*. 2010, in Essays in Biochemistry: Mitochondrial Function. Brown G.C., Murphy M.P., Eds. 47: 53-67.

Jornayvaz FR and Shulman GI. 2010. Regulation of mitochondrial biogenesis. Essays in Biochemistry 47: 69-84.

Kirkwood, T.B.L. 1977. Evolution of Ageing. Nature 270: 301-304.

Kirkwood, T.B.L. and Holliday, R. 1979. The Evolution of Ageing and Longevity. Proceedings of the Royal Society B 205 (1161).

Kleiber, M. 1975. The Fire of Life: An Introduction to Animal Energetics. Krieger Pub Co.

Klein, J. 1975. Natural History of the Mouse Histocompatibility-2 complex. Springer-Verlag, New York.

Lane, N. 2011. The Costs of Breathing. Science 334: 184-185.

Lane, N., and W. Martin. 2010. The energetics of genome complexity. Nature 467: 929-934.

Larsen, F.J. et al. 2011. Mitochondrial oxygen affinity predicts basal metabolic rate in humans. The FASEB Journal 25: 2843-2852.

Lipsky NG and Pedersen PI. 1981. Mitochondrial turnover in animal cells. The Journal of Biological Chemistry 256: 8652-8657.

Lobachev A. N. 1978. Role of mitochondrail processes in the development of aging of organism. Aging and cancer. Chemical Abstracts 91: 48.

Makinen M.W. and Lee, C.P. 1968. Biochemical studies of skeletal muscle mitochondria: I. Microanalysis of cytochrome content, oxidative and phosphorylative activities of mammalian skeletal muscle mitochondria. Arch Biochem Biophys 126: 75-82.

Maloney C.A. *et al*. 2013. A methyl-deficient diet fed to rats during pre- and peri-conceptions periods of development modifies the hepatic proteome in the adult offspring. Genes and Nutrition 8: 181-190.

Marcuello A. *et al*. 2009. Human mitochondrial variants influence on oxygen consumption. Mitochondrion 9: 27-30.

Messer, M. R. and Jackman, W. T. Willis. 2004. Pyruvate and citric acid cycle carbon requirements in isolated skeletal muscle mitochondria. American Journal of Physiology. Cell Physiology 286: C565-572.

Miquel J. *et al*. 1980. Mitochondrial role in cell aging. Experimental Gerontology 15: 575–591.

Metcalfe, N.B. and Alonso-Alvarez, C. 2010. Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. Functional Ecology 24: 984-996.

Monaghan, P., Metcalfe, N.B. and Torres, R. 2009. Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. Ecology Letters 12, 75-92.

Mootha VK et al. 2003. Integrated analysis of protein composition, tissue diversity, and gene regulation in mouse mitochondria. Cell 115, 629-640.

Oftedal, O.T. 1984. Milk composition, milk yield and energy output at peak lactation: a comparative review. pp. 33-85 in M. Peaker, R.G. Vernon and C.H. Knight, eds. Physiological Strategies in Lactation (Symposia of the Zoological Society of London) Oxford University Press, London.

Oldakowski L. *et al*. 2012. Is reproduction costly? No increase of oxidative damage in breeding bank voles. The Journal of Experimental Biology 215: 1799-1805.

Pichaud, N., *et al*. 2013. Physiological adaptations to reproduction. II. Mitochondrial adjustments in livers of lactating mice. Journal of Experimental Biology 216: 2889-2895.

Powers SK et al. 2010. Experimental guidelines for studies designed to investigate the impact of antioxidant supplementation on exercise performance. J Sport Nutr Exerc Metab 20: 2-14.

Promislow, D.E.L. and Harvey, P.H. 1990. Living fast and dying young: A comparative analysis of life history variation among mammals. Journal of Zoology 220:417-437.

R Core Team (2012). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/

Ricquier, D. and Bouillard, F. 2000. Mitochondrial uncoupling proteins: from mitochondria to the regulation of energy balance. Journal of Physiology 529: 3-10.

Rolfe, D.F.S. and Brown, G.C. 1997. Cellular energy utilization and molecular origin of standard metabolic rate in mammals. Physiological Reviews 77: 731-758.

Roff, D.A. 1992. *Evolution of Life Histories: Theory and Analysis.* Routledge, Chapman & Hall, London.

Salin, K. et al. 2012. Alteration of mitochondrial efficiency affects oxidative balance, development and growth in frog (Rana temporaria) tadpoles. Journal of Experimental Biology 215: 863-869.

Shigenaga MK, Hagen TM, Ames MN. 1994. Oxidative damage and mitochondrial decay in aging. Proc Natl Acad Sci 91: 10771-10778.

Speakman, J.R. 2008. The physiological costs of reproduction in small mammals. Philosophical Transactions of The Royal Society B 363: 375-398.

Speakman, J.R., and Garratt, M. 2014. Oxidative stress as a cost of reproduction: Beyond the simplistic trade-off model. Bioessays 36: 93-106.

Speakman, J.R., and E. Krol. 2005. Limits to sustained energy intake IX: a review of hypotheses. Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology 175: 375-394.

Speakman, J.R., *et al*. 2004. Uncoupled and Surviving: individual mice with high metabolism have greater mitochondrial uncoupling and live longer. Aging Cell 3: 87-95.

Stearns, S.C. 1992. *The Evolution of Life Histories*. Oxford University Press, Oxford.

Vernon RG. Endocrine control of metabolic adaptations during lactation. Proceedings of the Nutrition Society 48: 23-32 (1989).

Vidal-Puig AJ et al. 2000. Energy metabolism in Uncoupling Protein 3 gene knockout mice. The Journal of Biological Chemistry 275: 16258-16266.

Wanders, R.J., *et al*. 1984. Factors determining the relative contribution of the adenine-nucleotide translocator and the ADP-regenerating system to the control of oxidative phosphorylation in isolated rat-liver mitochondria. European journal of biochemistry / FEBS 142:417 424.

Williams, G. C. 1957. Pleiotropy, natural selection, and the evolution of senescence. Evolution 11:398-411.

Williamson, D.H. 1980. Integration of metabolism in tissues of the lactating rat. Febs Letters 117: K93-K105.

Xiao XQ, KL Grove, BE Grayson, and MS Smith. Inhibition of Uncoupling Protein Expression during lactation: Role of Leptin. Endocrinology 146: 830-838 (2004a).

Xiao XQ, KL Grove, and MS Smith. Metabolic adaptations in skeletal muscle during lactation: Complementary deoxyribonucleic acid microarray and real-time polymerase chain reaction analysis of gene expression. Endocrinology 146: 5344-5354 (2004b).

Zera A.J. and Harshman, L.G. 2001. The Physiology of Life History Trade-Offs in Animals. Annual Review of Ecological Systems 32: 95-126.

CHAPTER TWO

Reproduction does not adversely affect the mitochondrial efficiency of liver cells in the house moues (*Mus musculus*)

ABSTRACT

Investment in reproduction is costly and often comes at the cost of survival and future reproduction. The exact physiological mechanism driving this tradeoff is unknown. As mitochondria are the main source of energy in a cell, as well as the harmful byproduct of respiration, free radicals, oxidative damage has been implicated as the consequence of reproduction that would exact a cost on other life history traits. However, studies assessing this relationship by comparing reproductive individuals with non-reproductive controls have yet to yield consistent results. In this study, I reexamine the role of mitochondria in the energetic tradeoff between reproduction and longevity by evaluating the impact of reproduction on mitochondrial function, post-reproduction. In a post-reproductive, maintenance state the mitochondrial efficiency of mouse livers show no negative impacts of reproduction. In fact, there is a trend to suggest a higher maximal respiratory efficiency, post-reproduction. Furthermore, while post-reproductive mice have significantly higher antioxidants levels in liver mitochondria, there is also higher oxidative damage. Our results suggest that oxidative stress is higher post-reproduction, which is not managed by antioxidant production, but that the oxidative damage does not negatively impact mitochondrial function.

INTRODUCTION

The allocation of energy to reproduction occurs at a cost to other life history traits, including future reproduction and lifespan (Fisher, 1930; Williams, 1957; Promislow & Harvey, 1990; Zera & Harshman, 2001; Roff 1992; Stearns 1992; Flatt & Heyland, 2011; Kleiber, 1975). However, the physiological and intracellular mechanisms responsible for this tradeoff remain elusive. In what is now know as the disposable soma theory of aging (DST), Kirkwood (1977) suggested that the negative relationship between reproduction and longevity occurs because the allocation of energy to reproduction compromises the ability to allocate energy for tissue repair and maintenance, and as a result, longevity is reduced (Kirkwood and Holliday; 1979). Mitochondria are the main source of ATP production, supporting the energy demands of nearly all processes in the body, including those that those that fuel the high energy demand of reproduction. Yet, mitochondria are also a major source of damaging free radicals as a byproduct of cellular respiration. Free radicals and reactive oxygen species (ROS) produced by the electron transport chain (ETC) damage intracellular lipids, proteins, and importantly, mitochondrial DNA (mtDNA) (Harman 1956; Lobachev 1978; Miguel et al 1980). Since reproduction is energetically demanding, it is assumed that the increased metabolic demand of reproduction increases oxidative damage and that this damage will reduce the functional capacity of mitochondria and reduce the cell's ability to allocate energy to maintenance (Bottjie et al, 2002; Eya et al, 2012; Marcuello et al, 2009; Speakman, 2008; Bronson, 1989; Lane, 2011).

The aforementioned argument that mitochondria are responsible for the tradeoff between reproduction and longevity is based on the assumption that oxidative damage increases with increased energy expenditure. This assumption oversimplifies the process of respiration and ROS production and ignores the relevant nuances of the electron transport chain function. ROS are

created by the escape of electrons from the electron carriers in the inner mitochondrial membrane and mitochondrial matrix (Brand 2000; Speakman, 2004; Murphy 2009; Brand *et al* 2004; Brand & Nicholls 2011; Ricquer & Bouillard 2000). The slower the electrons move through the ETC, the greater the chance is that some will escape from electron carriers (creating free radicals) or come into contact with molecular oxygen (creating superoxides) either before entering the ETC, while associated with an ETC complex, or while being shuttled between complexes by electron carriers (Murphy 2009; Brand 2000; Ricquer & Bouillard 2000; Jastroch *et al* 2010;). The speed of electron movement is equivalent to the rate of respiration because it is directly tied to the rate of oxygen consumption at the end of the ETC. Thus, ROS production is negatively, not positively, correlated with mitochondrial respiration rate. Although the relationship between oxygen consumption and ATP production is indirect (Brand 2000; Ricquer & Bouillard 2000) energy expenditure is commonly measured by calorimetry or oxygen consumption rather than ATP and heat production (Poppitt et al 1993; Hickling et al 1991; Anantharaman-Barr and Decomba 1989; Parker et al 2001; Jéquier et al 1987; Ross 2000; Westbrook et al 2009)

To evaluate the role of oxidative stress in the tradeoff between reproduction and longevity, most investigators have compared oxidative damage in animals that are rearing offspring to the oxidative damage of non-reproductive individuals or, alternatively, compared the oxidative damage of individuals rearing varying numbers of young. Through meta-analysis, Blount et al (2015) reviewed the findings of studies following this design and found that oxidative damage was lower in the heart, ovarian follicles, and liver of breeding animals, but that there was no change in skeletal muscle and kidney. Although Speakman and Garratt (2014) also pointed out the error in assumption that oxidative damage should increase with energy expenditure, they concluded that the equivocal nature of the published data is a result of

insufficient data and poor experimental design. While I agree that greater caution is needed when choosing variables to include in experimental design, I disagree with Speakman's and Garratt's conclusion that comparing reproductive and virgin, non-reproductive individuals is the best approach to understand the impacts of reproduction on longevity. During the period of reproduction, many organ systems go through rapid proliferation that is reversed when reproduction ends (Akers 2002; Kennedy et al 1958; Hollister 1987; Smith and Baldwin 1974; Cripps and Williams 1975; Chatwin et al 1969). Because of this dramatic plasticity in organ phenotype, I argue that the most appropriate time to evaluate the impact of reproduction on self-maintenance is after the individual has returned to a non-reproductive state. It is the ability of the body to maintain maintenance functions that ultimately determines longevity.

Furthermore, most studies that have evaluated aspects of mitochondrial function in the context of determining the cost of reproduction have focused on oxidative damage and antioxidant levels. Although I have included both measures here, I also evaluated the respiratory efficiency of isolated mitochondria following Brand and Nicholls (2011). This measure is critical to understanding the long-term functional impact of reproduction on the tissue's maintenance ability. Mitochondria fuse and split constantly and are turned over every 2-4 days (Lipsky and Pedersen 1981). If mitochondrial damage, including damage from escaped ROS, is insufficient to signal autophagosomes then damaged or mutated mtDNA will continue to replicate and contribute to the tissues mitochondrial population, resulting in a decline in overall mitochondrial function (Jornayvaz and Shulman 2010; Kelly and Scarpulla 2004; Okamoto and Kondo-Okamoto 2012; Gomes and Scorrano 2013; Seo et al 2010). This decline in mitochondrial function is believed to a key player in senescence according to the mitochondrial theory of aging

(Bottjie et al 2002; Eya et al 2012; Marcuello et al 2009; Speakman 2008; Bronson 1989; Lane 2011).

The aim of this study is to reevaluate the role that mitochondria play in the energetic tradeoff between reproduction and longevity posited by DST by assessing the impact of reproduction on mitochondrial function, post-reproduction. Specifically, I compare non-reproductive individuals and experienced breeders in a post-reproductive, maintenance state. The DST predicts that the increased energetic demand of reproduction would decrease allocation to self-maintenance. If mitochondria are responsible for this tradeoff, post-reproductive animals should retain evidence of lipid-peroxidation and mtDNA and protein damage accumulated during reproduction, and thus mitochondrial respiratory efficiency would be reduced. To evaluate the impact of reproduction on maintenance, I compare mitochondrial efficiency, oxidative damage, and antioxidant capacity in 1-year-old females that reproduced from the onset of reproductive maturity until males were removed when they were 10-11 months of age to 1-year-old females that never bred.

METHODS

Animals

I evaluated mitochondrial function in house mice, *Mus musculus*. The parents of the mice included in this study were obtained from Dr. Wayne Potts and the University of Utah. These outbred lines of house mice were originally collected in Gainesville, FL. Those included in this study were 13 generations removed from the wild and were housed in semi-natural enclosures designed to mimic the environment, home range size, and social structure of wild mouse

populations living in a barn-like structure. The Institutional Animal Care and Use Committee of Auburn University approved the experimental set-up and research parameters (PRN 2012-2104, 2012-2106).

Experimental design

All samples included in this study were collected opportunistically at the termination of a study described by Sirman (2014). In the wild, mice are found in small social groups referred to as demes that typically include 7-12 adults. Each adult typically maintains a home range size of just a few meters (Klein 1975). For this study, mice were maintained in enclosures designed to mimic these conditions. Ten $5m^2$ enclosures were divided between 2 secure buildings that each had a roof and hardware-cloth windows that excluded predators but exposed the mice to ambient temperatures. At sexual maturity (~2 months of age), each population was founded with 3 male and 7 female mice. Within these enclosures, mice were allowed to maintain a natural social structure and bred at a natural pace. Pups were removed at day 28 maintain population densities. Females were allowed to breed for 7-8 months, each producing up to 7 litters. The females in two enclosures were classified as non-reproductive. These enclosures had several females, but ultimately only one male each due to intraspecific male aggression. Because no pups were ever observed in these enclosures, we made the assumption that it was more likely that the single male was sterile than that all of the females were sterile. All animals were offered *ad libitum* access to a 10 or 20 percent protein isocaloric diet (Sirman 2014). I found no effect of diet on mitochondrial function (see Results) and thus excluded this variable from further consideration in this study.

All adult mice were pit tagged (model number HPT12, Biomark, Inc., Boise, ID) and ear punched for identification purposes. Litters and putative mothers were censused daily. One month prior to collection, when the females were 10-11 months of age, males were removed from all enclosures to prevent further breeding and allow reproductive females to return to a maintenance state. At 1 year of age, females were sacrificed.

Sample Collection

In December 2013, females were euthanized. Immediately upon euthanasia, a sample of the liver (approximately 0.5g) was removed and placed in a buffer made of sucrose, HEPES, and EGTA (described below) to prepare for mitochondrial isolation. Once isolated, the mitochondria were divided. 0.5g of sample was used to immediately quantify respiratory function and the remaining sample was stored at -80°C to measure antioxidant capability and lipid peroxidation at a later date.

Mitochondrial isolation

Isolation was performed according to the methods of Makinen and Lee (1968) at 4°C. The liver was weighed and put into 10 volumes of a solution made up of 250mM sucrose, 5mM HEPES, and 1mM EGTA and minced with scissors. This minced tissue was further homogenized with a Potter-Elvhjem PTFE pestle and glass tube. The resulting homogenate was centrifuged for 10 minutes at 500g at 4°C, pelleting the cellular debris. The supernatant was decanted through cheesecloth and centrifuged for 10 minutes at 3,500g at 4°C, pelleting the mitochondrial fraction. The supernatant was removed and the pellet resuspended in the sucrose solution previously mentioned. This solution was centrifuged for 10 minutes at 3,500g at 4°C,

the supernatant discarded and the final mitochondrial pellet suspended in 250µl of a solution made up of 220mM mannitol, 70mM sucrose, 10mM Tris+HCl, and 1mM EGTA, at a pH of 7.4.

Respiratory Control Ratio

Liver tissue respiration was measured by mitochondrial oxygen consumption, measured by respiratory control ratio (RCR). This was measured by a technique described by Messer et al (2004), utilizing a respiration chamber maintained at 37°C to measure respiration polarographically (Oxytherm, Hansatech Instruments, United Kingdom). The isolated mitochondria were placed in 1ml respiration buffer adjusted from the solution described by Wanders et al (1984) (100mM KCL, 50mM MOPS, 10mM KH_2PO_4, 20mM glucose, 10mM MgCl2, 1mM EGTA, and 0.2% fatty acid free BSA; pH =7.0) in the respiratory chamber and spun constantly at 37°C. 2mM pyruvate and 2mM malate as complex I substrates and 0.25mM ADP were added to state 3 respiration, a measure of maximal respiratory capacity. State 4 respiration, a measure of basal respiratory rate, follows the phosphorylation of ADP to ATP, described by Estabrook (1967), and was then recorded. These measurements were recorded using O_2 View (Hansatech Instruments, United Kingdom). Respiratory control ratio (RCR) was then calculated as state 3 divided by state 4 respiration (O_2/mg mitochondrial protein, min) (Brand and Nicholls 2011).

Western blot analysis

Protein concentration of isolated mitochondrial pellet was measured following the technique of Bradford (1976). This value was used as an indicator of the number of mitochondria present. Copper-zinc superoxide dismutase (CuZnSOD), manganese superoxide dismutase

(MnSOD), glutathione peroxidase 1 (GPX-1), and catalase levels were quantified measures of antioxidant capacity. 4-Hydroxynonenal (4-HNE) levels were analyzed as a measure of lipid peroxidation, and indication of oxidative damage. α-Tubulin was measured as a loading and transfer control. Protein content of the isolated mitochondria was analyzed by Western blot. Proteins (12μg) were separated in 0.1% sodium dodecyl sulfate polyacrylamide gels by polyacrylamide gel electrophoresis. Proteins were then transferred to PVDF membranes. Non-specific proteins were blocked using a buffer of phosphate-buffered saline (PBS) with 0.05% Tween and 5% non-fat milk. Membranes were incubated with primary antibodies (GeneTex, Irvine, CA) for 1 hour at room temperature. Membranes were then washed thoroughly with PBS containing 0.05% Tween and incubated again with secondary antibodies (GeneTex, Irvine, CA) and then washed again. A chemiluminescent system was used to visualize the marked proteins (GE Healthcare). Luminescent membrane images were taken and visualized with the ChemiDocIt^2 Imaging System (UVP, LLC, Upland, CA).

Statistical analysis

All statistical analyses were done using R version 3.0.3. Comparisons between reproductive and non-reproductive groups were analyzed by two-sample t-test. Statistical significance was established at $p < 0.05$.

RESULTS

Dietary treatment

Mice were offered a high or low isocaloric diet while breeding to evaluate the effect of the individual diets during pregnancy and lactation on mitochondrial function. There was no

effect of diet on RCR (t_{17}=0.078, p=0.939), state 3 respiration (t_{17}=0.2945, p=0.773), or state 4 respiration (t_{17}=0.31, p=0.761). The diet treatment was removed from further consideration in this analysis.

Mitochondrial oxidative phosphorylation

To determine how multiple bouts of reproduction affect mitochondrial efficiency, RCR of isolated mitochondria was measured. The RCR of liver mitochondria showed breeding a trend suggesting that RCR may be greater in females that have reproduced relative to females who have never given birth (non-reproductive controls; t_{17}=1.80; p=0.089; Fig. 6a). When state 3 and state 4 respiration are considered independently, we see that the difference in RCR is driven by changes in state 3, or maximal respiration capacity. The livers of females that reproduced display significantly higher state 3 respiration than non-reproductive controls (t_{17}=2.02; p=0.059; Fig. 6b). In contrast, there was no difference in reproductive state 4 respiration, basal respiratory rate, between groups (t_{17}=0.602; p=0.555; Fig. 6c).

Antioxidants

To evaluate the effects of a lifetime of reproduction on the antioxidant capacity of the liver mitochondria, several antioxidant enzymes were assessed, including copper-zinc superoxide dismutase (CuZnSOD), manganese superoxide dismutase (MnSOD), glutathione peroxidase 1 (GPX-1), and catalase. The trend across all proteins measured was that females that reproduced had higher antioxidant levels than those that did not. Specifically, reproductive females had significantly higher CuZnSOD (t_{17}= 3.73; p= 0.0017; Fig. 7a) and catalase (t_{17}= 2.22; p= 0.04;

Fig. 7c). Similar patterns were also seen for MnSOD (t_{17}= 1.43; p= 0.169; Fig. 7b) and GPX-1 (t_{17}= 1.02; p= 0.32; Fig. 7d).

Oxidative damage

A measure of lipid peroxidation, 4-HNE, was used to assess the amount of damage accrued from oxidative stress. The liver mitochondria of females that reproduced exhibited significantly more lipid damage than non-reproductive controls (t_{17}= 2.07; p=0.05; Fig. 8).

DISCUSSION

The energetic demands of pregnancy and, in particular, lactation stimulates numerous changes to cellular metabolism throughout the body (Einstein et al 2008; Gutgesell et al 2009; Hamosh et al 1970; Jones et al 1984). Although these changes have long been assumed to exert a cost on longevity, how this cost contributes to senescence is still hotly debated. Here I show that despite high antioxidant projection, post-reproductive female mice display higher oxidative damage than females that never gave birth. Surprisingly, the higher levels of oxidative damage found in post-reproductive females did not result in reduced mitochondrial function of the liver tissue when females returned to a maintenance state. These findings question whether oxidative damage contributes to the changes in mitochondrial function that is associated with senescence.

Mitochondrial respiratory efficiency

The mitochondrial theory of aging suggests that the steady deterioration associated with ageing is caused by the accumulation of free radicals that damage mtDNA (Loeb et al 2005).

While most damaged mitochondria are destroyed through apoptosis, the remaining mitochondria replicate, including mitochondria with minor mutations in their mtDNA due to free radical damage (Okamoto and Kondo-Okamoto 2012; Galloway and Yoon 2012; Westermann 2010; Seo et al 2010). This turnover occurs every 3-4 days, contributing to an increasingly mutated mitochondrial population over the course of a lifetime (Seo et al 2010; Lipsky and Pedersen 1981). In this study, I found that post-reproductive and non-reproductive females displayed no significant difference in liver RCR (Fig. 6a) suggesting that reproduction did not reduce the respiratory capacity of mitochondria, as would be predicted by DST. Indeed, I found a trend suggesting that RCR was greater in many post-reproductive females than in non-reproductive females. This is supported by a higher maximum respiratory capacity in post-reproductive females (State 3; Fig. 6b) and no difference in basal respiratory capacity (State 4) between the groups (Fig. 6c). These findings counter the results of Pichaud et al (2013) who evaluated liver mitochondrial function in house mice at peak-lactation relative to non-reproductive controls and, thus, our findings highlight a plasticity in mitochondrial function that occurs as individuals transition between a reproductive and maintenance state. Pichaud et al (2013) found that liver RCR and state 3 respiration was not different between lactating and non-reproductive house mice, but state 4 respiration, i.e. uncoupled respiration, was significantly lower in lactating females. Lower state 4 respiration suggest that lactating females can respire at a lower, more efficient basal rate, which likely spares fatty acids for delivery to mammary glands (Fritz 1961).

Brand & Nicholls (2011) suggest that RCR is the most valuable measure of mitochondrial function because it responds to almost any change in variables of the electron transport chain. RCR is a ratio of state 3 (maximum metabolic rate) and state 4 (basal metabolic rate) respiration, representing the mitochondria's efficiency. An higher RCR represents the

mitochondria's ability to operate at a low basal rate (state 4), consuming very little oxygen to maintain a functional membrane dynamic, but being able to quickly achieve a maximum respiration (state 3) that effectively produces the largest quantity of ATP from available substrate and oxygen (Brand & Nicholls, 2011). State 3 respiration is diminished when there is lower ATP turnover, decreased substrate acquisition, smaller concentrations of necessary proteins, fewer available electrons, or less oxygen. State 4 respiration is controlled by the amount of proton leak and functional ATP synthases (Brand & Nicholls, 2011). With post-reproductive females displaying higher state 3 respiration and no difference in state 4 respiration compared to non-reproductive controls, our results show post-reproductive females maintain their basal respiration rate (Fig. 6c), while increasing their maximum respiratory capacity (Fig. 6b), suggesting that there is no negative impact of reproduction on mitochondrial function as would be predicted by the DST.

Antioxidants and Oxidative Damage

The mitochondrion and cell produce antioxidants to neutralize ROS and other free radicals produced by the ETC and prevent the damaging effects of ROS to macromolecules in mitochondria and the cell (Halliwell 1994; Bast et al 1991). In this study, antioxidants and oxidative damage were measured in mitochondria isolated from the liver. I found that post-reproductive females displayed both greater antioxidant levels (CuZnSOD and catalase; Fig. 7) and greater oxidative damage (4-HNE; Fig. 8) than non-reproductive controls. These results suggest that ROS production is higher in post-reproductive than in non-reproductive females, as high intra-mitochondrial antioxidants were insufficient to keep oxidative damage at the lower level observed in non-reproductive females. However, it should be noted that the concurrently

higher levels of superoxide dismutases and catalase and Gpx-1 is an indication of an upregulation of the complete free radical disposal process, as superoxides dismutases neutralize superoxides to hydrogen peroxide that is then disposed of by catalase and Gpx-1 (de Haan et al 1996). Given the short lifespan of individual mitochondria (Lipsky and Pedersen 1981) and that the mitochondria analyses were isolated from post-reproductive females several weeks after weaning their final litter, the oxidative damage that I quantified was produced by the liver cells after the mice had returned to a maintenance state.

During reproduction, Garratt et al (2011) reported higher antioxidant production, but lower oxidative damage in female house mice at peak-lactation compared to non-reproductive individuals. Reduced oxidative damage during reproduction is consistent with the results of a meta-analysis that reviewed studies that evaluated oxidative damage during reproduction in birds and mammals (Blount et al 2015). As females in our study were housed in groups and enclosures designed to reflect naturally occurring demes and home range sizes, it is possible that the accumulated oxidative damage seen in post-reproductive females was a results of the stress of regular social interaction. Although, Garratt et al (2011) found no effect of territorial defense on oxidative stress during lactation, their experimental design did not include the natural deme or range size or environmental conditions that our study included, which could have increased the stress of social interactions.

Future directions

Our results indicate that despite higher oxidative stress, post-reproductive females do not have the reduced mitochondrial respiratory function that would be expected by DST, and thought to contribute to senescence by the mitochondrial theory of aging. After comparing our results

from post-reproductive, maintenance state mitochondria to those reported at peak-lactation (Garratt et al 2011), it is clear that mitochondria display a high level of metabolic plasticity, and thus, I believe that future studies evaluating reproductive tradeoffs should compare individuals that are similar in age and life history stage. When the aim is to evaluate the impact of reproduction on self-maintenance capability, individuals should be compared when they are in a maintenance state.

Interestingly, our results show that post-reproductive females display both a higher state 3 respiration and higher oxidative damage than non-reproductive females. This is consistent with previous findings suggesting that oxidative damage may not be associated with reduced longevity or increased rate of senescence (Van Remmen et al 2003; Muller et al 2007; Kujoth et al 2005). While many studies show the effects of oxidative damage on increased incidence of disease and cancer (Van Remmen et al 2003) there is not evidence that it increases senescence. Kujoth et al (2005) found that mice deficient in the proofreading mitochondrial DNA polymerase γ (POLG) with accumulate mtDNA mutations not associated with increased levels of oxidative damage. While this did induce more rapid apoptosis and increase aging compared to control mice, it was unrelated to the levels of oxidative damage found in the tissue (Kujoth et al 2005), much like our results suggest.

With our unique experimental design, comparing non-reproductive and experienced breeders in a post-reproductive state, I determined that reproduction does not adversely impact the body's ability to maintain itself overtime, as is suggested by DST. Our results call into question the true nature of the tradeoff between reproduction and longevity, as well as the potential involvement of mitochondrial function in this process. Further research is certainly required to elucidate this problem.

It should be noted that there are several pitfalls to this study that could benefit from further research. As this was an opportunistic study, I have a small sample size and was limited on measurements of other tissues and a possible wider range of useful analyses. We require a more circumspect understanding of the organism-wide effects of reproduction on maintenance, including analyses of tissues such as skeletal muscle and heart. Furthermore, it is crucial that we understand the redox state of the tissues and the role that oxidative damage plays in the relationship between reproduction and maintenance. Yet, as future investigations in this direction proceed, it is imperative that we reflect on appropriate experimental designs, measuring the entire system of mitochondrial efficiency during appropriate time points and life phases.

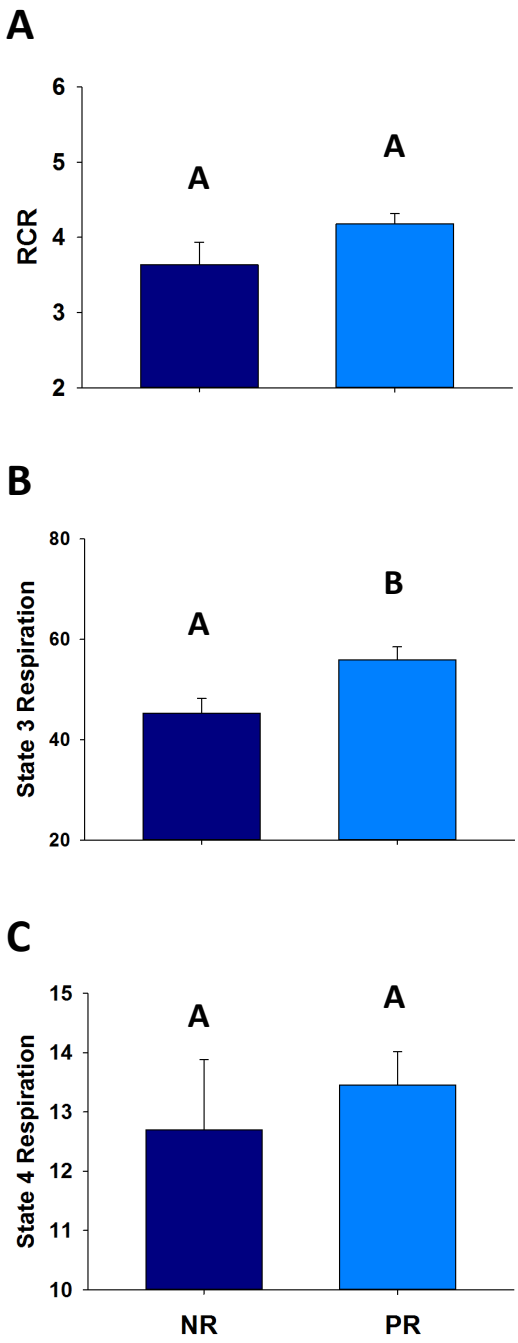

Figure 6. RCR (A), state 3 respiration (B), and state 4 respiration (C) in isolated liver mitochondria of non-reproductive (NR) and post-reproductive (PR) female mice. Bar graphs show means and standard error bars. Letters above bars indicate results of t-test with significant differences represented by different letters. Non-reproductive, n=4; Post-reproductive, n=15. Significance established at $P<0.05$

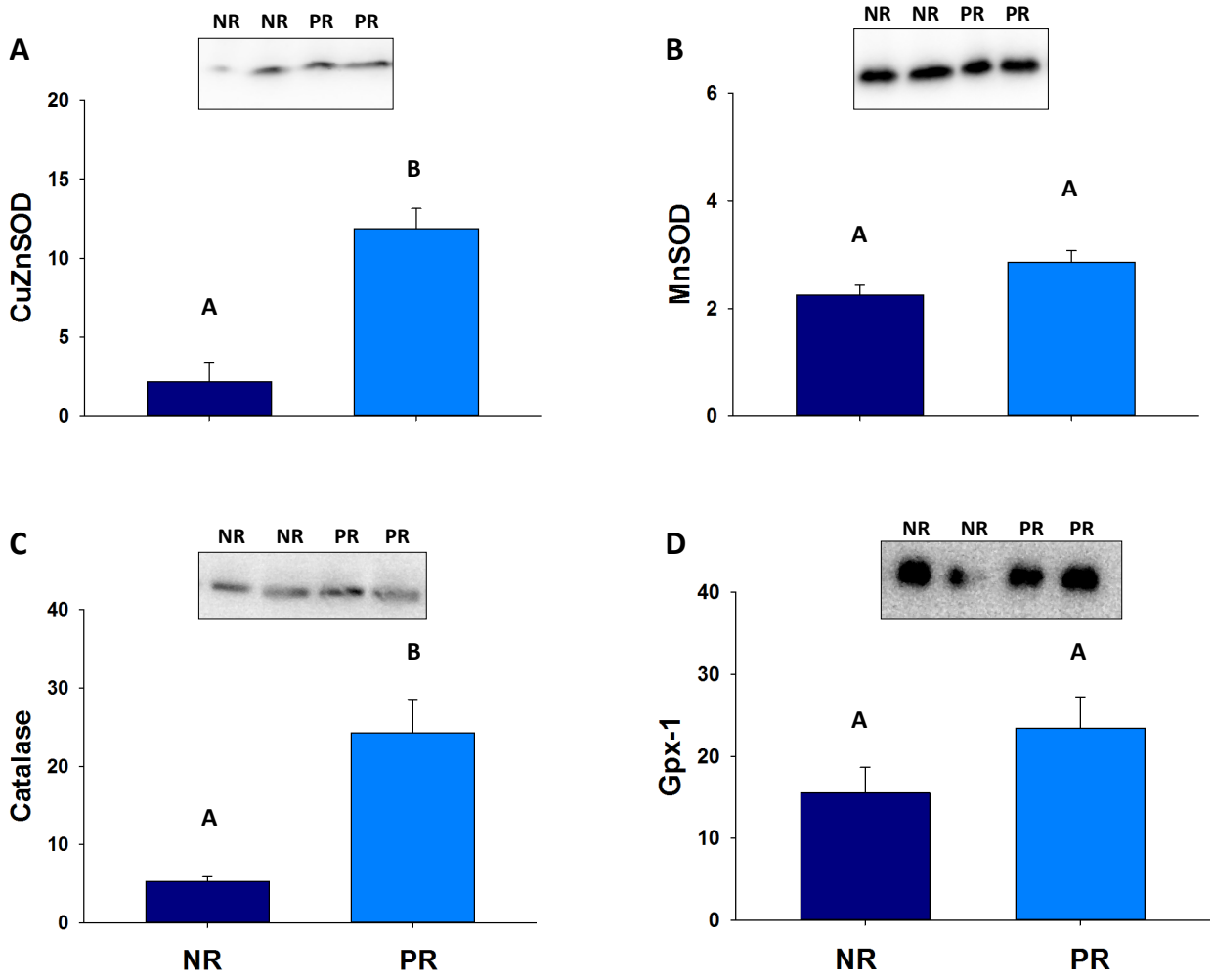

Figure 7. Copper-zinc superoxide dismutase (A), manganese-superoxide dismutase (B), catalase (C), and glutathione peroxidase 1 (D) protein levels in arbitrary units of isolated liver mitochondria of non-reproductive (NR) and post-reproductive (PR) female mice. Bar graphs show means and standard error bars. Letters above bars indicate results of t-test with significant differences represented by different letters. Significance established at $P<0.05$. Representative Western blots for each protein shown above graphs. Non-reproductive, n=4; Post-reproductive, n=15.

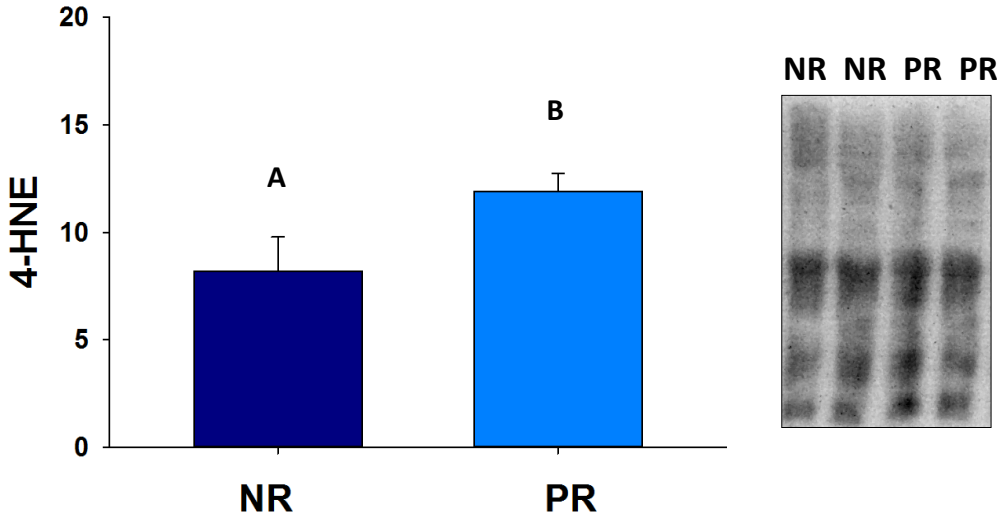

Figure 8. 4-hydroxynonenal (4-HNE) levels in arbitrary units from isolated liver mitochondria of non-reproductive (NR) and post-reproductive (PR) female mice. Bar graph show mean and standard error bars. Letters above bars indicate results of t-test with significant differences represented by different letters. Significance established at $P<0.05$. Representative Western blot shown above graphs. Non-reproductive, n=4; Post-reproductive, n=15.

LITERATURE CITED

Akers,RM. 2002. Lactation and the mammary gland. Iowa State University Press, Ames, Iowa, 2002.

Anantharaman-Barr HG and Decombaz J. 1989. The effect of wheel running and the estrous cycle on energy expenditure in female rats. Physiology & Behavior 46: 259-263.

Bast A, Haenen GR, and Doelman CJA. 1991. Oxidants and antioxidants: State of the art. The American Journal of Medicine 91: S2-S13.

Blount J.D. *et al*. 2015. Oxidative shielding and the cost of reproduction. Biological Reviews.

Bottje W. *et al*. 2002. Association of mitochondrial function with feed efficiency within a single genetic line of male broilers. Poultry Science 81: 546-555.

Bradford M.M. 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Analytical Biochemistry 72: 248-254.

Bradford M. 1976. Differential color change of a dye in response to various concentrations of protein. Analytical Biochemistry 72: 248-253.

Brand M.D. 2000. Uncoupling to survive? The role of mitochondrial inefficiency in ageing. Experimental Gerontology 35: 811-820.

Brand M.D. 2005. The efficiency and plasticity of mitochondrial energy transduction. Biochemical Society Transactions 33: 897-904.

Brand M.D. *et al*. 2004. Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. Free Radical Biology and Medicine 37: 755-767.

Brand M.D. and Esteves T.C. 2005. Physiological functions of the mitochondrial uncoupling proteins UCP2 and UCP3. Cell Metabolism 2: 85-93.

Brand, M.D. and Nicholls D.G. 2011. Assessing mitochondrial dysfunction in cells. Biochemical Journal 435: 297-312.

Bronson F.H. 1989. Mammalian Reproductive Biology. University of Chicago, Chicago.

Burton T. *et al*. 2011. What causes intraspecific variation in resting metabolic rate and what are its ecological consequences? Proceedings of the Royal Society B- Biological Sciences 278: 3465-3473.

Chatwin Al, Linzell JL, and Setchell BP. 1969. Cardiovascular changes during lactation in the rat. J Endocrinol 44: 247-254.

Cripps AW and Williams VJ. 1975. The effect of pregnancy and lactation on food intake, gastrointestinal anatomy and the absorptive capacity of the small intestine in the albino rat. British Journal of Nutrition 33: 17-32.

Cavaggioni A., Mucignat-Caretta C., and Redaelli M. 2008. Mice recognize recent urine scent marks by the molecular composition. Chemical Senses 33: 655-663.

Einstein, F.H. et al. 2008. Accretion of visceral fat and hepatic insulin resistance in pregnant rats. American Journal of Physiology-Endocrinology and Metabolism 294: E451-E455.

Erlanson-Albertsson C. 2003. The role of uncoupling proteins in the regulation of metabolism. Acta Physiol Scand. 178: 405-412.

Estabrook R.W. 1967. in *Methods in enzymology,* Ronald, M.E.P. and Estabrook, W., Eds. Academic Press. 10: 41-47.

Eya J.C. *et al*. 2012. Genetic variation in feed consumption, growth, nutrient utilization efficiency and mitochondrial function within a farmed population of channel catfish (Ictalurus punctatus). Comparative Biochemistry and Physiology Biochemistry & Molecular Biology 163: 211-220.

Fisher, R.A. 1930. *The Genetical Theory of Natural Selection*, Clarendon Press, Oxford.

Flatt, T. and Heyland, A. 2001. *Mechanisms of Life History Evolution, The Genetics and Physiology of Life History Traits and Trade-offs*. Oxford University Press, Oxford.

Fritz IB. 1961. Factors influencing the rates of long-chain fatty acid oxidation and synthesis in mammalian systems. Physiological Reviews 41: 52-129.

Galloway CA and Yoon Y. 2012. What comes first, misshape or dysfunction? The view from metabolic excess. J Gen Physiol 139: 455-463.

Garratt, M. *et al*. 2011. Is oxidative stress a physiological cost of reproduction? An experimental test in house mice. Proceedings of the Royal Society B-Biological Sciences 278: 1098-1106.

Garratt M. *et al*. 2013. Physiological adaptations to reproduction. I. Experimentally increasing litter size enhances aspects of antioxidant defence but does not cause oxidative damage in mice. The Journal of Experimental Biology 216: 2879-2888.

Gutgesell, A. et al. 2009. Downregulation of peroxisome proliferator-activated receptor alpha and its coactivators in liver and skeletal muscle mediates the metabolic adaptations during lactation in mice. Journal of Molecular Endocrinology 43: 241-250.

de Haan, J. et al. 1996. Elevation in the ration of Cu/Zn-superoxide dismutase to glutathione peroxidase activity indices features of cellular senescence and this effect is mediated by hydrogen peroxide. Human Molecular Genetics 5: 283-292.

Halliwell B. 1994. Free radicals and antioxidants: a personal view. Nutrition Reviews 52: 253-265.

Hamosh, M., T.R. Clary, S.S. Chernick and R.O. Scow. 1970. Lipoprotein lipase activity of adipose and mammary tissue and plasma triglyceride in pregnant and lactating rats. Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism 210: 473-482.

Harman, D. 1956. Aging: a theory based on free radical and radiation chemistry. Journal of Gerontology 11: 298-300.

Hickling GJ, Millar JS, and Moses RA. 1991. reproduction and nutrient reserves of bushy-tailed wood rats (Neotoma cinerea). Canadian Journal of Zoology 69: 3088-3092.

Hollister A et al. 1987. Reproduction in mice: Liver enlargement in mice during pregnancy and lactation. Life Sciences 40: 11-18.

Jastroch M. *et al*. 2010, in Essays in Biochemistry: Mitochondrial Function. Brown G.C., Murphy M.P., Eds. 47: 53-67.

Jéquier E, Acheson K, and Schutz Y. 1987. Assessment of energy expenditure and fuel utilization in man. Ann Rev Nutr 7: 187-208.

Jones, R.G., V. Ilic and D.H. Williamson. 1984. Physiological significance of altered insulin metabolism in the conscious rat during lactation. Biochem. J 220: 455-460.

Kennedy GC, Pearce WM, and Parrott DM. 1958. Liver growth in the lactating rat. J Endocrinology 17: 158-160.

Kirkwood, T.B.L. 1977. Evolution of Ageing. Nature 270: 301-304.

Kirkwood, T.B.L. and Holliday, R. 1979. The Evolution of Ageing and Longevity. Proceedings of the Royal Society B 205 (1161).

Kleiber, M. 1975. The Fire of Life: An Introduction to Animal Energetics. Krieger Pub Co.

Klein, J. 1975. Natural History of the Mouse Histocompatibility-2 complex. Springer-Verlag, New York.

Kujoth GC et al. 2005. Mitochondrial DNA mutations, oxidative stress and apoptosis in mammalian aging. Science 309: 481.

Lane, N. 2011. The Costs of Breathing. Science 334: 184-185.

Lipsky NG and Pedersen PI. 1981. Mitochondrial turnover in animal cells: half-lives of mitochondria and mitochondrial subfractions of rat liver based on [^{14}C]Bicarbonate incorporation. The Journal of Biological Chemistry 256: 8652-8657.

Lobachev A. N. 1978. Role of mitochondrail processes in the development of aging of organism. Aging and cancer. Chemical Abstracts 91: 48.

Loeb LA, Wallace DC, and Martin GM. 2005. The mitochondrial theory of aging and its relationship to reactive oxygen species damage and somatic tDNA mutations. PNAS 102: 18769-18770.

Makinen M.W. and Lee, C.P. 1968. Biochemical studies of skeletal muscle mitochondria: I. Microanalysis of cytochrome content, oxidative and phosphorylative activities of mammalian skeletal muscle mitochondria. Arch Biochem Biophys 126: 75-82.

Maloney C.A. *et al*. 2013. A methyl-deficient diet fed to rats during pre- and peri-conceptions periods of development modifies the hepatic proteome in the adult offspring. Genes and Nutrition 8: 181-190.

Marcuello A. *et al*. 2009. Human mitochondrial variants influence on oxygen consumption. Mitochondrion 9: 27-30.

Meagher, S., Penn, D.J. and Potts, W.K. 2000. Male-male competition magnifies inbreeding depression in wild house mice. Proceedings of the National Academy of Sciences of the United States of America 97: 3324-3329.

Meagher, S. and Potts, W.K. 1997. A microsatellite-based MHC genotyping system for house mice (Mus domesticus). Hereditas 127: 75-82.

Messer, M. R. and Jackman, W. T. Willis. 2004. Pyruvate and citric acid cycle carbon requirements in isolated skeletal muscle mitochondria. American Journal of Physiology. Cell Physiology 286: C565-572.

Miquel J. *et al*. 1980. Mitochondrial role in cell aging. Experimental Gerontology 15: 575–591.

Metcalfe, N.B. and Alonso-Alvarez, C. 2010. Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. Functional Ecology 24: 984-996.

Monaghan, P., Metcalfe, N.B. and Torres, R. 2009. Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. Ecology Letters 12, 75-92.

Muller FL et al. 2007. Trends in oxidative aging theories. Free Radical Biology & Medicine 43: 477-503.

Okamoto K and Kondo-Okamoto N. 2012. Mitochondria and autophagy: Critical interplay between two homeostats. Biochimicha et Biophysica Acta 1820: 595-600.

Oldakowski L. *et al*. 2012. Is reproduction costly? No increase of oxidative damage in breeding bank voles. The Journal of Experimental Biology 215: 1799-1805.

Parker GC et al. 2001. Whole-body metabolism varies across the estrous cycle in Sprague-Dawley rats. Physiology & Behavior 74: 399-403.

Pichaud, N., *et al*. 2013. Physiological adaptations to reproduction. II. Mitochondrial adjustments in livers of lactating mice. Journal of Experimental Biology 216: 2889-2895 (2013).

Poppitt SD, Speakman JR, and Racey PA. 1993. The energetics of reproduction in the common shrew (Sorex araneus): A comparison of indirect calorimetry and the doubly labelled water method. Physiological Zoology 66: 964-982.

Promislow, D.E.L. and Harvey, P.H. 1990. Living fast and dying young: A comparative analysis of life history variation among mammals. Journal of Zoology 220:417-437.

R Core Team (2012). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/

Ricquier, D. and Bouillard, F. 2000. Mitochondrial uncoupling proteins: from mitochondria to the regulation of energy balance. Journal of Physiology 529: 3-10.

Rolfe, D.F.S. and Brown, G.C. 1997. Cellular energy utilization and molecular origin of standard metabolic rate in mammals. Physiological Reviews 77: 731-758.

Roff, D.A. 1992. *Evolution of Life Histories: Theory and Analysis.* Routledge, Chapman & Hall, London.

Ross RE. 2000. Age-specific decrease in aerobic efficiency associated with increase in oxygen free radical production in Drosophila melanogaster. Journal of Insect physiology 46: 1477-1480.

Ruff, J.S., *et al*. 2013. Human-relevant levels of added sugar consumption increase female mortality and lower male fitness in mice. Nature Communications 4.

Seo AY et al. 2010. New insights into the role of mitochondria in aging: mitochondrial dynamics and more. Journal of Cell Science 123: 2533-2542.

Sirman AE. 2014. The developmental environment and metabolism in the house mouse (*Mus musculus*). Auburn University.

Speakman, J.R. 2008. The physiological costs of reproduction in small mammals. Philosophical Transactions of The Royal Society B 363: 375-398.

Speakman, J.R., and Garratt, M. 2014. Oxidative stress as a cost of reproduction: Beyond the simplistic trade-off model. Bioessays 36: 93-106.

Speakmam, J.R., *et al*. 2004. Uncoupled and Surviving: individual mice with high metabolism have greater mitochondrial uncoupling and live longer. Aging Cell 3: 87-95.

Smith NE and Baldwin RL. 1974. Effects of breed, pregnancy, and lactation on weight of organs and tissues in dairy cattle. Journal of Dairy Science 57: 1055-1060.

Stearns, S.C. 1992. *The Evolution of Life Histories*. Oxford University Press, Oxford.

Van Remmen H et al. 2003. Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging. Physiological Genomics 16: 29-37.

Wanders, R.J., *et al*. 1984. Factors determining the relative contribution of the adenine-nucleotide translocator and the ADP-regenerating system to the control of oxidative phosphorylation in isolated rat-liver mitochondria. European journal of biochemistry / FEBS 142:417 424.

Westbrook R et al. 2009. Alterations in oxygen consumption, respiratory quotient, and heat production in long-lived GHRKO and Ames Dwarf mice, and short-lived bGH transgenic mice. J Gerontol A Biol Sci Med Sci 64A: 443-451.

Westermann B. 2010. Mitochondrial fusion and fission in cell life and death. Nature Reviews 11: 872-884.

Williams, G. C. 1957. Pleiotropy, natural selection, and the evolution of senescence. Evolution 11:398-411.

Zera A.J. and Harshman, L.G. 2001. The Physiology of Life History Trade-Offs in Animals. Annual Review of Ecological Systems 32: 95-126.