The Mechanistic Basis for Improved Metabolic Health in Females Following Lactation

by

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A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
August 5, 2017

Keywords: Lactation, breastfeeding, mitochondria, metabolism, oxidative stress

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ABSTRACT

Human epidemiological data show that breastfeeding reduces the mother's prevalence of many disease conditions such as obesity, type II diabetes, and hypertension compared to mothers that give birth but do not breastfeed. The current research project seeks to delineate the changes in animals at different reproductive stages, and how these may confer health benefits to mothers that lactate. Markers of metabolism, mitochondrial function, and oxidative stress were measured in skeletal muscle, liver, and white adipose tissue in three experiments. In experiment 1, Sprague-Dawley rats were randomly assigned to one of four treatment groups (n = 8 per group): 1) non-pregnant animals (NP), 2) animals sacrificed at day 20 of gestation (P), 3) animals that gave birth, but did not lactate their pups (NL), and 4) animals that gave birth, and lactated their pups for 14 days (L). In experiment 2, rats were divided into three groups and were sacrificed at a time that corresponded to 28 days following parturition (n = 8 per group): 1) non-reproductive (NR), 2) those that were gave birth, but were not allowed to suckle their pups (P), and 3) those that gave birth, and suckled their young for 21 days (L). In experiment 3, rats were divided into three groups and sacrificed at a time that corresponded to 15 weeks after parturition (n = 8 per group): 1) rats that did not reproduce (NR), 2) rats that gave birth, but did not suckle their pups (NL), and 3) rats that give birth, and suckled their pups for 21 days before weaning (L). I found that metabolism and mitochondrial function is modulated in tissues in response to

pregnancy and lactation and that lactation improves glucose metabolism, increases peroxisome proliferator activated receptor delta (PPARδ) levels, and improves mitochondrial function in all experiments. I also found that animals that do not lactate may have a propensity for fat storage that could increase the risk of disease. These animal findings support human epidemiological data and provide systemic and tissue specific evidence of the maternal health benefits conferred via breastfeeding.

ACKNOWLEDGMENTS

This study was funded by a grant from the National Institutes of Health (Eunice Kennedy Shriver National Institute of Child Health & Human Development, RO3HD083654). Further support was provided through Auburn's University Summer Cell & Molecular Biosciences Graduate Research Fellowship. I especially thank my graduate advisor and committee chair, Dr. Andreas Kavazis for his mentorship and guidance. Drs. Wendy Hood and Andreas Kavazis were critical in the formation of the experimental protocol and formation of this idea. I also thank my co-author Yufeng Zhang for his assistance in data collection, as well as the Hood lab undergraduates that helped to monitor the rats and helped with dissections and tissue collection. Drs. Bruce Gladden Michael Roberts, and John Quindry also provided many helpful lessons and mentorship that were important in my ability to accomplish this work as well as my professional development.

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I. INTRODUCTION

A large body of evidence exists demonstrating that acute metabolic challenges often result in a hormetic response. Exercise, fasting, and caloric restriction are all examples of metabolic perturbations that induce physiological adaptations capable of promoting a healthy phenotype that is protective against various health disparities such as type II diabetes, obesity, various types of cancer, and even aging (4, 15, 24, 30, 31, 39, 61, 75). However, the metabolic toll of lactation is often an overlooked event in research populations and has not been observed to the same extent as the aforementioned metabolic challenges. Considering that breast-feeding can serve as one of the most metabolic demanding events in a female's lifetime, it would be logical that health benefits may also be associated with this event. Indeed, epidemiological studies in humans suggest that lactation is capable of benefitting the mother by reducing the risk of obesity, type II diabetes, hypertension, and several types of cancer (48, 56, 69-71). However, why these health benefits may accrue to lactation is largely unknown.

During reproduction, a female's metabolism goes through two dramatic changes. With the onset of pregnancy, a female's metabolic processes are primed to support glucose and amino acid transport to the fetus. Glucose transport is facilitated by an increase in maternal insulin production and as a result, maternal cells often display increased resistance to insulin. This change could also be associated with increases in

circulating fatty acids and increased visceral adiposity (18). In contrast, during lactation there is a drop in insulin secretion which is associated with a drop in β-cell proliferation, improved insulin sensitivity, and a shift in lipoprotein lipase and triacylglyceride levels that facilitate the mobilization of lipid precursors to the mammary gland for milk synthesis (23, 28). Based on current epidemiological data in humans and the limited physiological observations in animal models, Stuebe and Rich-Edwards proposed the reset hypothesis which posits that lactation plays a central role in mobilizing fat stores and resetting the risk of metabolic disease (71). Implicit to this theory is that females that lactate for an extended period experience a change in physiology that is sustained after lactation has ceased. However mechanistic research on the alterations that occur or are sustained following lactation are lacking.

The demands of white adipose tissue (WAT), liver, and skeletal muscle combined account for more than 50% of a non-reproductive adult's metabolic rate and also commonly display dysregulation in obese and T2DM individuals (36, 40, 60, 79). As such, physiological alterations regulating the metabolic handling in these tissues serve to conserve and provide metabolites for milk synthesis, but may also help explain the protective effects seen due to lactation. Gutgesell and collaborators showed that genes important in oxidative and lipid metabolism (i.e. PPAR-α, PGC-1α, and PGC-1β) displayed reduced mRNA expression in liver and skeletal muscle two weeks following the cessation of lactation compared to animals that gave birth but did not breast feed their young (22). Additionally, effects of lactation have been observed to also affect mitochondrial function in that mitochondrial coupling has been reported to be increased during peak lactation (52). Mediation of oxidative metabolism and thus mitochondrial

function also may have important health implications on states of oxidative stress. As such, the current research project seeks to delineate the potential effects that lactation has on markers of metabolism, mitochondrial functioning, and oxidative stress that may underlie protective health benefits conferred by lactation. Herein, this review will discuss the responses of metabolism, mitochondrial functioning, and oxidative stress in pregnancy and lactation.

II. REVIEW OF LITERATURE

EPIDEMIOLOGICAL EVIDENCE

Observational studies on large populations of mothers have provided evidence to suggest that long-term health benefits may be acquired through breast-feeding their young. A cross-sectional study based in Norway showed that women who did not breastfeed their young had an almost twofold higher risk of hypertension, more than threefold risk of obesity, and a fivefold increase of risk for diabetes compared to mothers who had breast-fed for longer than a cumulative 2 years (48). Furthermore, the duration of lactation has been shown to be associated with decreased incidence of the metabolic syndrome (56). A meta-analysis including data from 47 studies also suggested that protective effects of lactation reduced the risk of breast cancer in mothers who breast-fed their offspring (16). While there are many observational studies supporting the idea a protective effect due to lactation, there are potential criticisms that may offset the weight of the findings. There are often relationships to length of lactation with educational and smoking status that may complicate interpretation of findings. Also, other studies have seen null results, such as a recent study that observed no differences in maternal weight change over a three year period between mothers who formula-fed vs those who breast-fed their children (44). Nonetheless, a physiological mechanistic approach is necessary to better understand if and to what extent health benefits may be conferred to mothers by lactation.

METABOLISM

Lactation is the most demanding energetic event in a female's lifetime, and may provide a protective phenotype against disease due to the resulting metabolic alterations. While comparative studies are often difficult to manage, the rat model is a useful tool to observe biological occurrences that are relatable to humans, despite humans and rat species diverging 80 million years ago. Human mothers are also not ideal candidates for participation in basic research studies due to stress or time consumptions that may be required on top of the newly added responsibilities of childcare. Thus, many mechanisms involved in metabolic shifts due to pregnancy and lactation have currently been studied in animal models. While rats and humans share many similar biological processes, they do not share a similar timeline for breastfeeding their young and biological differences exist between the two species that must be considered. For instance, the energetic demand imposed by lactation differs rather drastically when normalized to body weight. For instance, relative to body weight the energy density of milk is much higher in the rat ~1000 kj/kg compared to 100 kj/kg in humans (55). However, it has been estimated that a human mother's metabolic expenditure can be increased by about 480 kcal/d due to lactation(12). This energetic demand is supported in humans by increasing food intake by 25% in order to meet the costs of lactation, while a rat with 8 pups can increase food intake by 300% (55). However the physiological toll imposed by lactation may be normalized, to some extent, due to total time of lactation being significantly longer in humans compared to rats. Human mothers are typically advised to breastfeed for at least 6 months compared to the three week

time period observed in rats. Give these observations, rat models are still an ideal tool for studying the physiological events that occur in pregnancy and lactation.

Maternal metabolic rate and food intake increases throughout pregnancy and even further during lactation. A large contribution to the increase in metabolic rate is the elevated demand of liver and other metabolically active tissues that are needed to support the energy demand induced by the growing offspring (66). Immediately after weaning, the differences in body composition and metabolism that is experienced across females is likely due to the propensity for food intake to match body composition. However when observed 21 days after weaning, animals that become pregnant but did not suckle their young (P) and animals that did not give birth (NR) have similar food intakes, yet P rats have higher total body fat than animals that gave birth and suckled their young (PL) and NR rats. The differences seen in body fat between P and PL rats after weaning is suggestive of a limited reversal of the cellular response to preferentially store fat during pregnancy.

During reproduction, a female's metabolism goes through two dramatic changes. Body fat accumulation occurs during early pregnancy allowing increased energy stores in order to support the growing offspring. This process is believed to occur primarily through hyperphagia and increases in lipogenesis (43, 46). With the onset of pregnancy, a female's metabolic processes are primed to support glucose and amino acid transport to the fetus. Glucose transport is facilitated by an increase in maternal insulin production and as a result, maternal cells often display increased resistance to insulin. This change could also be associated with increases in circulating fatty acids and increased visceral adiposity (18). In contrast, during lactation there is a decrease in

insulin secretion which is associated with a decline in β-cell proliferation, improved insulin sensitivity (28) and a shift in lipoprotein lipase and triacylglyceride levels that facilitate the mobilization of lipid precursors to the mammary gland for milk synthesis (23). For example at weaning, lactating mothers (rats) have smaller adipocytes and lower lipoprotein lipase than non-lactating controls (68). Three-weeks following the termination of lactation, rats that have undergone lactation (PL) experience reduced absolute fat mass associated with fewer visceral adipocytes relative to females that produced young but did not suckle them (P) and non-reproductive controls (NR) (41-43). Zhong and collaborators (84) showed that P female rats had higher body fat, higher fasting glucose, and higher rates of spontaneous abortion than PL and NR females following three reproductive bouts followed by a 12-week non-reproductive period. These findings are consistent with some but not all studies that examine the impacts of reproduction on adiposity with and without lactation in women (71). Inconsistencies are likely due to dramatic variation in the sample size between studies, the confounding effects of dieting and formula feeding, and differences in how each study dealt with variance in the duration that their subjects lactated (71). Despite complications in quantifying weight gain, women who breastfed had a reduced risk of metabolic syndrome and reduced hyperlipidemia (56, 71). However, little research has been conducted on PPARs role in lactation.

Studies evaluating cellular metabolism during reproduction have highlighted some key metabolic markers involved in the observed fat loss seen in lactating animals. Gutgesell and collaborators (22) showed that PPAR-α, PGC-1α, and PGC-1β were down-regulated in skeletal muscle at week 2 of lactation in PL compared to P mice.

Peroxisome proliferator activated receptors (PPARs) are a family of nuclear receptors that function as important regulators in cellular metabolism. PPARs modulate metabolism by acting as transcription factors regulating transcription of genes involved in the delivery of lipids and carbohydrates to the tricarboxylic acid cycle (TCA). The PPAR family has tissue specific functions, such that PPARδ upregulates lipid oxidation in skeletal muscle and enhances insulin sensitivity in the liver (33). PPARα is associated with the upregulation of genes involved in lipid oxidation (34), and is prominent in cell types that have active fatty acid oxidation such as in the liver. PPARγ and PPARδ interact in white adipose tissue to shift between cellular oxidization and storage of lipids in adipocytes (59). Additionally, PGC-1α and PGC-1β are critical transcription co-activators that are involved in cellular energy metabolism and the regulation of mitochondrial biogenesis (62).

More than 50% of the metabolic rate in non-reproductive adults is attributed to the energy demands needed to sustain liver, skeletal muscle, and white adipose tissue function (60). However physiological occurrences that drastically alter the metabolic needs of an organism, such as pregnancy and lactation, call for the ability to alter the metabolic load of these tissues. During lactation it is critical for the mother to be able to redirect energy toward her offspring. Thus, regulation of PPARs in white adipose tissue, skeletal muscle and liver likely serves as an important tool to conserve energy during lactation and to spare metabolic substrates needed for milk synthesis by reducing the amount of fatty acid utilization and thermogenesis in the respective tissues.

The importance of PPARα regulation for lactation was demonstrated by feeding rats an oxidized fat, a potent activator of PPARα in the liver (14), that increased fatty

acid uptake in the liver but decreased fatty acid uptake in the mammary gland (57, 58). Thus, PPARα activation during lactation would act to decrease the pool of fatty acids available to the mammary gland, reduce weight gains of suckling young by decreasing the lipid content and/or volume of milk produced by mothers. While this exemplifies the important role PPARs play in the mother's success of providing for her offspring, it also implicates the underlying role that PPARs have in respect to long-term regulation of the mother's metabolism. For instance, women who breastfeed their children are at lower risk for T2DM (72). Interestingly, a commonly prescribed insulin sensitizing drug used to treat T2DM, thiazolidinediones, targets a member of the PPAR family as a PPARγ agonists. Perhaps then, some of the metabolic abnormalities in women who are unable to breastfeed can be explained by a lack of PPAR down-regulation after birth.

Many of the systemic changes to metabolism that occur during pregnancy are mediated by hormones such as prolactin, growth hormone, and insulin. While insulin and growth hormone levels are elevated during pregnancy, insulin levels decrease post-partum and plasma growth hormone levels return to normal levels after delivery (19, 28). In contrast, serum prolactin levels remain elevated throughout lactation as long as suckling is maintained, but fall to levels seen during pregnancy within three hours of pup removal (1). While prolactin is traditionally considered to be primarily involved in the processes of mammary development during reproduction (i.e., mammogenesis, galactopoiesis, lactogenesis, and involution), numerous findings have demonstrated that prolactin also acts as a cytokine involved in metabolic processes.

Prolactin is implicated in several roles of metabolism for lipid and carbohydrate fuel sources. Specifically, prolactin has been shown to affect lipid utilization by

imposing inhibitory effects on lipoprotein lipase activity in adipose tissue, allowing for circulating lipid sources to be spared for milk synthesis (35). Additionally, prolactin is active in carbohydrate metabolism by improving insulin sensitivity, by interacting with pancreatic β-cells, and by stimulating the glucose sensing enzymes glucokinase and GLUT-2 (38, 64, 78). Prolactin is proposed to accomplish the effects on β-cell's activation through activation of Janus kinase-signal transducer and activator of transcription signaling (JAK/STAT) (9).The signaling cascade driven by prolactin's cytokine activity has the potential to exert systemic metabolic alterations in liver, white adipose tissue, and skeletal muscle that have yet to be discovered.

Due to the PPARs important role in metabolism, there are many pathways capable of modulating the activity and expression of these nuclear receptors. As such, the regulation of PPAR proteins during pregnancy and lactation are likely candidates as means of altering metabolic balance in support of milk synthesis. This is supported by findings demonstrating that PPARα is down-regulated in skeletal muscle after lactation (22). Importantly, signaling pathways that have been independently shown to regulate PPAR proteins, outside of the subject of lactation, are also activated by prolactin. Specifically, PPARα activity has been shown to be decreased by activation of the JAK2/STAT5b pathway in cell culture, and PPARγ is down regulated in adipocytes by mitogen activated protein kinase (MAPK)- mediated phosphorylation (83, 85). However despite prolactin cytokine actions and the ability to activate both of these pathways, very few studies have observed the role of prolactin on PPAR expression on liver, skeletal muscle, and white adipose tissue.

The first step of the signaling cascade brought on by prolactin occurs via its binding to the prolactin receptor (PRLR). PRLR has several different isoforms and exists differentially among all tissue types, contributing to the tissue-specific effects of prolactin (7, 47, 65). In a study quantifying the short and long isoforms of the prolactin receptor during pregnancy, liver was shown to have higher prolactin receptor mRNA expression than all tissues except mammary, while muscle was shown to display low levels of expression (25). However, it is important to note that skeletal muscle prolactin receptor measurements were taken during pregnancy and not during lactation. Because prolactin is believed to induce prolactin receptor expression (53), it is possible that despite a relative lower expression of PRLR in skeletal muscle, prolactin receptors could be increased during lactation in skeletal muscle in order for its effects to be propagated. Furthermore, studies utilizing knockout models of PRLR have demonstrated the effects on metabolism when the capacity for activating these pathways is removed. PRLR knockout mice were observed to have significantly less fat than wild type control animals after 16 weeks of age, furthermore this finding was more prominent in female knockout mice than in males (34% less in males and 49% less in females) (21). This evidence is not only indicative of the importance of the metabolic role of PRLR in females, but also could represent the inability to negatively regulate the metabolic demand in tissues such as the liver, skeletal muscle, and white adipose tissue. This hypothesis is further supported by a study showing that prolactin receptor knockout mice were resistant to high-fat-diet induced obesity. This was explained, at least in part, by an association with increased thermogenesis and oxygen consumption (2).

Once prolactin has been bound to the receptor, PRLR elicits a signaling cascade via activation of kinases associated to the receptor. The most prominent pathways activated by the PRLR are the JAK/STAT pathway, RAS-RAF-MAPK pathway, and Src tyrosine kinases (6). The JAK family consists of four tyrosine kinases: JAK1, JAK2, JAK3, and Tyk2. Prolactin signaling via PRLR predominantly activates the JAK2 member of the Janus kinase family, and is constitutively associated with PRLR (32). Once JAK2 is activated and has phosphorylated the PRLR tryosine, STAT associates with the complex and is consequently phosphorylated. The phosphorylation of STAT results in its dissociation and leads to its translocation to the nucleus where it activates promoters of the target genes. Prolactin's activation of STATs is of interest due to evidence that STAT5b, a member of the STAT family, has been shown to mediate the down regulation of PPARα activity in cell culture (85). Observing whether prolactin is capable of activating the JAK2/STAT5b pathway *in vivo* could explain the mechanism by which lipid metabolism is physiologically regulated in skeletal muscle and liver in regards to PPARα.

In white adipose tissue, PPARγ and PPARδ interact to alter the balance between lipid oxidation and storage (59). Specifically, PPARγ activation is characterized by fatty acid synthesis while PPARδ is associated with fatty acid oxidative metabolism. Thus, a down-regulation in PPARγ during lactation could be important for lipid avoidance in adipocytes, but PPARγ mRNA levels are unchanged across pregnancy and lactation (29). Importantly, this observation does not consider the post-translational modifications that can alter the function of PPARγ. For instance, MAPK can regulate PPARγ activity by phosphorylating serine 112 of PPARγ (13), such that the phosphorylation status of

serine 112 affects the affinity of PPARγ for its ligand as well as the ability of coactivator recruitment (63). Importantly, prolactin's signaling pathway activates the RAS-RAF-MAPK pathway, which could allow prolactin to mediate PPARγ function post-translationally in white adipose tissue in order to reduce fatty acid synthesis in adipocytes.

MILK SYNTHESIS

Milk synthesis is a critical component for survival of the offspring, and also provides a unique metabolic challenge to the mother. In order for milk production to occur, physiological and biochemical processes must be undergone to prime cellular function of the mammary gland to develop the capacity for milk secretion. There are many endocrine events important in the development of the mammary gland. At the onset of pregnancy, progesterone drives the occurrence of ductal branching that will provide for the development of the alveolar tissue. The importance of progesterone's action on ductal branching has been demonstrated in progesterone receptor knockout mice, in which mice lacking progesterone receptors in the mammary gland were not observed to have ductal proliferation (10, 37). While there are several potential downstream signaling mechanisms that progesterone can activate, one of interest is progesterone function through RANKL (receptor activator of NAFKB1 ligand) (74). One key role of RANKL's signaling pathway occurs in the regulation of osteoclastogenesis and bone-remodeling (81). Thus, it should not be surprising that RANKL also plays a role in mammary tissue development due to the role it has in calcium control in bone. Indeed, mice lacking either RANKL or another member of its superfamily, tumor necrosis factor ligand superfamily member 11 (RANK), were unable to undergo

alveologenesis during pregnancy (20). Prolactin also provides a signal to the mammary gland for alveolgenesis in coordination with progesterone. However, perhaps prolactin serves a larger role in the synthesis and composition of the mother's milk (50, 77).

Prolactin, as well as oxytocin, is secreted in a pulsatile fashion in response to suckling by the young. Prolactin is released by the anterior pituitary, but under normal conditions is inhibited by release of prolactin inhibitory hormone. Milk is composed of proteins, lactose, lipids, water and other ions, immunoglobulins, and leukocytes. The formation of milk proteins and lactose involves the transportation of calcium, phosphate, and citrate into golgi vesicles in cytoplasm. Within these vesicles, the calcium and phosphate combine with caseins to large micelles. Galactosyl transferase acts with a-lactalbumin within the golgi system to synthesize lactose (11). Golgi membrane is impermeable to lactose and water is drawn into the vesicle. The vesicle then fuses with the apical membrane and releases its contents into alveolar lumina. Milk fat contains primarily triglycerides and is synthesized through acyl-Coa:diacylglycerol actyltransferase (DGAT) and acyl-CoA:cholesterol acyltransferase enzymes located at the endoplasmic reticulum (73, 82). The components of milk fat are derived *de novo* from glucose or transferred into the cell from the serum (49). In humans, milk fat provides 50-60% of the caloric value of milk and this is even higher in rats (26, 27).

OXIDATIVE STRESS

The production of reactive oxygen species (ROS) is a naturally occurring phenomenon that can manifest itself through processes of energy production and metabolism; however an imbalance between ROS production and a counteracting antioxidant system can result in oxidative damage (54). More specifically, ROS can

result in the breakage of intact DNA strands, DNA base modification, protein oxidation, and lipid oxidation each having the potential to cause catastrophic cellular malfunctioning (5). The imbalance between ROS and the defending antioxidant pools is known as oxidative stress. Importantly however, cellular mechanisms have evolved capable of responding to ROS as a signaling event that induces cellular adaptations that can benefit parameters of health. Thus, acute doses of ROS exposure, such as during exercise (17), are capable of increasing endogenous antioxidant defense systems. While there are several antioxidant systems that protect against oxidative damage, key enzymes have been identified for ROS detoxification. One of the first lines of defense is through superoxide dismutase, mitochondrial (SOD2). SOD2 acts to detoxify ROS by catalyzing its conversion to hydrogen peroxide and diatomic oxygen. Hydrogen peroxide is then further detoxified to water and oxygen by reacting with either catalase (CAT) or glutathione peroxidase (GPX). Importantly, a large proportion of ROS production occurs as a consequence of oxidative metabolism at the sites of mitochondria. In this manner, better understanding of mitochondrial function serves to better comprehend conditions capable of inducing states of oxidative stress.

MITOCHONDRIA

The mitochondrial reticulum is a specialized organelle that functions to produce a large proportion of the energy needed to sustain cellular function in the majority of eukaryotic cells. When isolated for study, this reticulum presents as multiple vesicles called mitochondria. Mitochondria provide energy through the phosphorylation of adenosine diphosphate (ADP) into adenosine triphosphate. This act is accomplished through a series of electron pumps that harness the energy of electrons by oxidizing the

reduced form of either nicotinamide adenine dinucleotide (NADH) or flavin adenine dinucleotide (FADH). Oxidation of these substrates by mitochondrial complex enzymes helps to form an electrochemical potential across the inner membrane of the mitochondria. Complex I, also known as NADH dehydrogenase, transfers electrons from NADH to coenzyme Q10, located in the inner membrane, and uses this energy to shuttle four protons into the intermembrane space. Complex II, also known as succinate dehydrogenase, reduces a quinone, and transfers two protons into the intermembrane space. Complex III, also known as coenzyme Q: cytochrome C oxidoreductase, oxidizes coenzyme Q, reduces cytochrome c and in turn pumps four more protons across the inner membrane into the intermembrane space. Complex IV, also known as cytochrome c oxidase, oxidizes cytochrome c and in turn converts molecular oxygen into water and sends four more protons to the intermembrane space. Finally, ATP synthase acts to harness the energy of protons moving down the proton electrochemical gradient, created by the mitochondrial complexes, to phosphorylate ADP back to ATP.

Researchers have employed several techniques in order to better understand the functionality and alterations that can occur to mitochondria given a certain stimulus. Utilization of high-resolution respirometry has been utilized as a tool to determine the ability of the mitochondrial machinery to utilize oxygen and thus produce energy. This technique has been accomplished using both permeabilized cell and isolated mitochondria. While respiration in permeabilized cells allow for observation of mitochondrial function *in situ*, these measurements can be complicated due to the presence of ATPases and oxidases. In this regard, isolated mitochondria provided the ability to precisely measure mitochondrial function. In order to observe respiratory

capacities, substrates utilized in the TCA are added to a clark-electrode type chamber containing isolated mitochondria. Respiration with substrate added is termed state II respiration. Addition of ADP begins active respiration, as the mitochondria begin using oxygen to phosphorylate ADP into ATP. This is known as state III respiration. Following phosphorylation of the known amount of ADP added to the chamber, the mitochondria then enter state IV respiration. Diving state III respiration by state IV respiration is used as a proxy for mitochondrial function, as it represents the mitochondria's ability to respond to ADP concentration. In this manner RCR can be increased by a higher state III, representing a better ability to maximally respire, or a decrease in state IV, representing the ability to "turn off" and deal with a high electron potential gradient. Other measurements can also be utilized to determine parameters of mitochondrial function. P/O ratio is the known ratio of ADP added to the chamber divided by the amount of oxygen required for respiration to phosphorylate the ADP. However, it is unknown if this value is of physiological relevance, as the allosteric reactions occurring at ATP synthase is unlikely to be changed, however may represent experimental influence. Nonetheless, P/O is known as coupling. Coupling is the matching of oxygen usage to phosphorylation of ADP. Thus, some researchers employ uncoupling agents to determine the mitochondria's "maximal respiration". Interestingly, protein structures have been observed to employ uncoupling properties in the mitochondria.

Important to the topic of oxidative stress, mitochondria play a prominent role in the formation of ROS. While early reports demonstrated 1-2% of oxygen consumption being converted into ROS (8), it is now estimated that these values are much lower at around 0.15% (67), although still physiologically significant. The main sites of formation

of ROS in the mitochondria are at complex I and complex III (3, 45). Thus, some antioxidant enzymes, such as SOD2, are spatially located near mitochondria to quench ROS before damages occurs. Since PPAR-α and PGC-1α transcription has been reported to be decreased during lactation (22), this could have functional consequences on mitochondrial function due to their regulation of mitochondrial genes. Indeed, skeletal muscle has been demonstrated to have reduced expression of genes relating to the TCA cycle but increases in genes relating to glycolysis observed on the 11th day of lactation (80). Interestingly however, administration of the hormone leptin reversed many of the genes that were observed to be downregulated during lactation, suggesting that leptin may be an important hormone for regulating skeletal muscle metabolism during lactation (80). Conversely in the liver, mice that lactated appeared to have higher levels of mitochondrial density but a lower capacity for mitochondrial oxygen consumption in liver after lactation (52). Another group also observed that lactation increased state II and state IV respiration on day 18 of lactation, indicating a greater degree of uncoupling (76). This is an interesting observation as it has also been observed that uncoupling protein UCP-3 protein, which is activated by PPARa, is suppressed during lactation in mice (51).

AIM AND HYPOTHESES

Reproduction is a necessary event in order to ensure the survival of a species, but requires dramatic physiological alterations in maternal organs in order to support the growing fetuses and offspring. In this regard, lactation may confer protective health benefits against metabolic diseases that mother's may be pre-disposed to due to alterations that occur during pregnancy. However, the mechanistic basis for how

lactation could confer a protective phenotype against disease has not be elucidated. Given the aforementioned literature, we observed alterations in markers of metabolism, mitochondrial function, and oxidative stress at various periods of reproductive effort:

Purpose 1: Examine how markers of metabolism, mitochondrial function, and oxidative stress are altered during pregnancy, lactation, and if lactation were to not take place following parturition.

Hypothesis 1: Pregnancy will induce changes to metabolism, and these changes will again be altered during lactation.

Purpose 2: Examine the underlying events of how lactation may provide a healthy phenotype by observing markers of metabolism, mitochondrial function, and oxidative stress.

Hypothesis 2: Lactation will confer a phenotype protective against metabolic disease, and animals that did not lactate will still have alterations that occurred due to pregnancy.

Purpose 3: Determine what effects persist in alterations of metabolism, mitochondrial function, and oxidative stress after the cessation of lactation and a 12 week recovery period.

Hypothesis 3: Animals that partook in lactation will have lower fat mass and still demonstrate alterations in metabolic, mitochondrial, and oxidative markers that occurred due to lactation.

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III. TO BREASTFEED OR NOT: MITOCHONDRIAL, OXIDATIVE STRESS, AND METABOLIC BENEFITS OF LACTATION

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ABSTRACT

Lactation not only improves the health and development of the suckling young but it also confers lasting benefits to the health of mother including reduced incidence of obesity and type II diabetes. However, mechanistic data on this important women's health issue is lacking. Therefore, the current study seeks to evaluate putative mechanisms responsible for differences in maternal cellular metabolism in Sprague-Dawley rats in four groups (n = 8 per group): 1) non-pregnant rats (NP), 2) rats that were allowed to mate and became pregnant, and were sacrificed at day 20 of gestation (P), 3) rats that were allowed to mate and became pregnant, but were not allowed to suckle their pups (NL), and 4) those that were allowed to mate, became pregnant, and suckled their young for 14 days (L). Our data show that glucose metabolism is increased during lactation, facilitated, in part, by increased liver protein levels of peroxisome proliferator activated receptor delta (PPARδ) and peroxisome proliferator activated receptor gamma, coactivator 1 alpha (PGC-1α). Additionally, pregnancy predisposes WAT accumulation in animals that do not lactate via decreased expression of PPARδ in WAT and skeletal muscle. Reproduction did not result in increased oxidative damage in liver or WAT, however, lack of a lactation stimulus resulted in increased oxidative damage in skeletal muscle. The results of this experiment advance our understanding of how cellular physiology is improved by lactation and provide targets for future studies evaluating interventions for women that are unable to nurse their young for an extended period and women who don't reproduce.

INTRODUCTION

Gestation and lactation impose one of the most energetically demanding event in a female's lifetime, and as such, require physiological alterations in order to allocate adequate energy and nutrient requirements to her growing offspring. During pregnancy, the female's body not only maintains itself, but also supports fetal growth, and begins the preparation for the lactation period. In this regard, glucose transport to the fetus is facilitated (15), in part, by an increase in maternal insulin production, accompanied by a concordant increase in visceral adiposity (25). However, a metabolic shift occurs upon the onset of lactation in order to redirect energy substrates to the mammary gland to be used in milk production. As such, alterations in bioenergetic systems are necessary in order to support the dramatic metabolic alterations occurring between pregnancy and lactation.

Mitochondria meet the majority of energetic demands of cellular functions, as well as being implicated in a regulatory role in cellular metabolism (5, 30). Thus, modulation of mitochondrial function could serve as an important target for adjusting metabolic processes in energetically demanding tissues during reproduction. Indeed, markers of mitochondrial biogenesis and lipid metabolism (i.e. peroxisome proliferator activated receptor alpha [PPAR-α], peroxisome proliferator activated receptor gamma coactivator 1 alpha [PGC-1α], and peroxisome proliferator activated receptor gamma coactivator 1 beta [PGC-1β]) have previously been demonstrated to be down-regulated during peak lactation in skeletal muscle and liver in mice (12). Furthermore, the mitochondrial uncoupling protein UCP3 has been observed to be decreased in skeletal muscle during lactation (32, 45). Importantly, these cellular adaptations support the idea

that energy resources are allocated away from tissues less important in producing energy for the offspring toward those that are (40). In this regard, a reallocation of energy resources has been purported to indicate that reproductive effort may underlie a trade-off with longevity due to a reduction of resources being utilized for tissue maintenance in exchange for the production of healthy offspring (41, 46). As such, alterations in energetic demand and a consequential adjustment to mitochondrial function would likely impact degrees of oxidative stress during reproductive periods.

The production of reactive oxygen species (ROS) is a naturally occurring phenomenon that can manifest during processes of energy production in the mitochondria; however an imbalance between ROS production and a counteracting antioxidant system can result in oxidative damage (33). The balance between antioxidants and oxidants is often referred to as oxidative stress and has been purported as a cause in senescence and aging (14). In this regard, it has been proposed that reproduction would result in increased ROS production, due to increased metabolism, thus accelerating the process of aging and reducing longevity (8, 9, 24). Furthermore, this thought process has been used to support the disposable soma theory of aging, which posits that a trade-off exists between reproduction and longevity (19). Therefore, this study investigated the changes in metabolism, mitochondrial function, and oxidative stress that occur in the transition between pregnancy and peak lactation (i.e., day 14 post-parturition), and how these parameters are affected should lactation not take place following parturition.

METHODS

Animal husbandry.

All experimental procedures were approved by Auburn University's Institutional Animal Care and Use Committee. Ten-week old Sprague-Dawley rats were obtained from Harlan Laboratories, Inc. Animals were acclimated with their diet and facility ten days prior to experimental start. Rats were housed under standard laborartory conditions (46 x 25 x 20 cm boxes, 12L:12D cycle, 22˚C, 50% RH), and given *ad libitum* access to food (Tekland Global Diet 2018) and water. Animals (n = 8 per group) were randomly assigned to one of four treatment groups: 1) non-pregnant rats (NP), 2) rats that were allowed to mate and became pregnant, and were sacrificed at day 20 of gestation (P), 3) rats that were allowed to mate and became pregnant, but were not allowed to suckle their pups (NL), and 4) those that were allowed to mate, became pregnant, and suckled their young for 14 days (L). Female rats were paired with same-group counterpart, but animals in groups that underwent mating were separated during pregnancy and/or lactation. The NL animals had their pups removed within 12 hours of birth. The L animals had their litter size adjusted to eight on the day of parturition. NP animals were age-matched and sacrificed at a time that corresponded to P animals 20th day of pregnancy. NL and L animals were age-matched and sacrificed at a time that corresponded to the 14 days of lactation in PL animals.

Blood collection and analysis.

Rats were fasted four hours prior to blood collection. Animals were anesthetized using isoflurane vapors and body mass was quickly recorded. The anesthetized animals were than decapitated, and blood was collected, allowed to clot, and then centrifuged.

Following centrifugation the serum was frozen at -80ºC for subsequent analyses. Serum glucose (STA-680, Cell Biolabs, San Diego, CA, USA) and non-esterified fatty acids (NEFA) (STA-618, Cell Biolabs) were quantified using the manufacturer's specifications.

Tissue collection and handling.

After the decapitation, the following tissues were excised and weighed: liver, triceps surae ('calf' muscle), retroperitoneal white adipose tissue (RetroP WAT) and perirenal white adipose tissue (PR WAT) pads. After the mass of each tissue was recorded, a sample from calf skeletal muscle and liver was used for mitochondrial isolation and the remainder of tissues were frozen in liquid nitrogen and stored at -80ºC for subsequent analyses.

Mitochondrial isolation.

Mitochondrial isolations for muscle were performed as previously described (17). Excised muscles (~750 mg) were trimmed to remove fat and connective tissues, weighed, and placed in 10 volumes of solution I (100 mM KCl, 40 mM Tris HCl, 10 mM Tris base, 1 mM $MgSO_4$, 0.1 mM EDTA, 0.2 mM ATP, and 2% (wt/vol) free fatty acid bovine serum albumin (BSA), pH 7.40). Muscles were minced with scissors and the mince was homogenized for 15 seconds with a polytron. Protease (Trypsin) was added (5 mg/g wet muscle), and the digested mince was mixed continually for 7 minutes. Digestion was terminated by the addition of an equal volume of solution I. The homogenate was centrifuged at 500 *g* for 10 minutes at 4ºC and the supernatant was rapidly decanted through a double layer of cheesecloth and centrifuged at 3,500 *g* for 10 minutes. The supernatant was discarded and the mitochondrial pellet was resuspended in solution I. The suspension was centrifuged at 3,500 *g* for 10 minutes.

The supernatant was again discarded, and the pellet was resuspended in 10 volumes of solution II (similar to solution I, but without BSA). This resuspended pellet was subsequently centrifuged at 3,500 *g* for 10 minutes. The final mitochondrial pellet was suspended in 250 µl of a solution containing 220 mM mannitol, 70 mM sucrose, 10 mM Tris HCl, and 1 mM EGTA, pH 7.40. Mitochondria from liver were isolated as previously described (29). Briefly, liver (~750 mg) was weighed and placed in 10 volumes of solution III (250 mM sucrose, 5 mM HEPES, and 1 mM EGTA), minced with scissors and the mince was homogenized with a Potter-Elvehjem PTFE pestle and glass tube (2 passes). The homogenate was centrifuged at 500 g for 10 minutes at 4ºC. The supernatant was rapidly decanted through a double layer of cheesecloth and centrifuged at 3,500 g for 10 minutes. The supernatant was discarded and the mitochondrial pellet was resuspended in solution III. The suspension was centrifuged at 3,500 g for 10 minutes. The final mitochondrial pellet was suspended in 250 µl of a solution containing (in mM) 220 mannitol, 70 sucrose, 10 Tris HCl, and 1 EGTA, pH 7.40.

Isolated mitochondrial oxidative phosphorylation.

Mitochondrial oxygen consumption was measured as described by Messer et al. (23). Briefly, mitochondrial oxygen consumption was measured polarographically in a respiration chamber (Hansatech Instruments, United Kingdom). Isolated mitochondria (20 µL) were incubated with 1 ml of respiration buffer adapted from Wanders et al. (43) (100 mM KCL, 50 mM MOPS, 10 mM KH_2PO_4, 20 mM glucose, 10 mM $MgCl_2$, 1 mM EGTA, and 0.2% fatty acid free BSA; pH = 7.0) at 37°C in a respiratory chamber with continuous stirring. For state 3 respiration, 2 mM pyruvate and 2 mM malate (complex I

substrates) or 5 mM succinate (complex II substrate) was used in the presence of 0.25 mM ADP, and state 4 respiration was recorded following the phosphorylation of ADP as described by Estabrook (10). Respiratory control ratio (RCR) was calculated as state 3/state 4 oxygen consumption. Respiration values were expressed as a ratio to citrate synthase to compensate for mitochondrial enrichment in the samples

Mitochondrial oxidant emission.

Oxidant emission by mitochondria was determined using the oxidation of the fluorogenic indicator Amplex Red (Molecular Probes, Eugene, OR) in the presence of horseradish peroxidase (18). The assay was performed at 37°C in 96-well plates using succinate as the substrate. Specifically, this assay was developed based on the concept that horseradish peroxidase catalyzes the hydrogen peroxide-dependent oxidation of nonfluorescent Amplex Red to fluorescent Resorufin Red. Resorufin Red formation was monitored at an excitation wavelength of 545 nm and an emission wavelength of 590 nm using a multiwell plate reader fluorometer (Synergy H1, BioTek, Winooski, VT, USA). We recorded the level of Resorufin Red formation, and hydrogen peroxide production was calculated with a standard curve.

Enzymatic assays for electron transport chain complex activity.

Complex I (NADH dehydrogenase) enzyme activity (EC 1.6.5.3) was measured as a function of the decrease in absorbance from NADH oxidation by decylubiquinone before and after rotenone addition (42). Complex II (succinate dehydrogenase) activity (EC 1.3.5.1) was measured as a function of the decrease in absorbance from 2,6-dichloroindophenol reduction (42). Complex III (ubiquinol cytochrome *c* oxidoreductase) activity (EC 1.10.2.2) was determined as a function of the increase in absorbance from

cytochrome *c* reduction (42). Complex IV (cytochrome *c* oxidoreductase) activity was determined as a function of the decrease in absorbance from cytochrome *c* oxidation (42). Specificity of complex IV activity was determined by monitoring changes in absorbance in the presence of KCN (42). Citrate synthase (EC 4.1.3.7) was measured as a function of the increase in absorbance from 5,5'-dithiobis-2-nitrobenzoic acid reduction (42). Enzyme activities were expressed as a ratio to citrate synthase to compensate for mitochondrial enrichment in the cell samples.

Protein abundance.

The relative concentration of proteins was quantified by Western blot analysis (18). To accomplish this, tissue was homogenized 1:10 (wt/vol) in 5 mM Tris HCl (pH 7.5) and 5 mM EDTA (pH 8.0), and protease inhibitor cocktail (14224-396, VWR, Radnor, PA, USA) and was centrifuged at 1500 *g* for 10 minutes at 4°C. Protein content of the supernatant was quantified by the method of Bradford (6). Proteins were separated by polyacrylamide gel electrophoresis via 4-20% polyacrylamide gels (BioRad, Hercules, CA, USA). After electrophoresis, the proteins were transferred to PVDF membranes. Non-specific sites were blocked in phosphate-buffered saline (PBS) solution containing 0.1% Tween 20 and 5% non-fat milk. Membranes were then incubated overnight at 4ºC with primary antibodies purchased from GeneTex (Irvine, CA, USA) directed against peroxisome proliferator activated receptor alpha (PPARα, GTX101096, 1:1000), peroxisome proliferator activated receptor delta (PPARδ, GTX113250, 1:2000), peroxisome proliferator activated receptor gamme, coactivator 1 alpha (PGC-1α, GTX37356, 1:1000), superoxide dismutase 1 (SOD1, GTX100554 1:2000), superoxide dismutase 2 (SOD2, GTX116093, 1:2000), catalase (CAT,

GTX110704, 1:2000), and glutathione peroxidase (GPX, GTX116040, 1:2000). Following incubation with primary antibodies, membranes were washed (five minutes x 3) with PBS-Tween and then incubated with secondary antibodies for one hour in room temperature. After washing (five minutes x 3), a chemiluminescent system was used to detect labeled proteins (GE Healthcare, Buckinghamshire, UK). Images of the membranes were captured and analyzed by using the ChemiDoc-It2 Imaging System (UVP, LLC, Upland, CA). Protein expression was normalized to Ponceau staining.

Assessment of indices of oxidative damage.

To determine the relative amount oxidative damage, we measured protein oxidation and lipid peroxidation. Lipid peroxidation was assessed by determining 4-hydroxynoneal (4-HNE; *trans*-4-hydroxy-2-nonenal, $C_9H_{16}O_2$) expression via western blotting. Primary antibody for 4-HNE was purchased from Abcam (ab46545; 1:1000 dilution, Cambridge, MA, USA). Protein oxidation was measured by comparing relative expression of protein carbonyls using a commercially available kit (Oxy-Blot protein oxidation detection kit; Intergen, Purchase, NY, USA) via western blotting as described by the manufacturer's instructions.

Statistics.

Comparison between groups for each dependent variable were made by a one-way one-way analysis of variance (ANOVA), with a Tukey post hoc test being used to determine significance differences between groups when the ANOVA indicated statistical significance. However, in the case of PR WAT mass, state 3 succinate liver, RCR muscle, state 3 succinate muscle, serum NEFA, SOD2 liver, CAT liver, PGC-1a muscle, 4-HNE RetroP WAT, the Brown-Forsythe test was significant and thus the

Kruskal-Wallis test was performed followed by the Dunn's for multiple comparisons post-hoc. Data are presented as means ± SD, and significance was established at $p < 0.05$.

RESULTS

Body and tissue mass.

The transition into pregnancy and into lactation periods is matched with large variations in mass. Body mass was the highest in pregnant animals compared to all groups ($p < 0.05$) since the pregnant mother was also carrying the fetuses. Body mass of both non-lactating and lactating animals dropped after parturition, but was still higher compared to non-pregnant animals ($p < 0.05$) (Figure 1A). Liver mass was increased in pregnant animals compared to non-pregnant animals ($p < 0.05$). Following parturition, liver mass of non-lactating animals decreased ($p < 0.05$), but the liver mass of lactating animals increased and had the highest mass of all four groups ($p < 0.05$) (Figure 1B). The combined mass of the triceps surae calf muscle (henceforth skeletal muscle) was the highest in non-lactating animals compared to all groups ($p < 0.05$) (Figure 1C). RetroP WAT mass was higher in non-lactating animals compared to all groups ($p < 0.05$), and lactating animals had lower RetroP and PR WAT mass compared to pregnant and non-lactating animals ($p < 0.05$) (Figure 1D and 1E). The combined mass of RetroP and PR was highest in non-lactating animals, while also being higher in pregnant animals compared to non-pregnant and lactating animals ($p < 0.05$) (Figure 1F).

Serum glucose and NEFA concentrations.

Gestation and lactation require the mobilization of energy substrates to metabolically active tissues to provide for the growing fetuses/offspring. Importantly, glucose and NEFA are transported in the blood to help accomplish this. The ability of the body to regulate concentrations of NEFA and glucose is important, and dysregulation is indicative of metabolic disease**.** Serum glucose concentration was lower in pregnant animals compared to all groups ($p < 0.05$). Lactating animals also experienced lower serum glucose compared to non-pregnant animals ($p < 0.05$) (Figure 1G). Pregnant animals had high serum NEFA, albeit not significant, and lactating animals had lower free fatty acids compared to pregnant animals ($p < 0.05$) (Figure 1H).

Mitochondrial function and oxidant emission.

In order to determine how mitochondrial function is altered during pregnancy and lactation, we measured mitochondrial respiration utilizing complex I and complex II substrates in isolated mitochondria from liver and skeletal muscle. Specifically, the ratio of state 3 respiration to state 4 respiration is termed the respiratory control ratio and can be used as an indicator of mitochondrial function. Our data show that the RCR of liver mitochondria with complex I substrate was decreased in pregnant animals compared to all groups ($p < 0.05$) and had lower RCR with complex II substrate compared to non-pregnant animals ($p < 0.05$) (Figure 2). No differences were detected between groups in RCR in mitochondria isolated from skeletal muscle with complex I substrate ($p > 0.05$). However, RCR was higher in skeletal muscle mitochondria isolated from lactating animals compared to pregnant animals when using complex II substrate ($p < 0.05$) (Figure 3).

ROS are a potential by-product that can occur at complex I and III sites in mitochondria during respiration (2). Oxidant emission in isolated liver mitochondria was lower in non-lactating animals compared to pregnant animals ($p < 0.05$) (Figure 2G). No differences were detected in oxidant emission from skeletal muscle mitochondria ($p > 0.05$) (Figure 3G).

Mitochondrial complex activity.

We also measured parameters of mitochondrial function by determining the enzymatic activity of complex I, II, III, and IV. In this regard, several enzymatic complexes are present in mitochondria that aide in the generation of an electropotenial gradient that is harnessed in order to phosphorylate ADP. Specifically, complex I transfers electrons from NADH to coenzyme Q10. Complex II transfers electrons from FADH. Complex III passes electrons down the chain by reducing cytochrome c. And, complex IV converts molecular oxygen into water and further shuttles protons into the intermembrane space. Enzymatic activity of liver complex I was higher in non-lactating animals compared to all groups ($p < 0.05$) (Figure 4A). Conversely, non-lactating and lactating animals both had lower liver complex II activity compared non-pregnant and pregnant animals ($p < 0.05$) (Figure 4B). No differences were detected for complex III and IV activity in liver mitochondria ($p > 0.05$) (Figure 4C and 4D). Complex I activity of skeletal muscle mitochondria was lower in lactating animals compared to pregnant animals ($p < 0.05$) (Figure 4E). Additionally, complex III activity in skeletal muscle was higher in non-lactating and lactating animals compared to non-pregnant animals ($p < 0.05$) (Figure 4G). No differences were detected at complex II and IV in mitochondria isolated from skeletal muscle ($p > 0.05$) (Figure 4F and 4H).

Markers of metabolism.

The PGC-1α and PPARδ are members of the PPAR superfamily, which is associated with the regulation of genes involved in oxidative metabolism. Specifically, PGC-1α regulates genes involved in mitochondrial biogenesis (44) and PPARδ is associated with regulation of genes involved in lipid and glucose metabolism (21, 36). We measured relative protein levels via western blotting to assess markers of metabolism in liver, WAT, and skeletal muscle. PGC-1α protein levels were higher in the liver of lactating animals compared to non-pregnant and pregnant animals ($p < 0.05$) (Figure 5A). Liver PPARδ protein expression was lower in pregnant animals compared to all groups ($p < 0.05$) and was higher in lactating animals compared to non-lactating animals ($p < 0.05$) (Figure 5B). PPARδ protein levles were lower in non-lactating animals compared to non-pregnant and pregnant animals in skeletal muscle and in RetroP WAT ($p < 0.05$) (Figure 5D and 5E). No differences were detected for PGC-1α protein levels in skeletal muscle ($p > 0.05$) (Figure 5C) or PPARδ in PR WAT ($p > 0.05$) (Figure 5F).

Markers of oxidative stress.

Oxidative stress is considered the balance between oxidant and antioxidant sources. In this regard, cellular oxidants detoxified by endogenous antioxidant mechanisms. For example, SOD2 and SOD1 act to detoxify superoxide into the lesser harmful hydrogen peroxide. Hydrogen peroxide can be then detoxified by CAT and GPX. We also measured two markers of oxidative stress in liver, skeletal muscle, and WAT. GPX protein levels were lower in the liver of lactating animals compared to pregnant and non-lactating animals ($p < 0.05$). No differences were detected in

antioxidant protein expression in liver of SOD2, SOD1, and CAT ($p > 0.05$). Additionally, in the liver, no differences were detected in markers of lipid peroxidation (4-HNE) or protein oxidation (oxyblot) ($p > 0.05$) (Figure 6). SOD2 protein levels were higher in skeletal muscle of non-lactating and lactating animals compared to pregnant and non-pregnant animals ($p < 0.05$). Additionally, 4-HNE expression was higher in non-lactating animals compared to pregnant animals ($p < 0.05$). No differences were detected in antioxidant protein expression of SOD1, CAT, GPX, or oxyblot in skeletal muscle ($p > 0.05$) (Figure 7). In RetroP WAT, SOD1 protein expression was decreased in non-lactating animals compared to pregnant animals ($p < 0.05$), and CAT was decreased in lactating animals compared to pregnant and non-lactating animals ($p < 0.05$). No differences were detected in SOD2, GPX, or 4-HNE in RetroP WAT ($p > 0.05$) (Figure 8). In PR WAT, SOD2 protein levels were decreased in lactating animals compared to non-pregnant and non-lactating animals ($p < 0.05$), and CAT protein levels were decreased in lactating animals compared to pregnant and non-lactating animals ($p < 0.05$) (Figure 9).

DISCUSSION

The demanding act of reproduction is a necessary event for the survival of a species. Here, we report that mitochondrial function, oxidative stress, and markers of metabolism were different in animals at different reproductive stages. Specifically, serum glucose and serum NEFA concentrations were dramatically altered during pregnancy and lactation. We also show for the first time that this, in part, is regulated by modulation of PPARδ expression in liver, skeletal muscle, and WAT. Additionally, suppression of mitochondrial function occurs in the liver during pregnancy, but it is

reversed following parturition. We also highlight the alterations in lipid metabolism during pregnancy that may predispose adipose tissue accumulation in animals that do not lactate their pups. Finally, we also demonstrate that gestation and lactation did not result in increased markers of oxidative damage, but that a lack of lactation following parturition resulted in increased oxidative damage in skeletal muscle. The following segments provide a detail discussion of our findings.

Physiological alterations in liver and WAT metabolism.

Not surprisingly, pregnant animals were significantly heavier than all groups due to the mass of the unborn offspring. Interestingly, there were no significant differences in body mass between non-lactating and lactating animals despite a higher energetic demand being imposed by lactation. However, further observation of relative organ masses lends insight into why body mass was not altered between non-lactating and lactating animals. Liver mass was the highest in lactating animals and second highest in pregnant animals. Organs involved in nutritional processing are hypertrophied during gestation and lactation in order to meet the demands of higher energy utilization (39). The increased energetic demand imposed by lactation is met, in part, by hyperphagia. Food uptake is observed to increase 300% during lactation in rats (34), thus a larger capacity for enzymatic processing is required in order to absorb and utilize additional nutrients. During lactation, the liver plays a large role in producing the lipids that are converted into triacylglycerides in the mammary gland (1). Indeed transcription of genes involved in solute transport and lipid biosynthesis in the liver are demonstrated to be increased during pregnancy, and even further so during lactation (31). This increased

enzymatic processing is also demonstrated by an increased metabolic rate in the liver during lactation: contributing to the heightened overall metabolism (39).

In this regard, our results show that liver PPARδ protein expression was elevated in lactating animals and was suppressed during pregnancy. PPARδ is a member of a nuclear receptor superfamily that function to regulate transcriptional control of genes involved in cellular metabolism. Importantly, PPARδ has been demonstrated to have a critical role in glucose metabolism and insulin sensitivity in the liver (20, 38). Thus, modulation of liver PPARδ protein expression is likely an important event in the liver during pregnancy in order to conserve glucose for the fetus. Our results show that liver PPARδ protein expression was lowest during pregnancy compared to all groups, being almost 30% reduced compared to non-pregnant control animals. During gestation, the liver is known to increase rates of gluconeogenesis, however the mother often experiences hypoglycemia during late pregnancy (3, 16). This hypoglycemic state in late pregnancy is driven by increased glucose utilization by the fetus and insulin resistance in maternal cells (22, 35). Indeed, our results agree with these findings as serum glucose concentrations were decreased in pregnant animals compared to all groups. Thus, decreased PPARδ in the liver of pregnant animals would aid to spare glucose for fetal consumption as well as explain findings of decreased insulin sensitivity in maternal cells. In contrast, relative liver PPARδ protein expression in the liver of lactating animals was 40% higher than pregnant animals and 16% higher compared to non-lactating animals.

Additionally, we also report higher liver protein levels of PGC-1α in lactating animals compared to non-pregnant and pregnant animals. PGC-1α is a well-known

transcriptional regulator of genes involved in oxidative metabolism and mitochondrial biogenesis (44). Thus, increased PGC-1α protein levles could serve as a means for driving mitochondrial biogenesis to support enhanced glucose metabolism via increased PPARδ protein expression. Indeed, our results show that reproductive status yielded alterations in mitochondrial function between groups. RCR, a metric used to determine mitochondrial function, was decreased when using both complex I and complex II substrates in liver mitochondria. This is perhaps another mechanism for sparing resources for fetal consumption during pregnancy. Further, liver mitochondrial complex activity of complex I was higher in non-lactating animals compared to all groups and this may be indicative of an undefined compensatory mechanism for reverting the suppressed mitochondrial function that occurs during pregnancy when the stimulus of lactation is not provided following birth. Thus, it is possible that upregulation of PGC-1α and PPARδ during lactation may also be important in reversing mitochondrial alterations that occurred during pregnancy via driving transcriptional events involved in mitochondrial biogenesis and oxidative metabolism.

Visceral adiposity is often observed to increase during pregnancy in order to aid during the energetically demanding time of lactation, as well as to provide lipid stores should food sources become scarce (25). Interestingly, we show that WAT (RetroP+PR) mass was highest in non-lactating animals compared to all groups with the lowest mass being in lactating animals. Thus, it would appear that pregnancy predisposes the accumulation of WAT should lactation not take place, yet participating in lactation is capable of significantly reducing WAT mass. Indeed previous evidence has shown that this occurrence persists 21 days following the cessation of lactation as data show

reduced absolute fat mass and fewer visceral adipocytes in animals that lactated compared to those that did not (25-27). Converse to PPARδ's role in liver, PPARδ in white adipose tissue is involved in fat catabolism through oxidation of fatty acids (13, 36). Thus our findings of suppressed PPARδ protein levels in RetroP WAT of non-lactating animals indicates another potential mechanism that could underlie the propensity for fat storage in animals lacking the stimulus of breastfeeding. Indeed, we show that serum NEFA were the lowest in lactating animals, with non-lactating values being more than 2-fold higher than lactating animals, thus, exemplifying lipid utilization of the mammary gland for milk synthesis in lactating animals. In contrast, serum NEFA were highest during pregnancy, which agrees with a previous study showing that serum NEFA were over 4-fold higher than non-reproductive animals that is part due lipolytic placental hormones (4).

Physiological alterations in skeletal muscle.

Liver, WAT, and skeletal muscle attribute to 50% of the body's basal metabolic rate in a non-reproductive individual (37). Thus, reducing the metabolic cost imposed by skeletal muscle during reproductive states would be useful in order to conserve energy for the offspring. Indeed, previous reports have shown that skeletal muscle gene expression of proteins involved in glycolysis, citric acid cycle, and lipid metabolism are downregulated during lactation (12, 45). In our study, measurements of mitochondrial function and metabolism were unchanged in pregnant animals, suggesting that these parameters are not altered in skeletal muscle during pregnancy. However we do report differences in animals 14 days following parturition. Thus the energetic demand due to pregnancy may not be robust enough to require alterations in skeletal muscle

metabolism and mitochondrial function. Previously, Gutgessel et al. showed that PPARα and PGC-1α mRNA expression were decreased at day 15 of lactation in mice, serving to reduce fatty acid oxidation and energetic demand of skeletal muscle (12). However, we report that no differences were detected in PGC-1α protein levels in skeletal muscle. Thus, transcriptional observations of PGC-1α during lactation may not lead into translational outcomes. Conversely, we found that PPARδ protein levels was decreased in skeletal muscle of non-lactating animals. This finding is important since PPARδ expression in skeletal muscle regulates genes involved in lipid oxidation (20), and this could indicate a decrease in the capacity for lipid metabolism in skeletal muscle leading to increased adiposity and higher serum NEFA. In terms of skeletal muscle mitochondrial function, we observed that RCR was higher during lactation when using complex II substrate compared to pregnant animals. This appears to be driven by a lower state 4 respiration. This finding agrees with previous work by our lab, in which RCR was increased in lactating mice in skeletal muscle being driven by a decrease in state 4 respiration (28). State 4 can be a proxy for leak respiration, and thus a reduction in leak may be useful for reducing metabolic cost of skeletal muscle. This finding may be further supported by previous findings of decreased UCP3 expression in skeletal muscle during lactation (32, 45), as increased UCP3 expression in skeletal muscle is associated with increased metabolism (7).

Oxidative stress in pregnancy and lactation.

The disposable soma theory postulates that a limited pool of resources are available for allocation toward processes involved in reproduction and somatic maintenance (e.g. protection against oxidative damage) (19). Importantly, ROS

formation can occur during the processes of mitochondrial production of ATP leading to oxidative damage; however endogenous antioxidant proteins within the cell exist in order to protect against oxidative damage (33). An imbalance between antioxidant defense mechanisms and oxidant production is known as oxidative stress. In this regard, the free radical theory of aging has also been used to explain aging, in that oxidative damage across a lifetime causes disruption to cellular function causal in the processes of aging (14). Due to the disposable soma theory and free radical theory of aging, researchers have posited that the increased energetic demands imposed by reproduction would result in elevated oxidant production, leading to cellular senescence, and thus impacting longevity (8, 9, 24). However, little empirical evidence exists on the physiological mechanisms that could support this claim. On the contrary, reports have demonstrated that markers of oxidative stress were decreased in liver during lactation compared to non-reproductive controls (11, 47). Our results show no differences in markers of lipid peroxidation and protein oxidation in liver or WAT. Conversely, markers of lipid peroxidation were higher in the skeletal muscle of non-lactating animals compared to non-reproductive animals. This is an interesting finding, considering that according to the disposable soma theory non-lactating animals would be expending less energy toward milk synthesis and thus would be better able to allocate resources toward combating oxidative damage.

Conclusions.

Reproduction is associated with many physiological alterations. In this study, we observed alterations in glucose and lipid metabolism that occur in the shift between pregnancy and lactation. Specifically, we show that glucose sparing occurs during

pregnancy, however glucose metabolism is increased during lactation. We show for the first time that this is, in part, regulated by modulation of PPARδ expression in liver, skeletal muscle, and WAT. Additionally, suppression of mitochondrial function occurs in the liver during pregnancy, however is reversed following parturition. Importantly, it would appear that improvements in mitochondrial function in the liver following parturition can occur without the stimulus of lactation, albeit through different mechanisms. We also highlight that the alterations in lipid metabolism that occur during pregnancy and the accumulation of WAT in animals that do not participate in lactation. Finally, we demonstrate that gestation and lactation did not result in increased markers of oxidative damage, but that a lack of lactation follow parturition resulted increased oxidative damage in skeletal muscle. This finding adds to the growing evidence against the idea of oxidative damage being causal of the disposable soma theory of aging.

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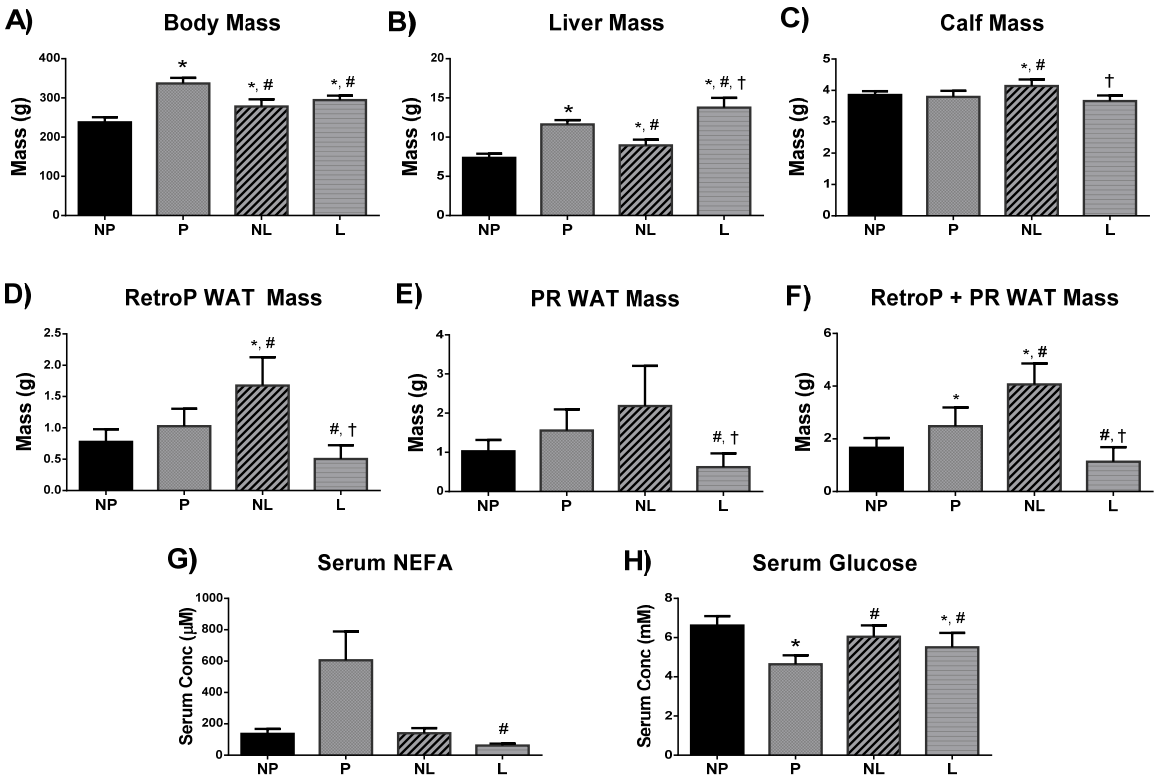
Figure 1. Body mass, tissue mass, and serum metabolites for non-pregnant rats (NP), rats that were allowed to mate and became pregnant, and were sacrificed at day 20 of gestation (P), rats that were allowed to mate and became pregnant, but were not allowed to suckle their pups (NL), and those that were allowed to mate, became pregnant, and suckled their young for 14 days (L). A) Body mass, B) liver mass, C) mass of both rear triceps surae (calf muscle mass), D) retroperitoneal white adipose tissue (RetroP WAT) mass, E) perirenal (PR) WAT mass, F) combined mass of RetroP and PR WAT, G) serum concentration of non-esterified fatty acids (NEFA), and H) serum concentration of glucose. Data shown are mean ± SD. * different from NP ($p < 0.05$), # different from P ($p < 0.05$), and † different from NL ($p < 0.05$).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NL
L

Figure 2. Respiration and oxidant emission for non-pregnant rats (NP), rats that were allowed to mate and became pregnant, and were sacrificed at day 20 of gestation (P), rats that were allowed to mate and became pregnant, but were not allowed to suckle their pups (NL), and those that were allowed to mate, became pregnant, and suckled their young for 14 days (L). Data include liver A) state 3 respiration utilizing complex I substrates (pyruvate and malate; P/M), B) state 3 respiration utilizing complex II substrates (succinate; suc), C) state 4 respiration utilizing P/M, D) state 4 respiration utilizing suc, E) respiratory control ratio (RCR) utilizing P/M), F) RCR utilizing suc, and G) oxidant emission. Oxygen consumption and hydrogen peroxide (H_2O_2) rates were normalized to citrate synthase (CS). Data shown are mean ± SD. * different from NP ($p < 0.05$) and # different from P ($p < 0.05$).

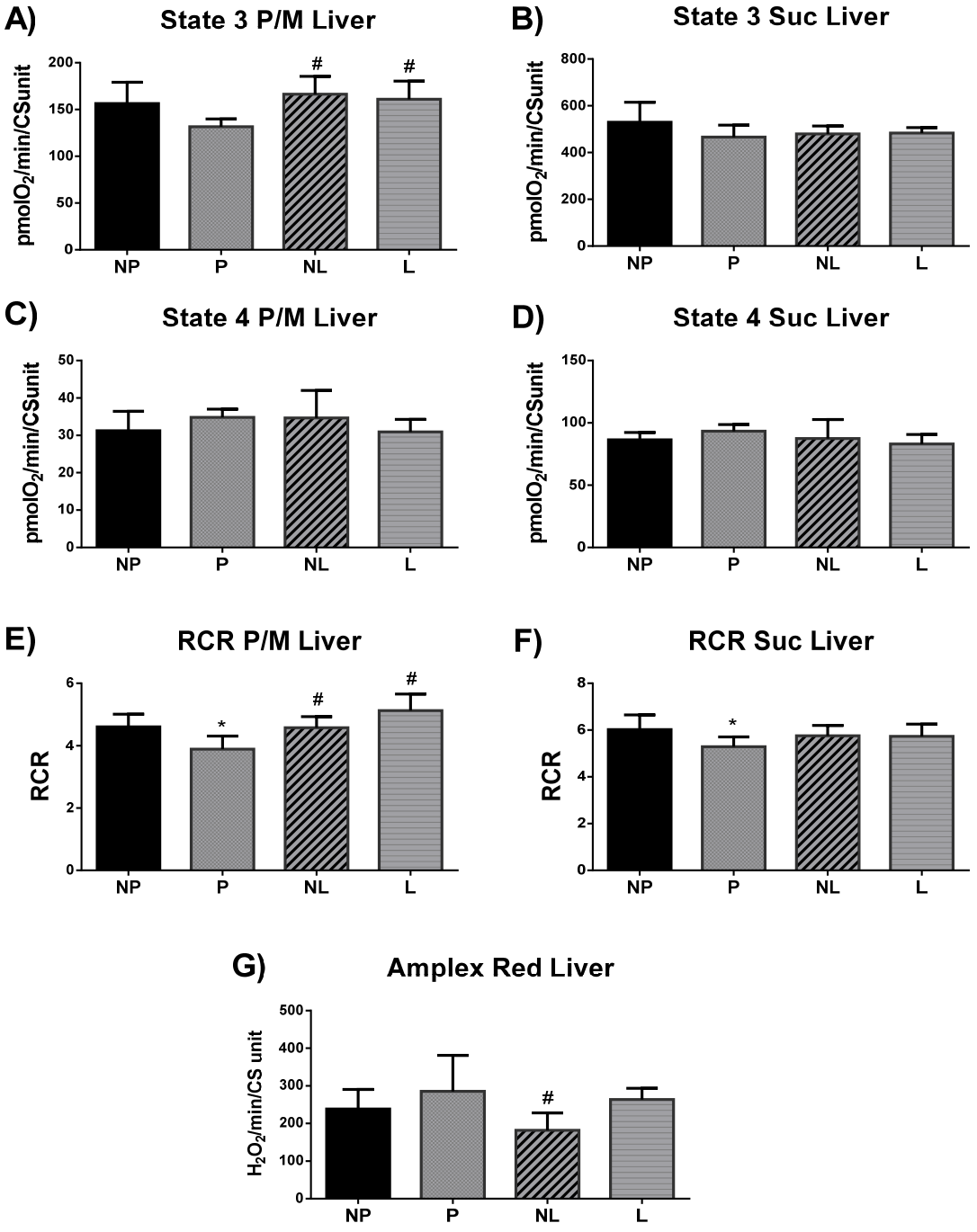

A)
State 3 P/M Liver
pmolO2/min/CSunit
200
150
100
50
0
#
#
NP
P
NL
L
B)
State 3 Suc Liver
pmolO2/min/CSunit
800
600
400
200
0
NP
P
NL
L
C)
State 4 P/M Liver
pmolO2/min/CSunit
50
40
30
20
10
0
NP
P
NL
L
D)
State 4 Suc Liver
pmolO2/min/CSunit
150
100
50
0
NP
P
NL
L
E)
RCR P/M Liver
RCR
6
4
2
0
*
#
#
NP
P
NL
L
F)
RCR Suc Liver
RCR
8
6
4
2
0
*
NP
P
NL
L
G)
Amplex Red Liver
H2O2/min/CS unit
500
400
300
200
100
0
#
NP
P
NL
L

Figure 3. Respiration and oxidant emission for non-pregnant rats (NP), rats that were allowed to mate and became pregnant, and were sacrificed at day 20 of gestation (P), rats that were allowed to mate and became pregnant, but were not allowed to suckle their pups (NL), and those that were allowed to mate, became pregnant, and suckled their young for 14 days (L). Data include skeletal muscle A) state 3 respiration utilizing complex I substrates (pyruvate and malate; P/M), B) state 3 respiration utilizing complex II substrates (succinate; suc), C) state 4 respiration utilizing P/M, D) state 4 respiration utilizing suc, E) respiratory control ratio (RCR) utilizing P/M), F) RCR utilizing suc, and G) oxidant emission. Oxygen consumption and hydrogen peroxide (H_2O_2) rates were normalized to citrate synthase (CS). Data shown are mean ± SD. # different from P ($p < 0.05$).

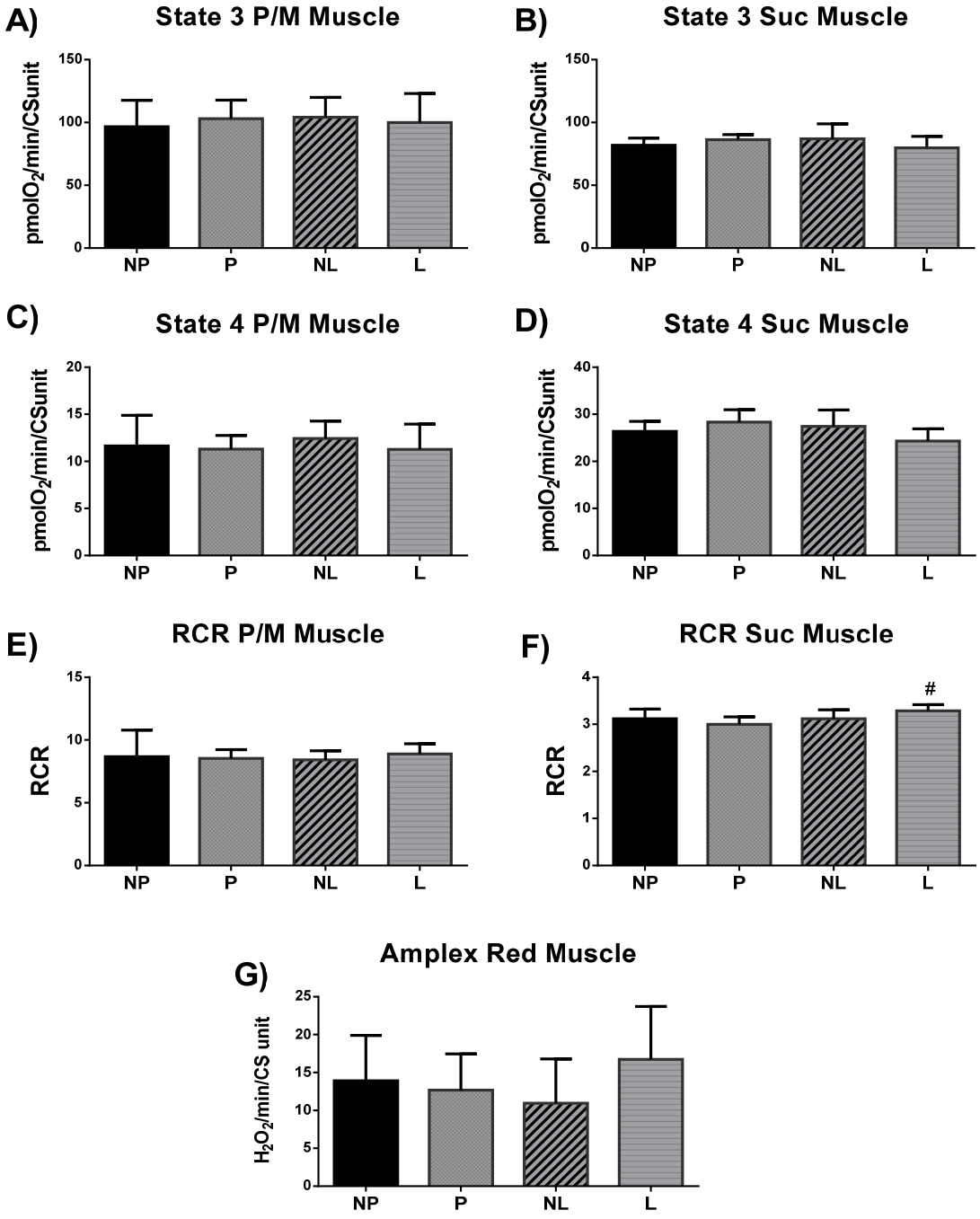

A)
State 3 P/M Muscle
pmolO2/min/CSunit
150
100
50
0
NP
P
NL
L
B)
State 3 Suc Muscle
pmolO2/min/CSunit
150
100
50
0
NP
P
NL
L
C)
State 4 P/M Muscle
pmolO2/min/CSunit
20
15
10
5
0
NP
P
NL
L
D)
State 4 Suc Muscle
pmolO2/min/CSunit
40
30
20
10
0
NP
P
NL
L
E)
RCR P/M Muscle
RCR
15
10
5
0
NP
P
NL
L
F)
RCR Suc Muscle
RCR
4
3
2
1
0
#
NP
P
NL
L
G)
Amplex Red Muscle
H2O2/min/CS unit
25
20
15
10
5
0
NP
P
NL
L

Figure 4. Enzymatic activity of the mitochondrial complexes for non-pregnant rats (NP), rats that were allowed to mate and became pregnant, and were sacrificed at day 20 of gestation (P), rats that were allowed to mate and became pregnant, but were not allowed to suckle their pups (NL), and those that were allowed to mate, became pregnant, and suckled their young for 14 days (L). Data include A) complex I, B) complex II, C) complex III, and D) complex IV activity in the liver. In addition, data include E) complex I, F) complex II, G) complex III, and H) complex IV activity in skeletal muscle. Complex activity data are normalized to citrate synthase (CS). Data shown are mean ± SD. * different from NP ($p < 0.05$), # different from P ($p < 0.05$), and † different from NL ($p < 0.05$).

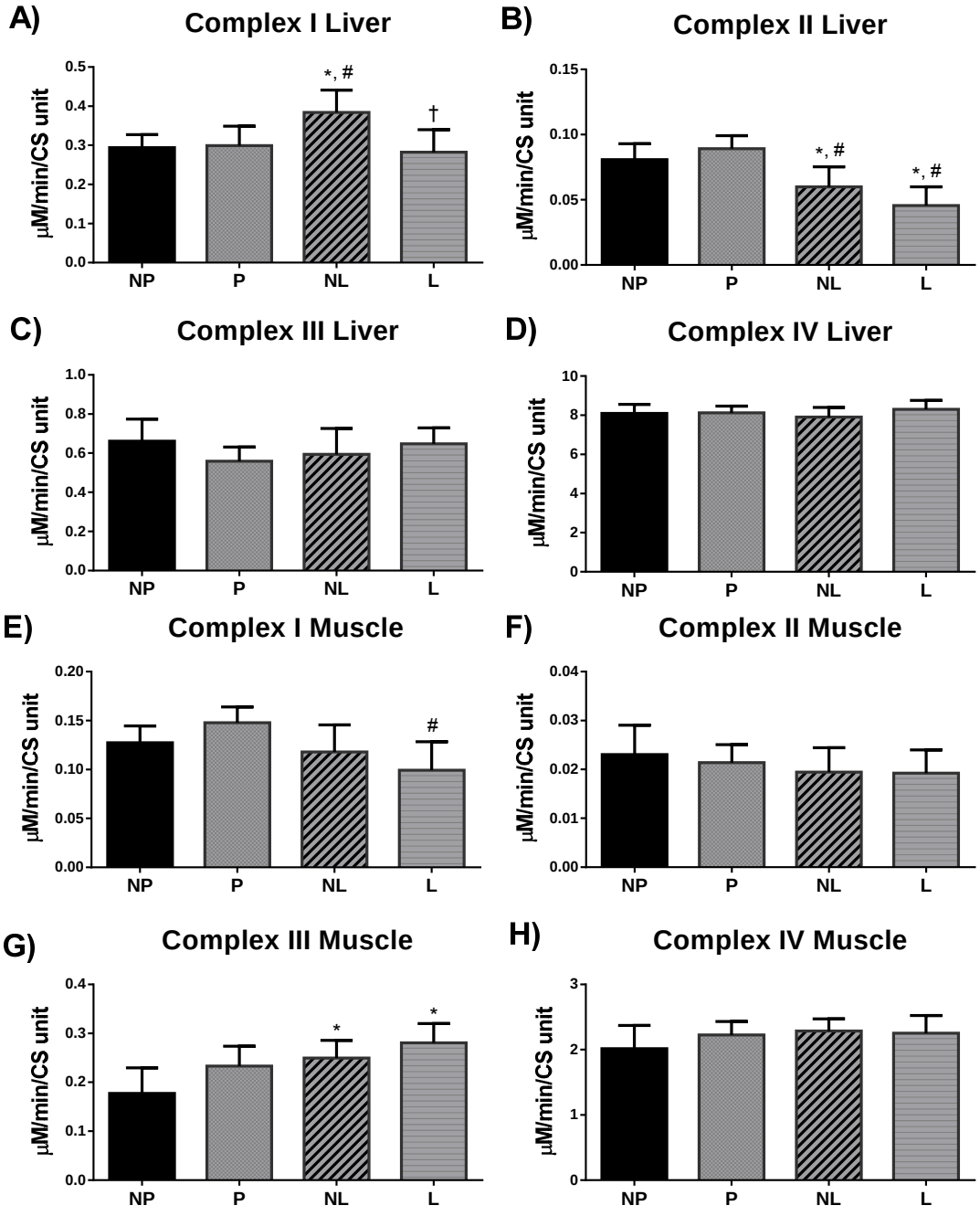

A)
Complex I Liver
µM/min/CS unit
0.5
0.4
0.3
0.2
0.1
0.0
*, #
†
NP
P
NL
L
B)
Complex II Liver
µM/min/CS unit
0.15
0.10
0.05
0.00
*, #
*, #
NP
P
NL
L
C)
Complex III Liver
µM/min/CS unit
1.0
0.8
0.6
0.4
0.2
0.0
NP
P
NL
L
D)
Complex IV Liver
µM/min/CS unit
10
8
6
4
2
0
NP
P
NL
L
E)
Complex I Muscle
µM/min/CS unit
0.20
0.15
0.10
0.05
0.00
#
NP
P
NL
L
F)
Complex II Muscle
µM/min/CS unit
0.04
0.03
0.02
0.01
0.00
NP
P
NL
L
G)
Complex III Muscle
µM/min/CS unit
0.4
0.3
0.2
0.1
0.0
*
*
NP
P
NL
L
H)
Complex IV Muscle
µM/min/CS unit
3
2
1
0
NP
P
NL
L

Figure 5. Markers of metabolism in liver, skeletal muscle, and white adipose tissue (WAT) for non-pregnant rats (NP), rats that were allowed to mate and became pregnant, and were sacrificed at day 20 of gestation (P), rats that were allowed to mate and became pregnant, but were not allowed to suckle their pups (NL), and those that were allowed to mate, became pregnant, and suckled their young for 14 days (L). Data include A) PGC-1α and B) PPARδ protein levels in liver. C) PGC-1α and D) PPARδ protein levels in skeletal muscle. Also, PPARδ protein levels in E) retroperitneal (RetroP) and F) perirenal (PR) WAT are shown. Representative blots are shown under the graphs. Data shown are mean ± SD. * different from NP ($p < 0.05$), # different from P ($p < 0.05$), and † different from NL ($p < 0.05$).

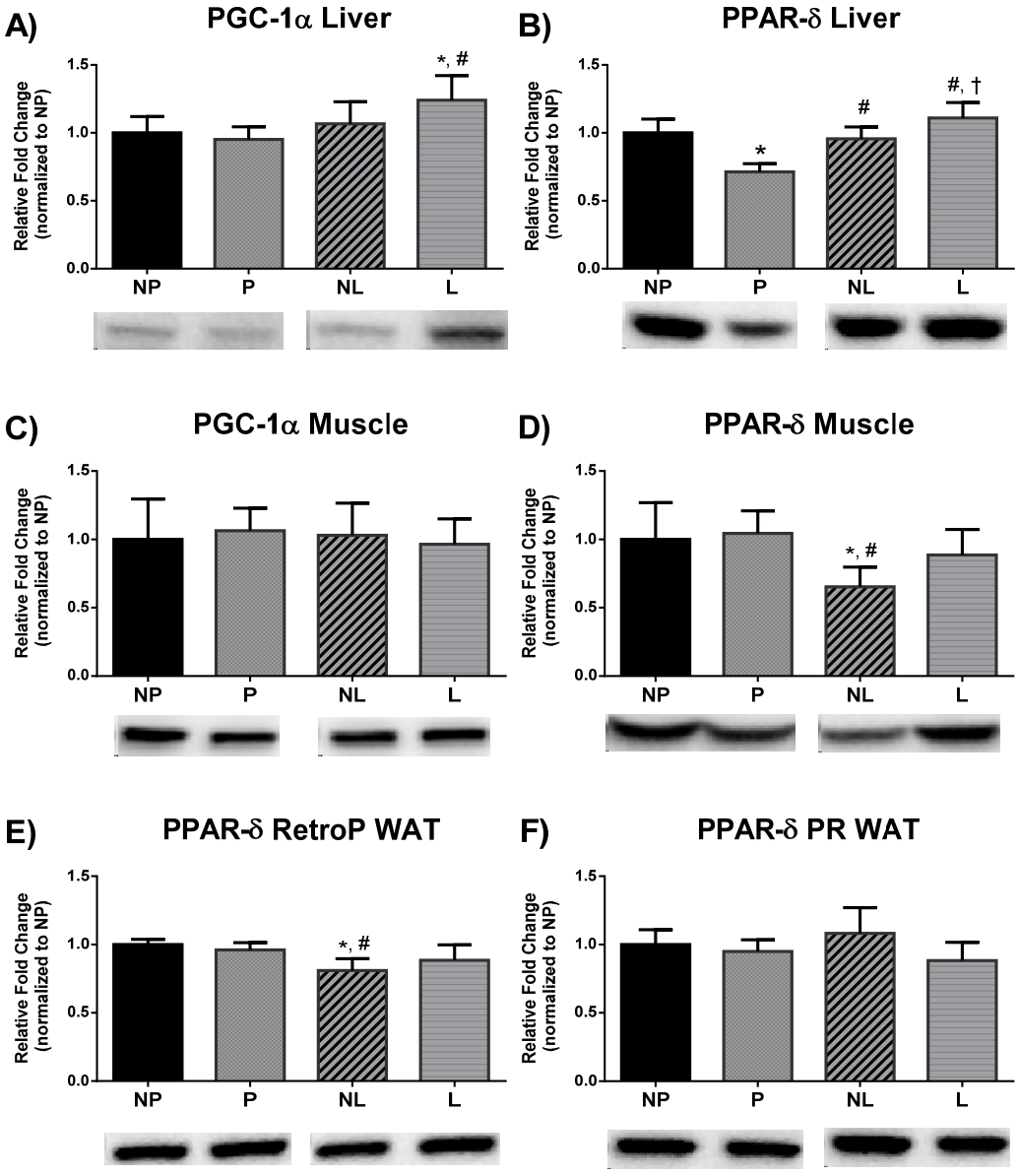

A)
PGC-1α Liver
Relative Fold Change (normalized to NP)
1.5
1.0
0.5
0.0
*, #
NP
P
NL
L
B)
PPAR-δ Liver
Relative Fold Change (normalized to NP)
*
#
#, †
C)
PGC-1α Muscle
D)
PPAR-δ Muscle
E)
PPAR-δ RetroP WAT
F)
PPAR-δ PR WAT

Figure 6. Markers of oxidative stress in liver for non-pregnant rats (NP), rats that were allowed to mate and became pregnant, and were sacrificed at day 20 of gestation (P), rats that were allowed to mate and became pregnant, but were not allowed to suckle their pups (NL), and those that were allowed to mate, became pregnant, and suckled their young for 14 days (L). Data include protein level of the antioxidants A) SOD2, B) SOD1, C) CAT, and D) GPX. In addition, data include markers of oxidative damage including E) lipid peroxidation determined by 4-HNE, and F) protein carbonyls levels determined by using oxyblot. Representative blots are shown under the graphs (A-D) or to the right of the graphs (E and F). Data shown are mean ± SD. # different from P ($p < 0.05$), and † different from NL ($p < 0.05$).

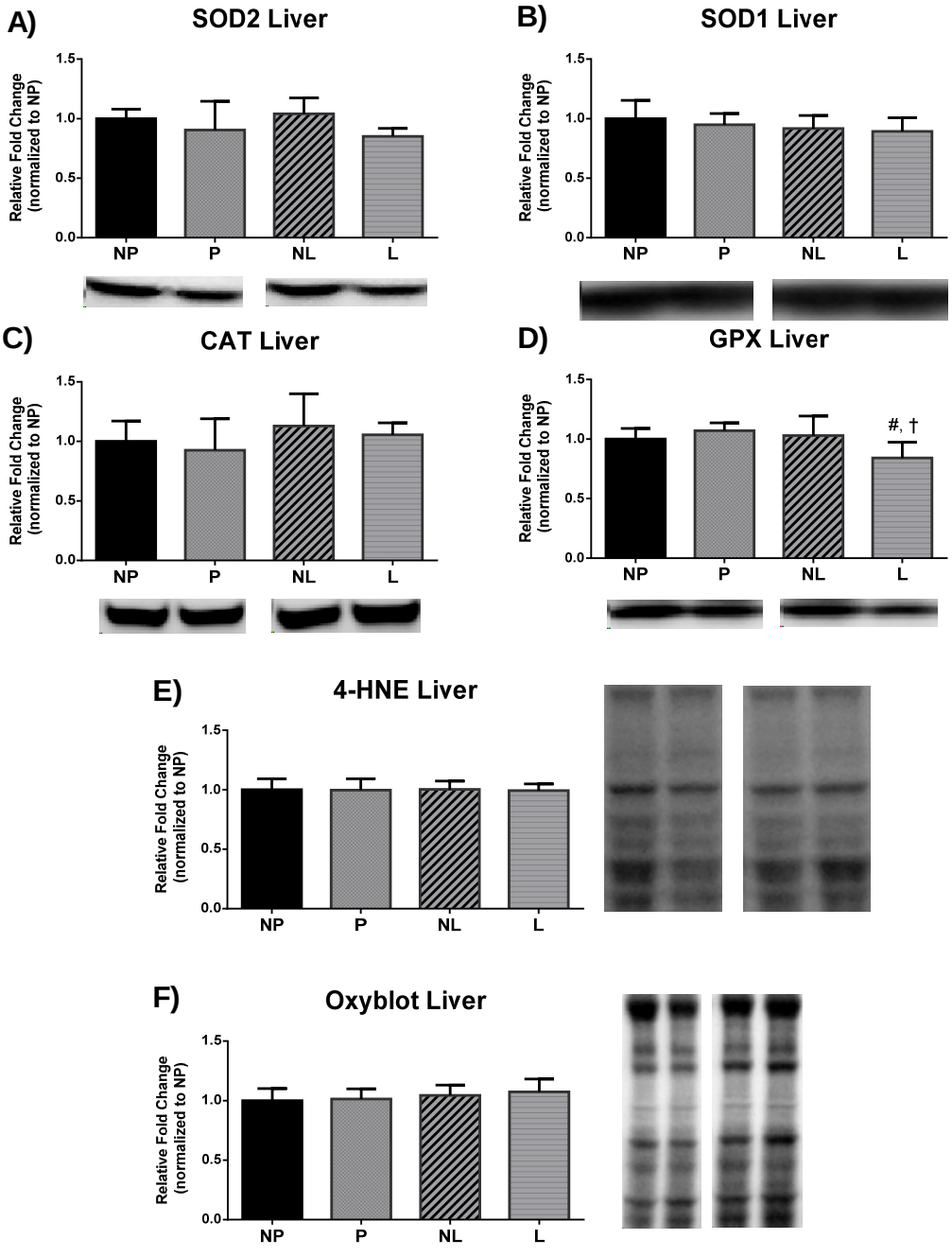

A)
SOD2 Liver
Relative Fold Change (normalized to NP)
1.5
1.0
0.5
0.0
NP
P
NL
L
B)
SOD1 Liver
Relative Fold Change (normalized to NP)
1.5
1.0
0.5
0.0
NP
P
NL
L
C)
CAT Liver
Relative Fold Change (normalized to NP)
1.5
1.0
0.5
0.0
NP
P
NL
L
D)
GPX Liver
Relative Fold Change (normalized to NP)
#, †
1.5
1.0
0.5
0.0
NP
P
NL
L
E)
4-HNE Liver
Relative Fold Change (normalized to NP)
1.5
1.0
0.5
0.0
NP
P
NL
L
F)
Oxyblot Liver
Relative Fold Change (normalized to NP)
1.5
1.0
0.5
0.0
NP
P
NL
L

Figure 7. Markers of oxidative stress in skeletal muscle for non-pregnant rats (NP), rats that were allowed to mate and became pregnant, and were sacrificed at day 20 of gestation (P), rats that were allowed to mate and became pregnant, but were not allowed to suckle their pups (NL), and those that were allowed to mate, became pregnant, and suckled their young for 14 days (L). Data include protein level of the antioxidants A) SOD2, B) SOD1, C) CAT, and D) GPX. In addition, data include markers of oxidative damage including E) lipid peroxidation determined by 4-HNE, and F) protein carbonyls levels determined by using oxyblot. Representative blots are shown under the graphs (A-D) or to the right of the graphs (E and F). Data shown are mean ± SD. * different from NP ($p < 0.05$), and # different from P ($p < 0.05$).

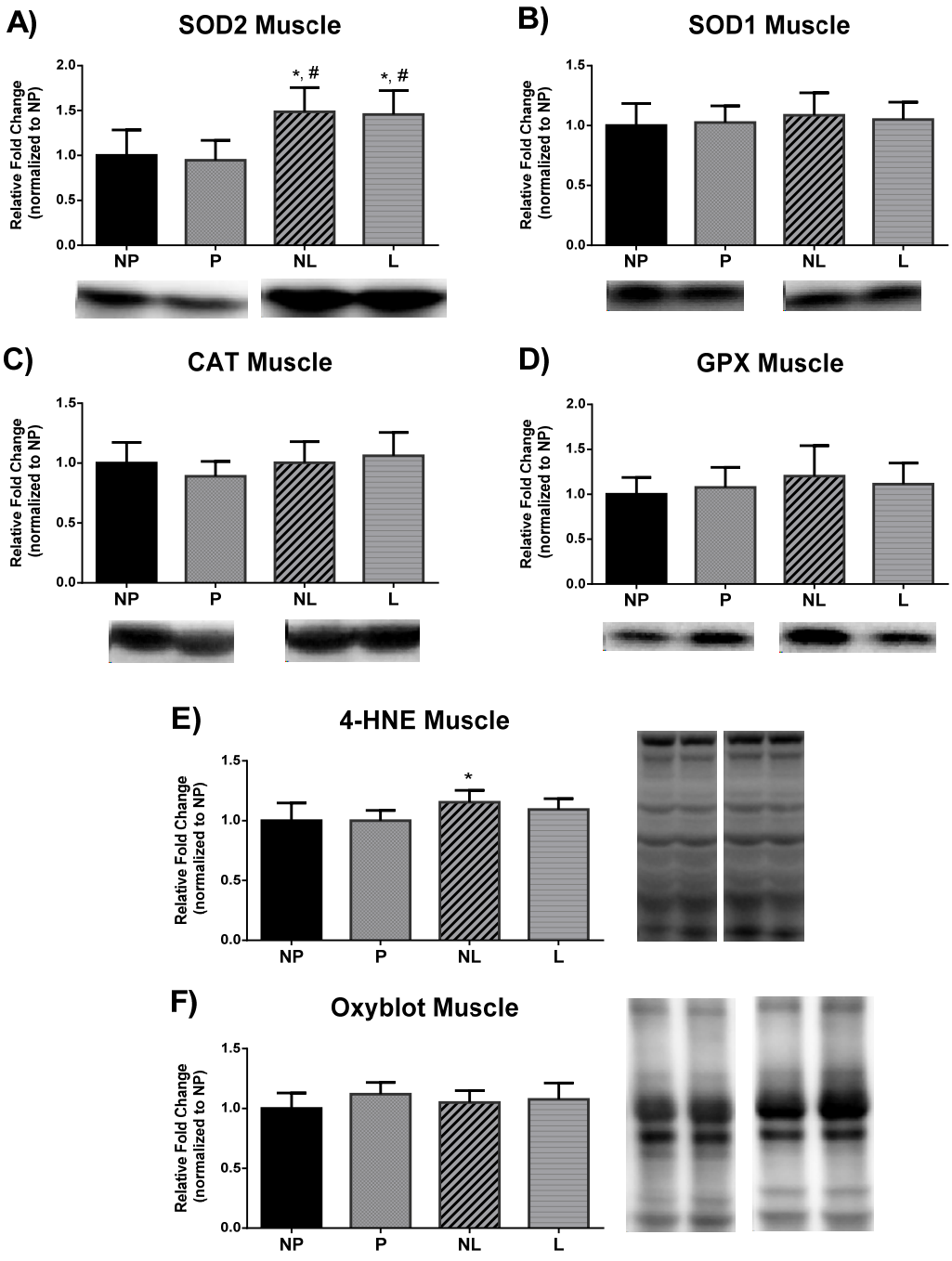

A)
SOD2 Muscle
Relative Fold Change (normalized to NP)
2.0
1.5
1.0
0.5
0.0
*, #
*, #
NP
P
NL
L
B)
SOD1 Muscle
Relative Fold Change (normalized to NP)
1.5
1.0
0.5
0.0
NP
P
NL
L
C)
CAT Muscle
Relative Fold Change (normalized to NP)
1.5
1.0
0.5
0.0
NP
P
NL
L
D)
GPX Muscle
Relative Fold Change (normalized to NP)
2.0
1.5
1.0
0.5
0.0
NP
P
NL
L
E)
4-HNE Muscle
Relative Fold Change (normalized to NP)
1.5
1.0
0.5
0.0
*
NP
P
NL
L
F)
Oxyblot Muscle
Relative Fold Change (normalized to NP)
1.5
1.0
0.5
0.0
NP
P
NL
L

Figure 8. Markers of oxidative stress in retroperitneal (RetroP) white adipose tissue (WAT) for non-pregnant rats (NP), rats that were allowed to mate and became pregnant, and were sacrificed at day 20 of gestation (P), rats that were allowed to mate and became pregnant, but were not allowed to suckle their pups (NL), and those that were allowed to mate, became pregnant, and suckled their young for 14 days (L). Data include protein level of the antioxidants A) SOD2, B) SOD1, C) CAT, and D) GPX. In addition, data include E) lipid peroxidation determined by 4-HNE, Representative blots are shown under the graphs. Data shown are mean ± SD. # different from P ($p < 0.05$), and † different from NL ($p < 0.05$).

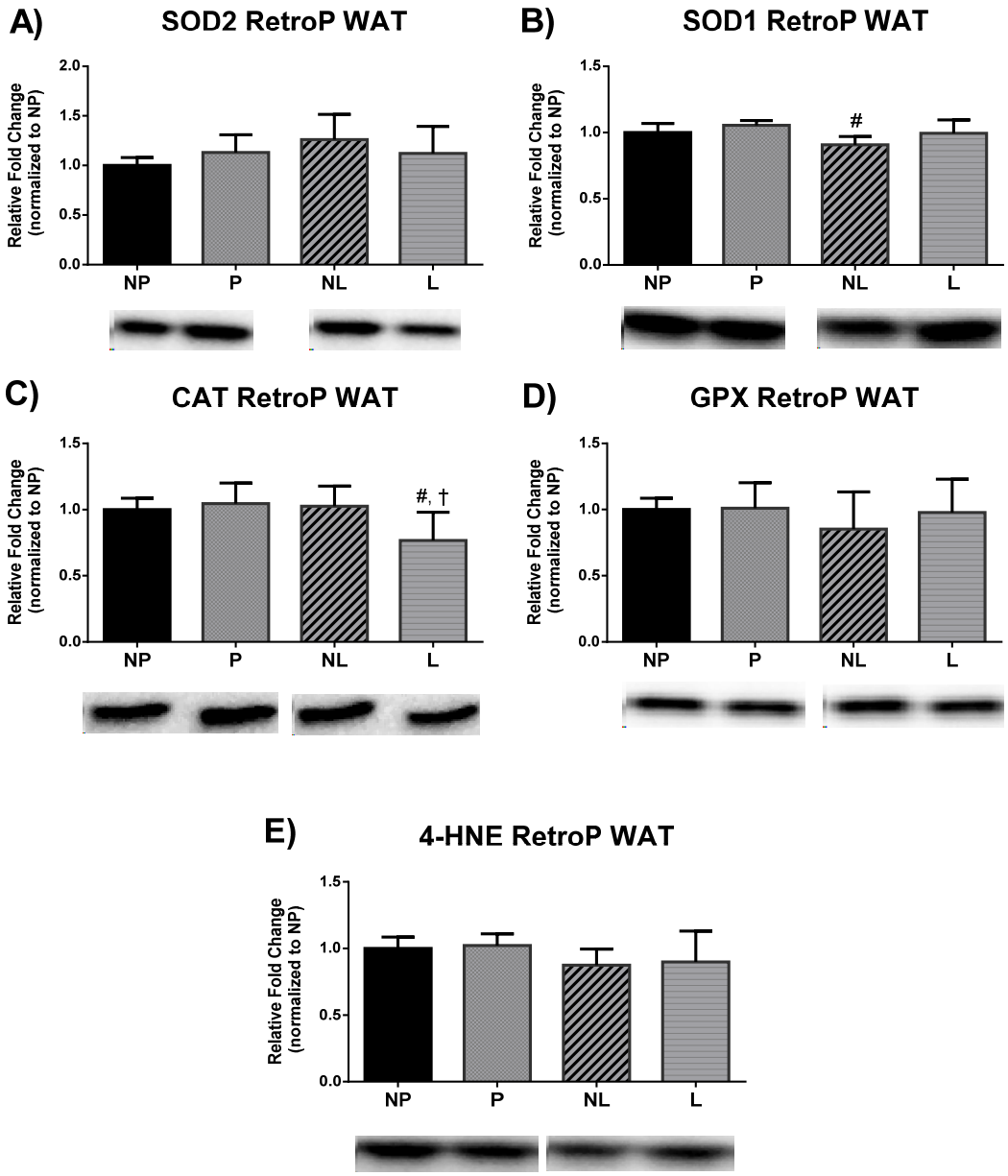
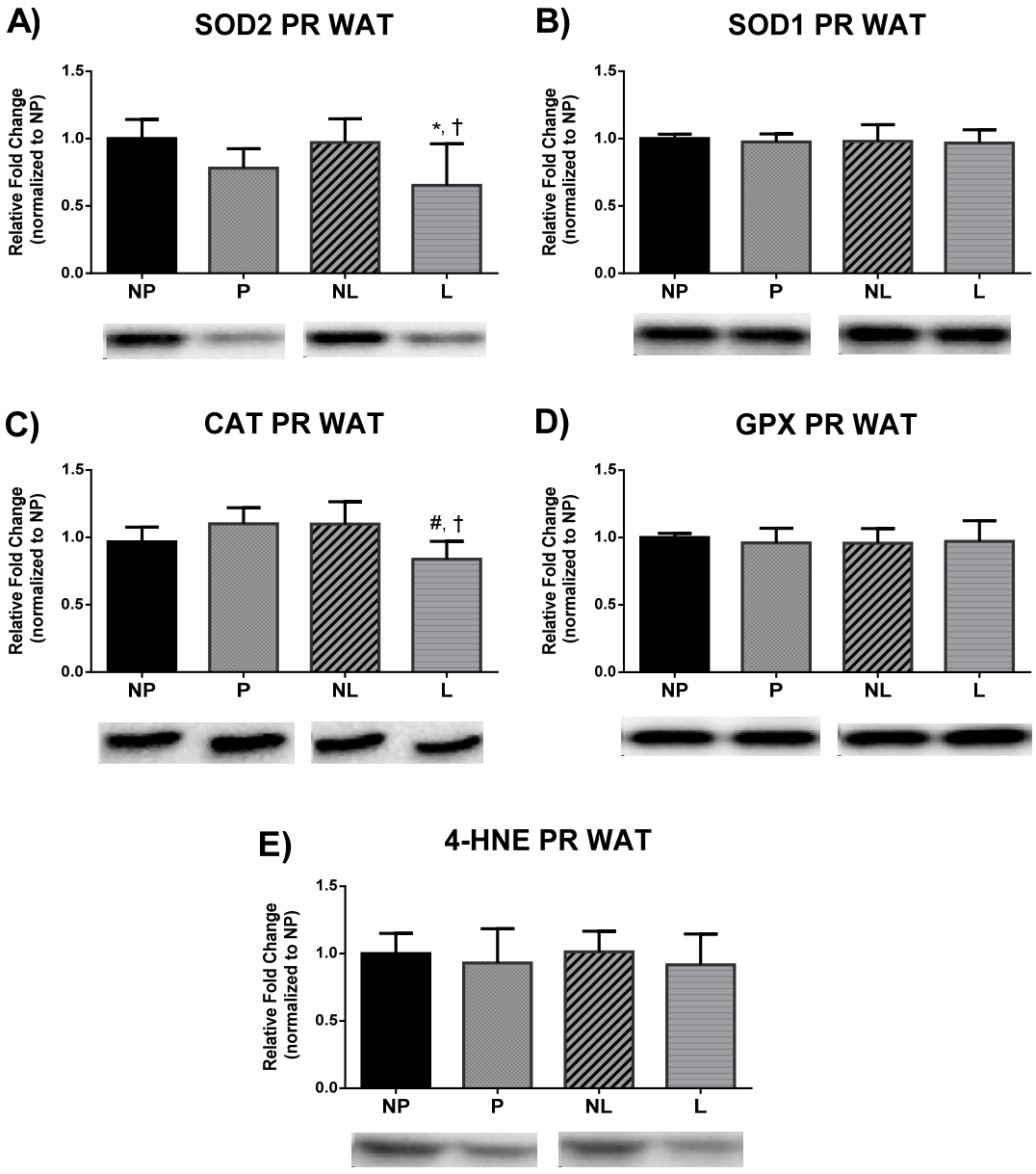

A)
SOD2 RetroP WAT
Relative Fold Change (normalized to NP)
2.0
1.5
1.0
0.5
0.0
NP
P
NL
L
B)
SOD1 RetroP WAT
Relative Fold Change (normalized to NP)
1.5
1.0
0.5
0.0
#
NP
P
NL
L
C)
CAT RetroP WAT
Relative Fold Change (normalized to NP)
1.5
1.0
0.5
0.0
#, †
NP
P
NL
L
D)
GPX RetroP WAT
Relative Fold Change (normalized to NP)
1.5
1.0
0.5
0.0
NP
P
NL
L
E)
4-HNE RetroP WAT
Relative Fold Change (normalized to NP)
1.5
1.0
0.5
0.0
NP
P
NL
L

Figure 9. Markers of oxidative stress in perirenal (PR) white adipose tissue (WAT) for non-pregnant rats (NP), rats that were allowed to mate and became pregnant, and were sacrificed at day 20 of gestation (P), rats that were allowed to mate and became pregnant, but were not allowed to suckle their pups (NL), and those that were allowed to mate, became pregnant, and suckled their young for 14 days (L). Data include protein level of the antioxidants A) SOD2, B) SOD1, C) CAT, and D) GPX. In addition, data include E) lipid peroxidation determined by 4-HNE, Representative blots are shown under the graphs. Data shown are mean ± SD. * different from NP ($p < 0.05$), # different from P ($p < 0.05$), and † different from NL ($p < 0.05$).


A)
SOD2 PR WAT
Relative Fold Change (normalized to NP)
1.5
1.0
0.5
0.0
*, †
NP
P
NL
L
B)
SOD1 PR WAT
Relative Fold Change (normalized to NP)
1.5
1.0
0.5
0.0
NP
P
NL
L
C)
CAT PR WAT
Relative Fold Change (normalized to NP)
1.5
1.0
0.5
0.0
#, †
NP
P
NL
L
D)
GPX PR WAT
Relative Fold Change (normalized to NP)
1.5
1.0
0.5
0.0
NP
P
NL
L
E)
4-HNE PR WAT
Relative Fold Change (normalized to NP)
1.5
1.0
0.5
0.0
NP
P
NL
L

IV. FEMALE REPRODUCTIVE HEALTH: ANIMAL EVIDENCE OF THE MATERNAL BENEFITS OF BREASTFEEDING

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ABSTRACT

Human epidemiological data show that breastfeeding reduces the mother's prevalence of many disease conditions such as obesity, type II diabetes, hypertension, and several types of cancer compared to mothers that give birth but do not breastfeed. The current study seeks to delineate the early beneficial mechanisms of breastfeeding on body composition, markers of metabolism, mitochondrial function, and oxidative stress. Ten-week old female Sprague-Dawley rats were divided into three groups (n = 8 per group): 1) non-reproductive (NR), 2) those that were allowed to mate and became pregnant, but were not allowed to suckle their pups (P), and 3) those that were allowed to mate, became pregnant, and suckled their young for 21 days (L). All animals were sacrificed at a time corresponding to seven days following the weaning of pups (i.e., day 28 postpartum). We report that at 28 days postpartum, the body mass of L rats was similar to NR rats, but the body mass of P rats was higher than NR rats. Importantly, L rats had lower retroperitoneal white adipose tissue mass compared to both NR and P rats, and that difference was accompanied by higher protein levels of peroxisome proliferator activated receptor delta (PPARδ) and superoxide dismutase 2 (SOD2) and reduced oxidative damage (assessed using the lipid peroxidation marker of 4-hydroxynoneal (4-HNE)). Furthermore, L rats had higher liver mitochondrial function of NADH-linked substrates, and protein expression of peroxisome proliferator activated receptor gamma, coactivator 1 alpha (PGC-1α), PPARδ, and SOD2. These animal findings support human epidemiological data and provide systemic and tissue specific evidence of the maternal health benefits conferred via breastfeeding.

INTRODUCTION

Exercise, fasting, and calorie restriction are examples of metabolic perturbations that induce physiological adaptations capable of promoting a healthy phenotype that is protective against various health disparities (6, 13, 17, 47). However, investigators often overlook lactation as a challenging metabolic event and thus, the physiological benefits of breastfeeding for human health have not been studied to the same extent as the aforementioned examples. Indeed, epidemiological studies in humans suggest that breastfeeding benefits the mother by reducing the risk of obesity, type II diabetes, hypertension, and several types of cancer (31, 35, 43-45). However, why these health benefits may accrue to breastfeeding are largely unknown.

During reproduction, a female's metabolism goes through two dramatic changes. With the onset of pregnancy, a female's metabolic processes are primed to support glucose and amino acid transport to the fetus. Glucose transport is facilitated by an increase in maternal insulin production and as a result, maternal cells often display increased resistance to insulin (22, 36). This change could also be associated with increases in circulating fatty acids and increased visceral adiposity (8). In contrast, during breastfeeding there is a decrease in insulin secretion, which is associated with a drop in β-cell proliferation, improved insulin sensitivity, and a shift in lipoprotein lipase and triacylglyceride levels that facilitate the mobilization of lipid precursors to the mammary gland for milk synthesis (12, 15). Based on current epidemiological data in humans and the limited physiological observations in animal models, Stuebe and Rich-Edwards proposed the reset hypothesis which posits that breastfeeding plays a central role in mobilizing fat stores and resetting the risk of metabolic disease (45). Implicit to

this theory is that females that breastfeed for an extended period experience a change in physiology that is sustained after breastfeeding has ceased. However, research evaluating the mechanistic differences between females that give birth and lactate versus those that give birth and do not participate in lactation are lacking.

White adipose tissue (WAT), liver, and skeletal muscle account for more than 50% of a non-reproductive adult's metabolic rate and also commonly display dysregulation in obese and individuals with type II diabetes mellitus (20, 25, 38, 51). Thus, any physiological changes that regulate metabolism in these organs during lactation could contribute to the protective effects that can be conferred by lactation. In this regard, Gutgesell and collaborators showed that genes important in oxidative and lipid metabolism (i.e. peroxisome proliferator activated receptor alpha [PPAR-α], peroxisome proliferator activated receptor gamma coactivator 1 alpha [PGC-1α], and peroxisome proliferator activated receptor gamma coactivator 1 beta [PGC-1β]) displayed reduced mRNA expression in liver and skeletal muscle two weeks following the cessation of lactation when compared to animals that gave birth but did not suckle their young (11). Additionally, Pichaud and collaborators reported decreased electron transport system activity in the liver during peak lactation in the mouse (32). Mediation of oxidative metabolism and thus mitochondrial function also may have important health implications on states of oxidative stress. As such, the current study seeks to delineate the potential effects that breastfeeding has on markers of metabolism, mitochondrial function, and oxidative stress. Specifically, we hypothesized that females that give birth and lactate their pups would have lower body mass due to enhanced lipid metabolism and lower blood glucose concentrations driven, in part, by enhanced mitochondrial

signaling in liver and skeletal muscle. In addition, we hypothesized that the higher metabolic demand in lactating animals would not impose an increase in markers of oxidative damage as suggested by the theory of trade-offs between life-history traits (e.g., reproduction).

METHODS

Animal husbandry.

All experimental procedures were approved by Auburn University's Institutional Animal Care and Use Committee (PRN 2014-2591). Ten-week old Sprague-Dawley rats were obtained from Harlan Laboratories, Inc. Animals were acclimated with their diet and facility ten days prior to the beginning of the experiment. Rats were housed under standard laboratory conditions (46 x 25 x 20 cm boxes, 12L:12D cycle, 22˚C, 50% RH), and given *ad libitum* access to food (Tekland Global Diet 2018) and water. Female rats (n = 8 per group) were randomly assigned to one of three treatment groups: 1) non-reproductive (NR), 2) those that were allowed to mate and became pregnant, but were not allowed to suckle their pups (P), and 3) those that were allowed to mate, became pregnant, and suckled their young for 21 days (L). Female rats were paired in their boxes with same-group counterpart, but separated during late pregnancy and lactation to prevent cross-fostering. Pups were removed from females in the P group within twelve hours of birth. Litter size adjusted to eight on the day of parturition for females in the L group. All animals were sacrificed at a time corresponding to seven days following the weaning of pups (i.e., day 28 postpartum).

Blood collection and analysis.

Rats were fasted for four hours and then were anesthetized using isoflurane vapors and body mass was quickly recorded. The anesthetized animals were than decapitated, and blood was collected, allowed to clot, and then centrifuged. Following centrifugation the serum was frozen at -80ºC for subsequent analyses. Serum glucose (STA-680, Cell Biolabs, San Diego, CA, USA) and non-esterified fatty acids (NEFA) (STA-618, Cell Biolabs) were quantified using the manufacturer's specifications.

Tissue collection and handling.

After the decapitation, the following tissues were excised and weighed: liver, triceps surae (calf muscle), and the retroperitoneal white adipose tissue (WAT). After the mass of each tissue was recorded, a sample of tissue from calf skeletal muscle and liver was used for mitochondrial isolation and the remainder of tissues were frozen in liquid nitrogen and stored at -80ºC for subsequent analyses. Upon gross evaluation, mammary tissue was not observed for females in any of the groups suggesting that the regression of mammary tissue was nearly or fully complete for females in the P and L groups at the time of tissue collection.

Mitochondrial isolation.

Mitochondrial isolations for muscle were performed as previously described (14). Excised muscles (~750 mg) were trimmed to remove fat and connective tissues, weighed, and placed in 10 volumes of solution I (100 mM KCl, 40 mM Tris HCl, 10 mM Tris base, 1 mM MgSO4, 0.1 mM EDTA, 0.2 mM ATP, and 2% (wt/vol) free fatty acid bovine serum albumin (BSA), pH 7.40). Muscles were minced with scissors and the mince was homogenized for 15 seconds with a polytron. Protease (Trypsin) was added

(5 mg/g wet muscle), and the digested mince was mixed continually for 7 minutes. Digestion was terminated by the addition of an equal volume of solution I. The homogenate was centrifuged at 500 *g* for 10 minutes at 4ºC and the supernatant was rapidly decanted through a double layer of cheesecloth and centrifuged at 3,500 *g* for 10 minutes. The supernatant was discarded and the mitochondrial pellet was resuspended in solution I. The suspension was centrifuged at 3,500 *g* for 10 minutes. The supernatant was again discarded, and the pellet was resuspended in 10 volumes of solution II (similar to solution I, but without BSA). This resuspended pellet was subsequently centrifuged at 3,500 *g* for 10 minutes. The final mitochondrial pellet was suspended in 250 µl of a solution containing 220 mM mannitol, 70 mM sucrose, 10 mM Tris HCl, and 1 mM EGTA, pH 7.40. Mitochondria from liver were isolated as previously described (30). Briefly, liver (~750 mg) was weighed and placed in 10 volumes of solution III (250 mM sucrose, 5 mM HEPES, and 1 mM EGTA), minced with scissors and the mince was homogenized with a Potter-Elvehjem PTFE pestle and glass tube (2 passes). The homogenate was centrifuged at 500 g for 10 minutes at 4ºC and the supernatant was rapidly decanted through a double layer of cheesecloth and centrifuged at 3,500 g for 10 minutes. The supernatant was discarded and the mitochondrial pellet was resuspended in solution III. The suspension was centrifuged at 3,500 g for 10 minutes. The final mitochondrial pellet was suspended in 250 µl of a solution containing (in mM) 220 mannitol, 70 sucrose, 10 Tris HCl, and 1 EGTA, pH 7.40.

Isolated mitochondrial oxidative phosphorylation.

Mitochondrial oxygen consumption was measured as described by Messer et al. (24). Briefly, mitochondrial oxygen consumption was measured polarographically in a respiration chamber (Hansatech Instruments, United Kingdom). Isolated mitochondria (20 µL) were incubated with 1 ml of respiration buffer adapted from Wanders et al. (49) (100 mM KCL, 50 mM MOPS, 10 mM KH_2PO_4, 20 mM glucose, 10 mM $MgCl_2$, 1 mM EGTA, and 0.2% fatty acid free BSA; pH = 7.0) at 37°C in a respiratory chamber with continuous stirring. For state 3 respiration, 2 mM pyruvate and 2 mM malate (complex I substrates) or 5 mM succinate (complex II substrate) was used in the presence of 0.25 mM ADP, and state 4 respiration was recorded following the phosphorylation of ADP as described by Estabrook (9). Respiratory control ratio (RCR) was calculated as state 3/state 4 oxygen consumption. Respiration values were expressed as a ratio to citrate synthase to compensate for mitochondrial enrichment in the samples.

Mitochondrial oxidant emission.

Oxidant emission by mitochondria was determined using the oxidation of the fluorogenic indicator Amplex Red (Molecular Probes, Eugene, OR) in the presence of horseradish peroxidase (16). The assay was performed at 37°C in 96-well plates using succinate as the substrate. Specifically, this assay was developed based on the concept that horseradish peroxidase catalyzes the hydrogen peroxide-dependent oxidation of nonfluorescent Amplex Red to fluorescent Resorufin Red. Resorufin Red formation was monitored at an excitation wavelength of 545 nm and an emission wavelength of 590 nm using a multiwell plate reader fluorometer (Synergy H1, BioTek, Winooski, VT,

USA). We recorded the level of Resorufin Red formation, and hydrogen peroxide production was calculated with a standard curve.

Enzymatic assays for electron transport chain complex activity.

Complex I (NADH dehydrogenase) enzyme activity (EC 1.6.5.3) was measured as a function of the decrease in absorbance from NADH oxidation by decylubiquinone before and after rotenone addition (48). Complex II (succinate dehydrogenase) activity (EC 1.3.5.1) was measured as a function of the decrease in absorbance from 2,6-dichloroindophenol reduction (48). Complex III (ubiquinol cytochrome *c* oxidoreductase) activity (EC 1.10.2.2) was determined as a function of the increase in absorbance from cytochrome *c* reduction (48). Complex IV (cytochrome *c* oxidoreductase) activity was determined as a function of the decrease in absorbance from cytochrome *c* oxidation (48). Specificity of complex IV activity was determined by monitoring changes in absorbance in the presence of KCN (48). Citrate synthase (EC 4.1.3.7) was measured as a function of the increase in absorbance from 5,5'-dithiobis-2-nitrobenzoic acid reduction (48). Enzyme activities were expressed as a ratio to citrate synthase to compensate for mitochondrial enrichment in the cell samples.

Protein abundance.

The relative concentration of proteins was quantified by Western blot analysis (16). To accomplish this, tissue was homogenized 1:10 (wt/vol) in 5 mM Tris HCl (pH 7.5) and 5 mM EDTA (pH 8.0), and protease inhibitor cocktail (14224-396, VWR, Radnor, PA, USA) and was centrifuged at 1500 *g* for 10 minutes at 4°C. Protein content of the supernatant was quantified by the method of Bradford (4). Proteins were separated by polyacrylamide gel electrophoresis via 4-20% polyacrylamide gels

(BioRad, Hercules, CA, USA). After electrophoresis, the proteins were transferred to PVDF membranes. Non-specific sites were blocked in phosphate-buffered saline (PBS) solution containing 0.1% Tween 20 and 5% non-fat milk. Membranes were then incubated overnight at 4ºC with primary antibodies purchased from GeneTex (Irvine, CA, USA) directed against peroxisome proliferator activated receptor alpha (PPARα, GTX101096, 1:1000), peroxisome proliferator activated receptor delta (PPARδ, GTX113250, 1:2000), peroxisome proliferator activated receptor gamma, coactivator 1 alpha (PGC-1α, GTX37356, 1:1000), superoxide dismutase 1 (SOD1, GTX100554 1:2000), superoxide dismutase 2 (SOD2, GTX116093, 1:2000), catalase (CAT, GTX110704, 1:2000), and glutathione peroxidase (GPX, GTX116040, 1:2000). Following incubation with primary antibodies, membranes were washed with PBS-Tween (five minutes x 3) and then incubated with secondary antibodies for one hour in room temperature. After washing (five minutes x 3), a chemiluminescent system was used to detect labeled proteins (GE Healthcare, Buckinghamshire, UK). Images of the membranes were captured and analyzed by using the ChemiDoc-It2 Imaging System (UVP, LLC, Upland, CA). Protein expression was normalized to Ponceau staining.

Assessment of indices of oxidative damage.

To determine the relative amount oxidative damage, we measured protein oxidation and lipid peroxidation. Lipid peroxidation was assessed by determining 4-hydroxynoneal (4-HNE; *trans*-4-hydroxy-2-nonenal, $C_9H_{16}O_2$) expression via western blotting. Primary antibody for 4-HNE was purchased from Abcam (ab46545; 1:1000 dilution, Cambridge, MA, USA). Protein oxidation was measured by comparing relative expression of protein carbonyls using a commercially available kit (Oxy-Blot protein

oxidation detection kit; Intergen, Purchase, NY, USA) via western blotting as described by the manufacturer's instructions.

Statistics.

Comparison between groups for each dependent variable were made by a one-way one-way analysis of variance (ANOVA), with a Tukey post hoc test being used to determine significance differences between groups when the ANOVA indicated statistical significance. However, in the case of state 3 respiration with succinate in muscle and serum FFA, the Brown-Forsythe test was significant and thus the Kruskal-Wallis test was performed, followed by the Dunn's post-hoc to determine significance differences between groups when the Kruskal-Wallis indicated statistical significance. Data are presented as mean ± SD, and significance was established at $p < 0.05$.

RESULTS

Body and tissue mass

During pregnancy and lactation, the body of females undergoes a large fluctuation in mass to accommodate the developing fetuses and active mammary tissue. The regression of these tissues 28 days following pregnancy and lactation varied between groups. Body mass was higher in P compared to NR rats ($p = 0.005$) and the body mass of P rats had a higher mass than the L rats, but this was not statistically different ($p = 0.145$) (Figure 1A). Liver mass was higher in L than NR rats ($p = 0.004$) (Figure 1B). L rats had lower retroperitoneal fat mass compared to both P ($p = 0.041$) and NR rats ($p < 0.001$) (Figure 1C). The combined mass of the rear triceps surae calf muscle was higher in P than in NR rats ($p = 0.027$) (Figure 1D).

Serum glucose and NEFA concentrations.

The high energetic demand of lactation requires energy substrates to be delivered to metabolically active tissues via the blood stream. Two important metabolites transported in blood are glucose and NEFAs. The ability of the body to regulate blood glucose is indicative of several disease types, particularly type II diabetes. Additionally, elevated NEFA concentrations in the blood can indicate metabolic dysregulation. Serum glucose concentration was lower in L compared to NR ($p = 0.029$), but no statistical differences were detected in serum NEFA concentrations ($p = 0.802$) (Figure 1E and 1F).

Mitochondrial respiration and oxidant emission.

RCR is a useful tool in assessing mitochondrial function, and can be used to measure respiration capacity of the mitochondria with different available substrates. Measuring RCR using isolated motichondria provides the ability to observe ATP phosphorylation via electron shuttling at complex I and complex II. High resolution respirometry was used in isolated mitochondria from liver and skeletal muscle. The RCR of liver mitochondria was higher in L compared to P and NR rats ($p = 0.029$ and $p = 0.002$, respectively) when using pyruvate and malate as the substrate (Figure 2C). No statistical differences were detected in liver RCR between groups when succinate was used as the substrate ($p = 0.234$; Figure 2F). In addition, no statistical differences were detected in skeletal muscle RCR between groups when pyruvate and malate or succinate were used as the substrate ($p = 0.792$ and $p = 0.996$, respectively) (Figure 2I and 2L).

Oxidant emission occurs during oxidative phosphorylation in the mitochondria, specifically at complex I and complex III sites (1). No statistically differences ($p = 0.191$) were detected in oxidant emission from mitochondria isolated from liver (Figure 2M). Oxidant emission from mitochondria isolated from skeletal muscle was lower in P and L compared to NR ($p = 0.017$ and $p = 0.008$, respectively) (Figure 2N).

Mitochondrial complex activity.

Phosphorylating ADP in the mitochondria is accomplished through electron pumps that harness the energy of electrons by oxidizing the reduced form of either NADH or FADH. Specifically, complex I transfers electrons from NADH to coenzyme Q10. Complex II transfers electrons from FADH. Complex III passes electrons down the chain by reducing cytochrome c. And, complex IV converts molecular oxygen into water and further shuttles protons into the intermembrane space. Activity of complex I in liver mitochondria was higher for both P and L rats compared to NR rats ($p = 0.027$ and $p = 0.046$, respectively) (Figure 3A). Conversely, enzymatic activity of complex II in liver mitochondria was lower in BF rats than both NR and P rats ($p = 0.004$ and $p = 0.0029$, respectively) (Figure 3B). Complex III activity in liver mitochondria of L rats was lower, albeit not statistically different, than P and NR rats ($p = 0.065$ and $p = 0.075$, respectively) (Figure 3B). Complex IV activity was lower in liver mitochondria of L compared to P rats ($p = 0.025$; Figure 3D). Enzymatic activity of complex I and II were lower in mitochondria isolated from skeletal muscle in L compared to NR rats ($p = 0.044$ and $p = 0.032$, respectively). (Figure 3E and 3F).

Markers of oxidative and lipid metabolism.

The PPAR superfamily is associated with the regulation of genes involved in oxidative metabolism. Specifically PGC-1α is associated with regulation of genes involved in mitochondrial biogenesis (52) and PPARδ is associated with genes involved in lipid and glucose metabolism (18, 37). PGC-1α liver protein expression was higher in L rats compared to P and NR rats ($p = 0.035$ and $p = 0.001$, respectively) (Figure 4A). Also, PPARδ liver protein expression was higher in L compared to NR rats ($p = 0.009$) (Figure 4B). No statistically differences were detected for PGC-1α and PPARδ in skeletal muscle ($p = 0.331$ and $p = 0.691$, respectively) (Figure 4C and 4D). PPARδ protein expression in WAT was higher in L compared to P and NR rats ($p < 0.001$) (Figure 4E).

Markers of oxidative stress.

The balance between oxidants and antioxidants is often referred to as oxidative stress. Endogenous antioxidant defense mechanisms exist to protect and detoxify oxidants. Specifically, SOD2 and SOD1 act to detoxify superoxide into the lesser harmful hydrogen peroxide. Hydrogen peroxide can be then further detoxified by CAT and GPX. The protein levels of the antioxidants (SOD2, SOD1, CAT, and GPX) and markers of oxidative damage were compared between groups. Liver SOD2 protein levels were higher in L compared to NR and P rats ($p = 0.001$ and $p = 0.025$, respectively) (Figure 5A). Liver CAT protein expression was higher in L and P rats compared to NR rats ($p = 0.015$ and $p = 0.001$, respectively) (Figure 5C). No statistical differences ($p > 0.05$) were detected for SOD1, GPX, 4-HNE or protein carbonyls in liver (Figure 5B, 5D, 5E, and 5F). SOD2 protein levels were higher in WAT of L compared to

NR rats ($p = 0.039$) (Figure 6A), and 4-HNE was lower in WAT of L rats compared to NR rats ($p = 0.004$) (Figure 6E). No statistical differences ($p > 0.05$) were detected for SOD2, SOD1, CAT, GPX, 4-HNE, or protein carbonyls in skeletal muscle (Figure 7).

DISCUSSION

The metabolic changes that a female experiences during lactation are often the most dramatic changes that she will experience in her lifetime. In humans, it has been estimated that metabolic expenditure can be increased by 480 kcal/day in women that are breastfeeding (5). Many metabolic challenges (e.g., exercise, calorie restriction, etc.) have demonstrated to provide protective health benefits against diseases such as obesity and type II diabetes (2, 6, 17, 39). Epidemiological data suggests that maintaining lactation by breastfeeding also contributes to a lower prevalence of obesity and type II diabetes, in addition to other health disparities (31, 35, 43-45). Yet despite this knowledge, our understanding of the metabolic differences between females that maintain lactation and those that do not following lactation is limited. Our data show the maternal benefits of lactation that exist 28 days postpartum. Specifically, we observed a reduced body and WAT mass and lower serum glucose concentration in animals that lactated. Furthermore, our data indicate that glucose metabolism was improved, in part, due to improved liver mitochondria function.

Change in body and tissue mass.

The process of milk synthesis presents a high energetic demand to the mother. In mice resting metabolic rate during peak lactation has been recorded as being 2-fold higher than controls (41). A large contribution to this increase during lactation is imposed by the liver and mammary gland that is actively synthesizing milk. Our findings

show that P rats had a higher body mass 28 days post-partum compared to NR, while no statistical differences were detected between L and NR rats. While the high energy demand of breastfeeding is often supported through means of hyperphagia, seen by an increase of 300% food intake in rats and 25% in humans (34), our results show that breastfeeding still serves as an important event in decreasing body mass gains observed during pregnancy. Furthermore, our results indicate that while body mass was not significantly different between NR and L rats, changes in body composition do occur. WAT mass was lower in L rats compared to both P and NR rats. Of note, our findings of decreased WAT mass would agree with previous findings showing lactation leads to a reduction in absolute fat mass associated with fewer visceral adipocytes (27-29). In addition, liver is hypertrophied during gestation and lactation in order to meet the demands of higher energy utilization. Our data support this, and liver mas was still higher in L rats 28 days postpartum which in fact may have masked some of the body mass differences between the groups. These findings highlight the extent of anatomical changes that occur in response to the metabolic perturbations imposed by breastfeeding, but also underscore the extensive physiological changes that must occur within these tissues in order for these phenotypic changes to take place.

Lactation and glucose metabolism.

Systemic changes in numerous metabolic processes must occur during pregnancy in order for the female's body to support the growing fetus. One such change is an increase in maternal insulin production. This change facilitates an increase in glucose transport to the fetus, however this can often result in increased insulin resistance in maternal cells. In some cases, this may manifest itself in the form of

gestational diabetes. This change also appears to have longer lasting effects that dispose the mother to a higher risk of developing type II diabetes later in life if she does not breastfeed her child. This idea is supported through epidemiological findings of decreased prevalence of type II diabetes in women that breastfed their children compared to those that did not (31, 46). In contrast to pregnancy, there is a decrease in insulin secretion during lactation which is associated with a decline in β-cell proliferation and improved insulin sensitivity (15). This finding alone supports the idea that breastfeeding may serve to protect against the development of type II diabetes.

Importantly, our finding of higher liver protein levels of PPARδ in L rats may help explain increased glucose metabolism. PPARδ plays a significant role in the regulation of glucose metabolism and insulin sensitivity (18) and Sanderson and collaborators demonstrated its importance in the liver. Specifically, knockout models of PPARδ had lower expression of genes relating to glucose metabolism and higher plasma glucose in a fasted state compared to wild type mice (40). The studies described above and our data, in which fasted serum glucose was decreased and liver PPARδ protein expression was higher in L rats, suggest that the resulting modulation of PPARδ expression that occurred during lactation and persisted thereafter is at least one mechanistic that may be beneficial in conferring a protective phenotype against type II diabetes. In addition, we report that mitochondrial function was enhanced in L rats when using complex I substrates, as shown by a higher RCR. When considering that complex I respiration plays a larger role through the utilization of glucose due to a larger NADH:$FADH_2$ production occurring in glycolysis compared to beta-oxidation, our finding of increased complex I function in mitochondria provides a mechanistic outcome for enhancing the

ability to metabolize glucose. Interestingly, measurements of enzymatic activity of the electron transport system show that both P and L rats had higher complex I activity. Our findings of increased liver complex I mitochondrial function in L animals may be better explained by the increased protein expression of PGC-1α and PPARδ. While PPARδ knockout models have demonstrated PPARδ is involved in the expression of genes relating to oxidative metabolism, PGC-1α is a well-known regulator of mitochondrial biogenesis and coactivator of genes involved in oxidative phosphorylation (52). Thus, increased expression of these proteins likely results in the improved mitochondrial function observed. Alternatively, L rats did express lower liver complex II activity compared to both P and NR, as well as lower respiration when using complex II substrates. As stated above, fat metabolism via beta oxidation produces a lower NADH:$FADH_2$ ratio compared to carbohydrate utilization, and perhaps this can be viewed as a result of fat sparing by the liver in order to divert lipid sources for milk synthesis.

Lactation drives lipolysis.

During pregnancy, the mother's body experiences increasing visceral adiposity (8). During breastfeeding however, a shift in lipoprotein lipase and triacylglyceride levels facilitate the mobilization of fats to the mammary gland to be used for milk synthesis (12). While our findings support previous findings of reduced WAT depots in L rats, we provide evidence of how this may occur. Specifically, our data show that PPARδ protein expression is increased in WAT of L compared to NR and P rats. In contrast to PPARδ's role in liver, PPARδ in WAT is involved in catabolic effects through oxidation of fatty acids (37). Our findings of increased PPARδ protein expression in two functionally

different metabolic tissues also demonstrate that a systemic signaling event is a likely candidate for driving these changes. One potential systemic stimulus that may be capable of inducing these changes is the hormone prolactin. While often associated with its role in mammary development, prolactin is also implicated in signaling changes in lipid and carbohydrate metabolism. For instance, prolactin has been shown to affect lipid utilization by imposing inhibitory effects on lipoprotein lipase activity in adipose tissue, allowing for circulating lipid sources to be spared for milk synthesis (19). Furthermore, prolactin is active in carbohydrate metabolism by improving insulin sensitivity, by interacting with pancreatic β-cells, and by stimulating the glucose sensing enzymes glucokinase and glucose transporter 2 (23, 50). However, the extent of prolactin's role in PPARδ expression has not been elucidated.

Oxidative stress in liver and WAT.

The production of reactive oxygen species (ROS) is a naturally occurring phenomenon that can manifest itself through processes of energy production and metabolism; however an imbalance between ROS production and a counteracting antioxidant system can result in oxidative damage (33). In regards to the field of biology, a central tenet is that reproductive investment increases oxidative stress that has been implicated as a driving force in the development of diabetes and other health disparities (3, 10). Researchers have supported this theory through the prediction that heightened energy expenditure during reproduction would lead to increased oxidative damage in tissues and thus shortening lifespan (7, 26). However, data supporting these claims remains largely equivocal (42) and our data show no differences in markers of liver lipid peroxidation and protein oxidation between groups. In this regard, our finding of higher

liver SOD2 in L rats can be, at least, explained by increased PGC-1α, as SOD2 is a downstream target of PGC-1α (21). Increased liver SOD2 could explain why oxidative damage was not different between groups. Systemic effects of lactation are further demonstrated by our finding that SOD2 protein levels were higher in WAT of L rats. However, oxidative damage was lower in WAT of L as shown by decreased 4-HNE. Reduced oxidative damage in WAT following breastfeeding and lactation may serve as a beneficial adaptation, as oxidative stress in WAT has been proposed to disrupt adipokine secretion by WAT, which may play a role in the development of metabolic syndrome (10). This finding is yet another potential mechanisms of how breastfeeding may be beneficial, as the prevalence of metabolic syndrome is lower in human populations who participate in breastfeeding following parturition (35).

Conclusions.

Breastfeeding reduces the prevalence of many disease conditions (e.g., obesity, type II diabetes, metabolic syndrome, and cardiovascular disease) in reproductive females (31, 35, 44, 46). However, the mechanistic basis of how lactation provides long-term health benefits is largely unknown. We report that animals who experienced lactation had reduced body mass, reduced WAT mass, and exhibited changes in mitochondrial function and select markers of metabolism and oxidative stress after lactation had ended and mammary tissue had regressed. Collectively these data advance our understanding of the health benefits of breastfeeding. However, future research is necessary to observe the extent at which these changes persist beyond our experimental model. In closing, our findings help to lay the groundwork of understanding

the protective maternal health benefits conferred via breastfeeding seen in human populations.

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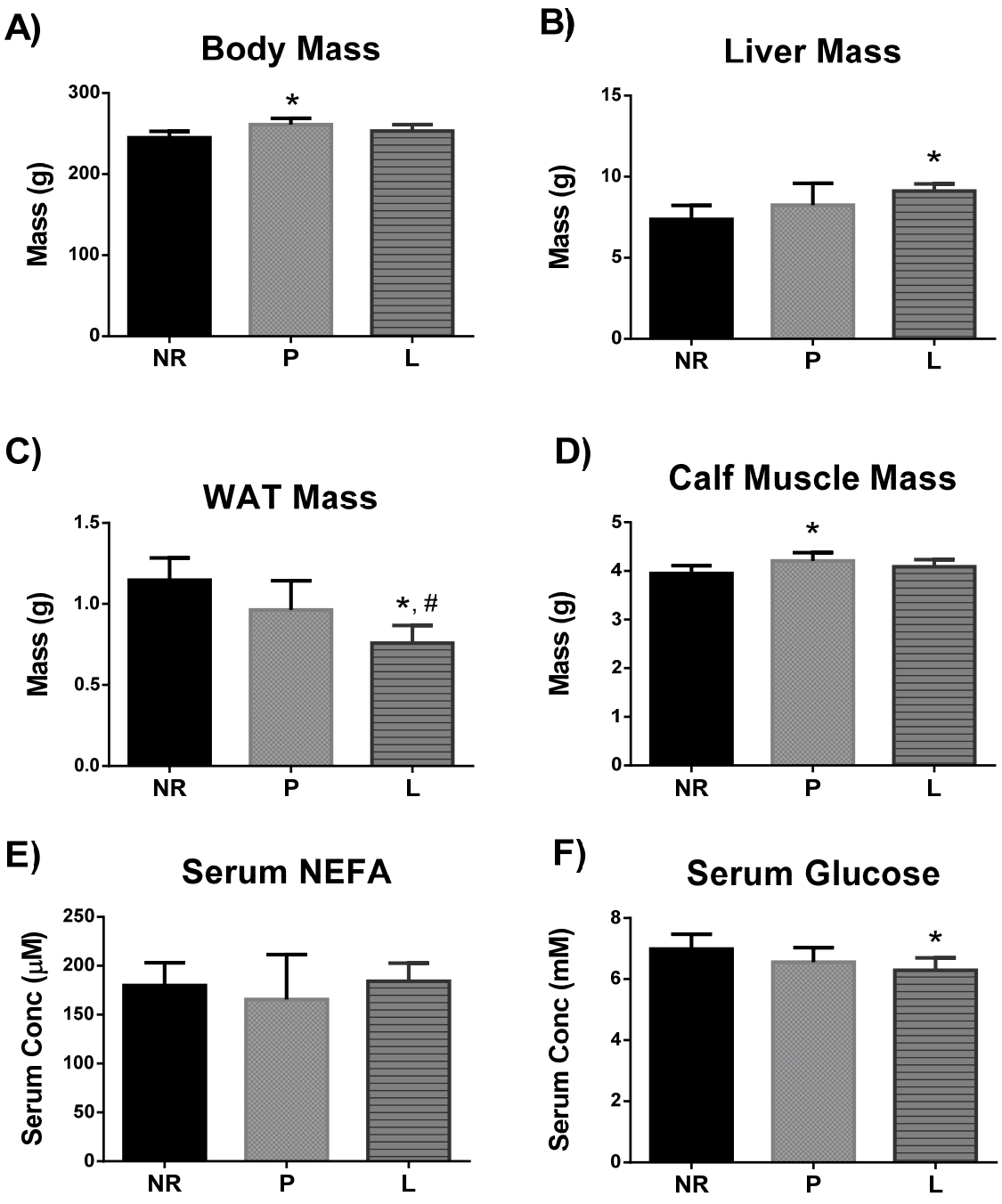
Figure 1. Body mass, tissue mass, and serum metabolites for age-matched rats that did not reproduce (NR), and rats that did not (P) or did (L) suckle their young for 21 days postpartum. A) Body mass, B) liver mass, C) retroperitoneal white adipose tissue (WAT) mass, D) mass of both rear triceps surae (calf muscle mass), E) serum concentration of non-esterified fatty acids (NEFA), and F) serum concentration of glucose. Data shown are mean ± SD. * indicates different from NR ($p < 0.05$), and # indicates different from P ($p < 0.05$).


A)
Body Mass
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300
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B)
Liver Mass
Mass (g)
15
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NR
P
L
C)
WAT Mass
Mass (g)
1.5
1.0
0.5
0.0
*, #
NR
P
L
D)
Calf Muscle Mass
Mass (g)
5
4
3
2
1
0
*
NR
P
L
E)
Serum NEFA
Serum Conc (μM)
250
200
150
100
50
0
NR
P
L
F)
Serum Glucose
Serum Conc (mM)
8
6
4
2
0
*
NR
P
L

Figure 2. Respiration and oxidant emission from mitochondria isolated from the liver and skeletal muscle of age-matched rats that did not reproduce (NR), and rats that did not (P) or did (L) suckle their young for 21 days postpartum. Data include liver A) state 3 respiration utilizing complex I substrates (pyruvate and malate; P/M), B) state 4 respiration utilizing P/M, C) respiratory control ratio (RCR) utilizing P/M), D) state 3 respiration utilizing complex II substrates (succinate; suc), E) state 4 respiration utilizing Suc, F) RCR utilizing suc. Data also include skeletal muscle G) state 3 respiration utilizing P/M, H) state 4 respiration utilizing P/M, I) RCR utilizing P/M, J) state 3 respiration utilizing suc, K) state 4 respiration utilizing suc, L) RCR utilizing suc. Finally M) oxidant emission from liver and N) oxidant emission from skeletal muscle are also presented. Oxygen consumption and hydrogen peroxide (H_2O_2) rates were normalized to citrate synthase (CS). Data shown are mean ± SD. * indicates different from NR ($p < 0.05$), and # indicates different from P ($p < 0.05$).

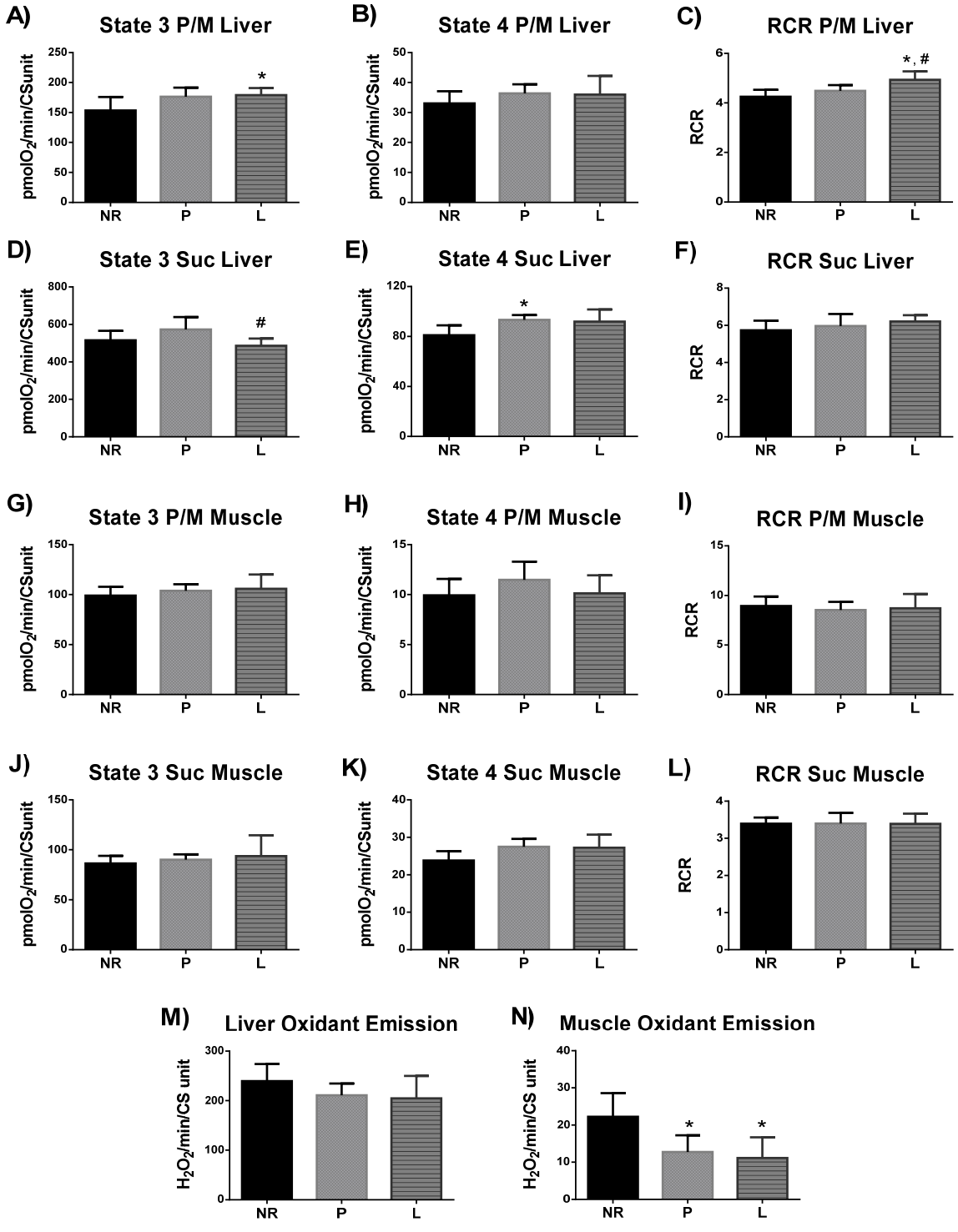

A)
State 3 P/M Liver
pmolO2/min/CSunit
NR
P
L
B)
State 4 P/M Liver
C)
RCR P/M Liver
RCR
D)
State 3 Suc Liver
E)
State 4 Suc Liver
F)
RCR Suc Liver
G)
State 3 P/M Muscle
H)
State 4 P/M Muscle
I)
RCR P/M Muscle
J)
State 3 Suc Muscle
K)
State 4 Suc Muscle
L)
RCR Suc Muscle
M)
Liver Oxidant Emission
H2O2/min/CS unit
N)
Muscle Oxidant Emission

Figure 3. Enzymatic activity of the mitochondrial complexes for liver and skeletal muscle of age-matched rats that did not reproduce (NR), and rats that did not (P) or did (L) suckle their young for 21 days postpartum. Data include A) complex I, B) complex II, C) complex III, and D) complex IV activity in the liver. In addition, data include E) complex I, F) complex II, G) complex III, and H) complex IV activity in skeletal muscle. Complex activity data are normalized to citrate synthase (CS). Data shown are mean ± SD. * indicates different from NR ($p < 0.05$), and # indicates different from P ($p < 0.05$).

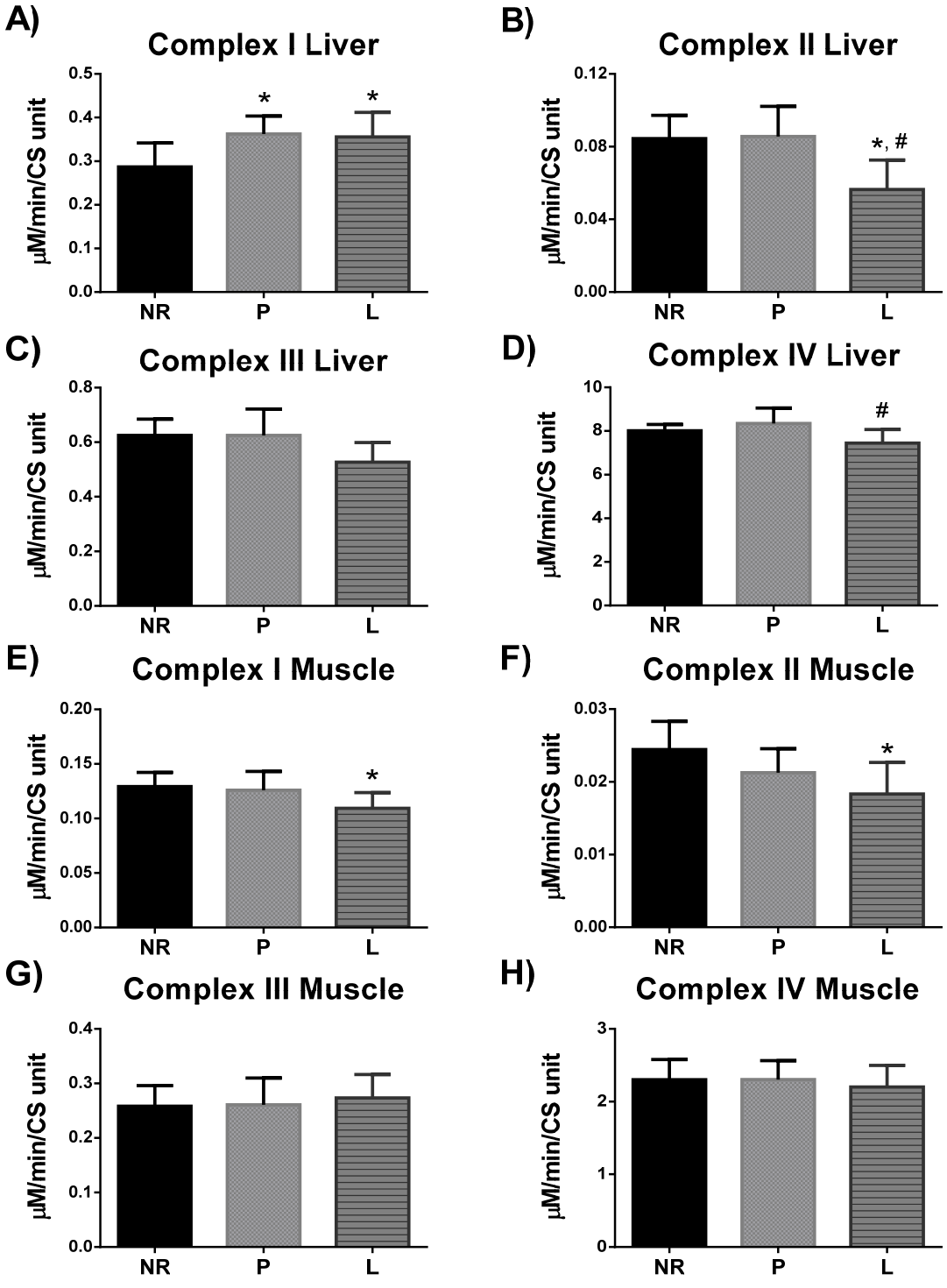

A)
Complex I Liver
µM/min/CS unit
0.5
0.4
0.3
0.2
0.1
0.0
*
*
NR
P
L
B)
Complex II Liver
µM/min/CS unit
0.12
0.08
0.04
0.00
*, #
NR
P
L
C)
Complex III Liver
µM/min/CS unit
0.8
0.6
0.4
0.2
0.0
NR
P
L
D)
Complex IV Liver
µM/min/CS unit
10
8
6
4
2
0
#
NR
P
L
E)
Complex I Muscle
µM/min/CS unit
0.20
0.15
0.10
0.05
0.00
*
NR
P
L
F)
Complex II Muscle
µM/min/CS unit
0.03
0.02
0.01
0.00
*
NR
P
L
G)
Complex III Muscle
µM/min/CS unit
0.4
0.3
0.2
0.1
0.0
NR
P
L
H)
Complex IV Muscle
µM/min/CS unit
3
2
1
0
NR
P
L

Figure 4. Markers of metabolism in liver, skeletal muscle, and white adipose tissue (WAT) of age-matched rats that did not reproduce (NR), and rats that did not (P) or did (L) suckle their young for 21 days postpartum. Data include A) PGC-1α and B) PPARδ protein levels in liver. C) PGC-1α and D) PPARδ protein levels in skeletal muscle. E) PPARδ protein levels in WAT. Representative blots are shown under the graphs. Data shown are mean ± SD. * indicates different from NR ($p < 0.05$), and # indicates different from P ($p < 0.05$).

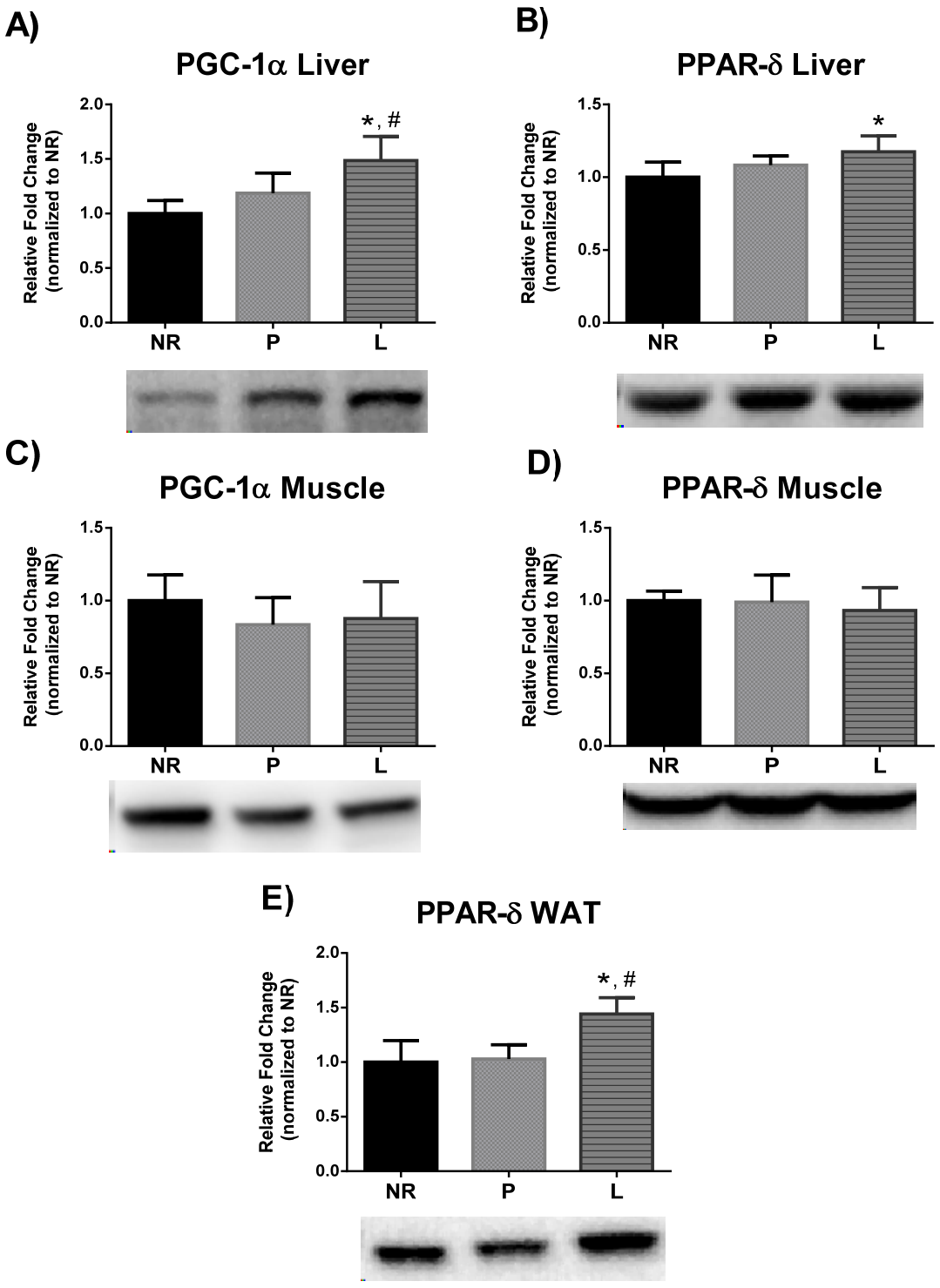

A)
PGC-1α Liver
Relative Fold Change (normalized to NR)
2.0
1.5
1.0
0.5
0.0
*, #
NR
P
L
B)
PPAR-δ Liver
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
*
NR
P
L
C)
PGC-1α Muscle
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
P
L
D)
PPAR-δ Muscle
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
P
L
E)
PPAR-δ WAT
Relative Fold Change (normalized to NR)
2.0
1.5
1.0
0.5
0.0
*, #
NR
P
L

Figure 5. Markers of oxidative stress in liver of age-matched rats that did not reproduce (NR), and rats that did not (P) or did (L) suckle their young for 21 days postpartum. Data include protein level of the antioxidants A) SOD2, B) SOD1, C) CAT, and D) GPX. In addition, data include markers of oxidative damage including E) lipid peroxidation determined by 4-HNE, and F) protein carbonyls levels determined by using oxyblot. Representative blots are shown under the graphs (A-D) or to the right of the graphs (E and F). Data shown are mean ± SD. * indicates different from NR ($p < 0.05$), and # indicates different from P ($p < 0.05$).

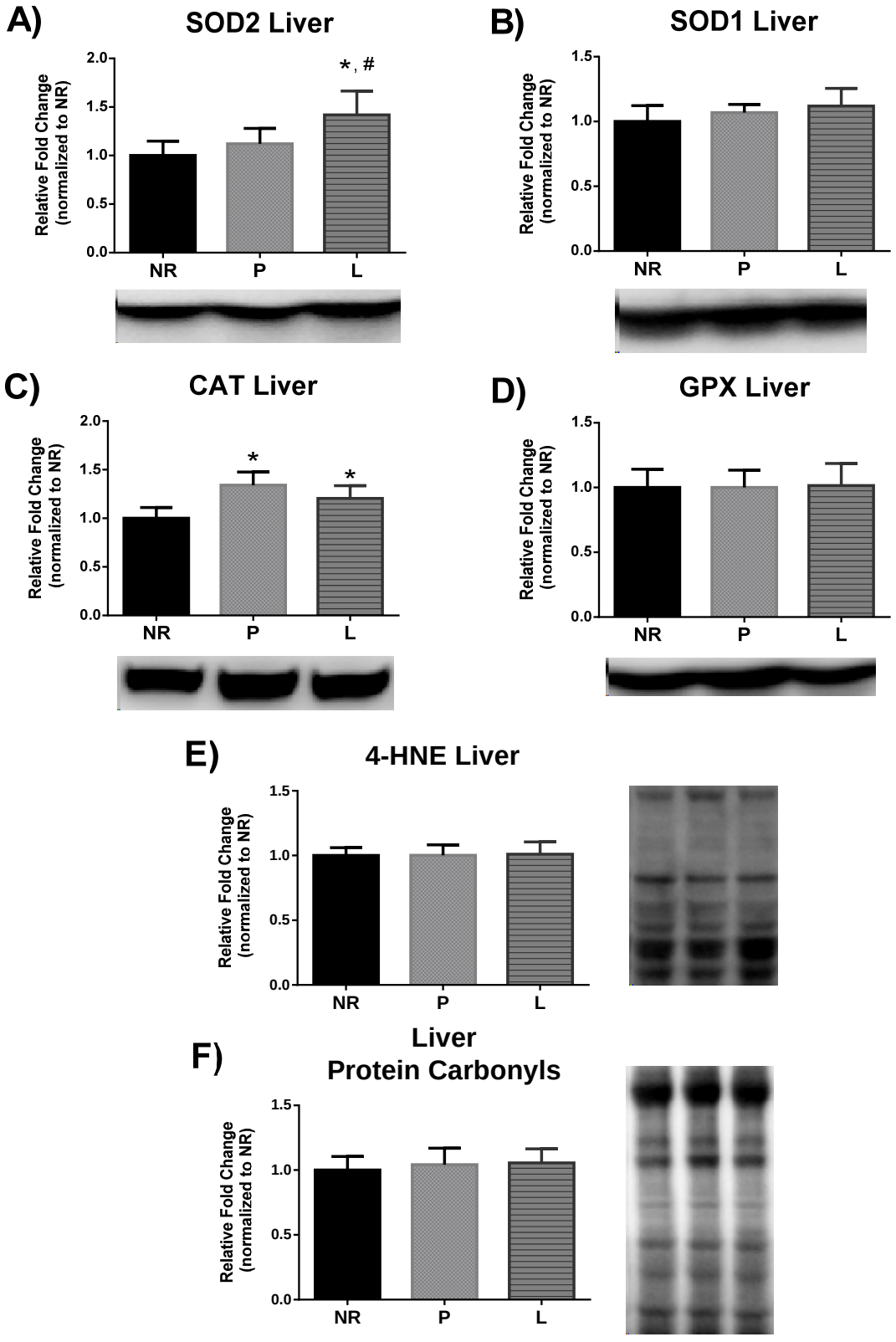

A)
SOD2 Liver
Relative Fold Change (normalized to NR)
2.0
1.5
1.0
0.5
0.0
*, #
NR
P
L
B)
SOD1 Liver
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
P
L
C)
CAT Liver
Relative Fold Change (normalized to NR)
2.0
1.5
1.0
0.5
0.0
*
*
NR
P
L
D)
GPX Liver
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
P
L
E)
4-HNE Liver
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
P
L
F)
Liver
Protein Carbonyls
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
P
L

Figure 6. Markers of oxidative stress in white adipose tissue (WAT) of age-matched rats that did not reproduce (NR), and rats that did not (P) or did (L) suckle their young for 21 days postpartum. Data include protein level of the antioxidants A) SOD2, B) SOD1, C) CAT, and D) GPX. In addition, data include E) lipid peroxidation determined by 4-HNE, Representative blots are shown under the graphs. Data shown are mean ± SD. * indicates different from NR ($p < 0.05$).

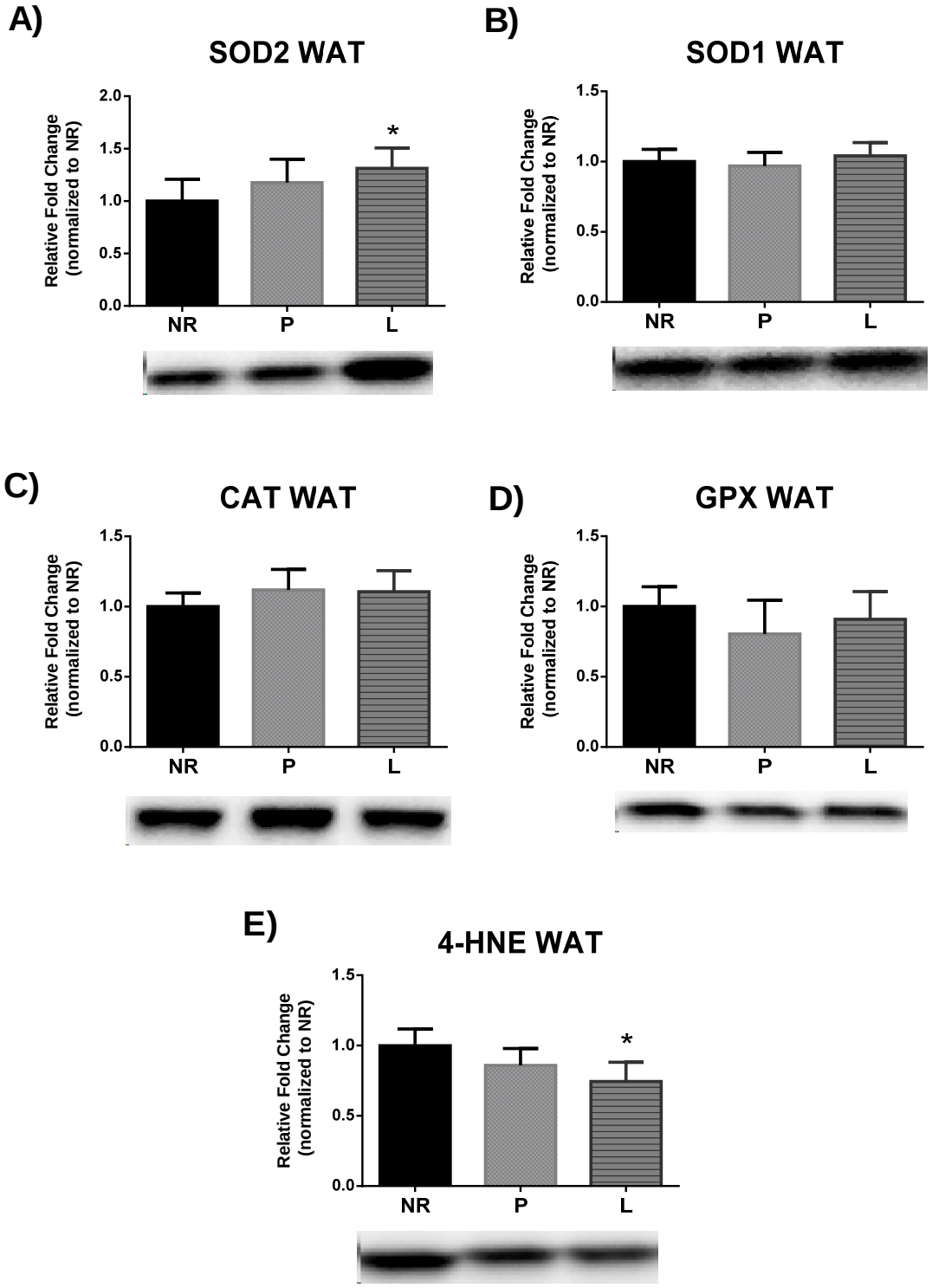
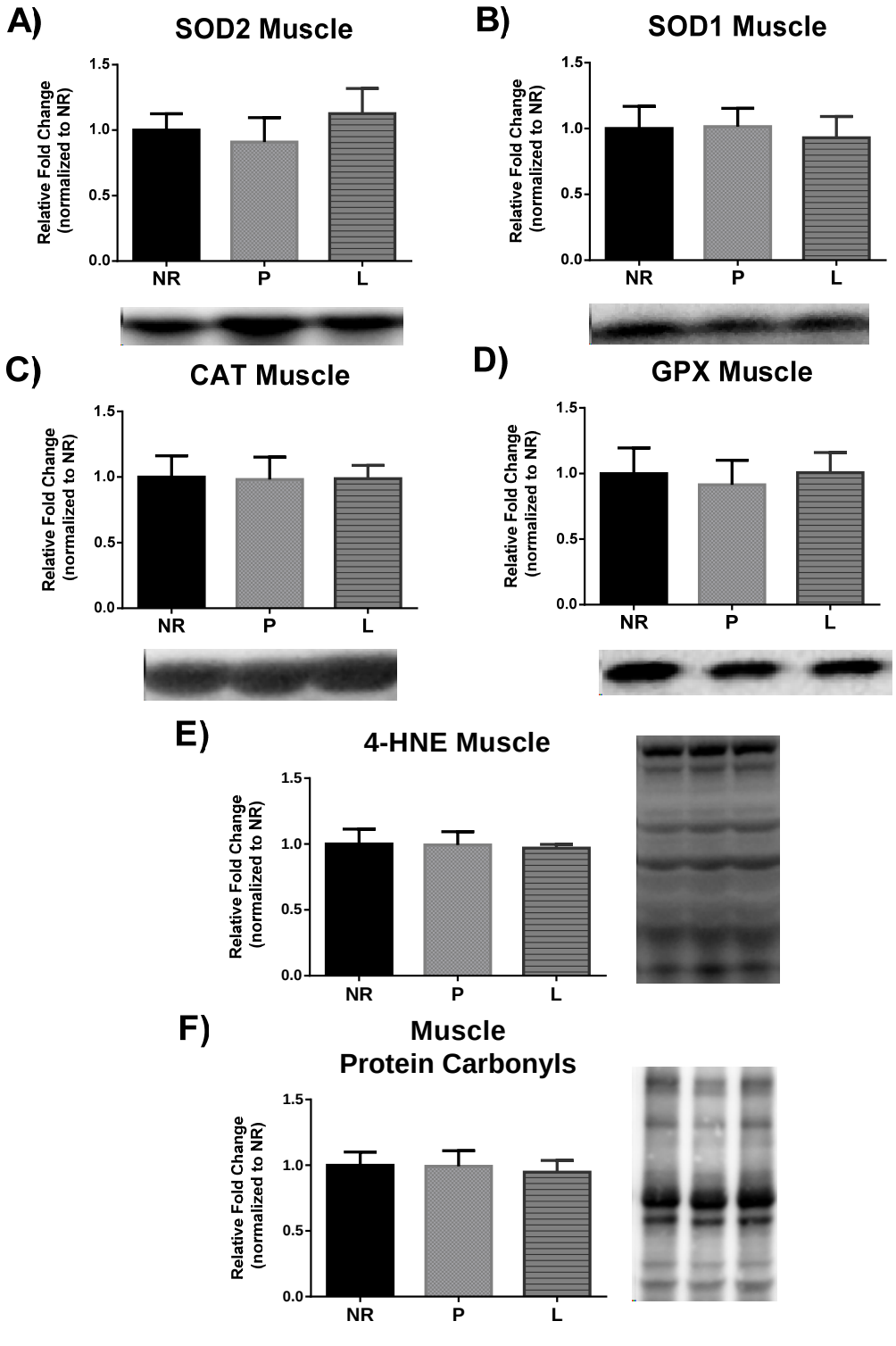

A)
SOD2 WAT
Relative Fold Change (normalized to NR)
2.0
1.5
1.0
0.5
0.0
*
NR
P
L
B)
SOD1 WAT
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
P
L
C)
CAT WAT
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
P
L
D)
GPX WAT
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
P
L
E)
4-HNE WAT
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
*
NR
P
L

Figure 7. Markers of oxidative stress in muscle of age-matched rats that did not reproduce (NR), and rats that did not (P) or did (L) suckle their young for 21 days postpartum. Data include protein level of the antioxidants A) SOD2, B) SOD1, C) CAT, and D) GPX. In addition, data include markers of oxidative damage including E) lipid peroxidation determined by 4-HNE, and F) protein carbonyls levels determined by using oxyblot. Representative blots are shown under the graphs (A-D) or to the right of the graphs (E and F). Data shown are mean ± SD.


A)
SOD2 Muscle
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
P
L
B)
SOD1 Muscle
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
P
L
C)
CAT Muscle
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
P
L
D)
GPX Muscle
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
P
L
E)
4-HNE Muscle
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
P
L
F)
Muscle
Protein Carbonyls
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
P
L

V. ALTERATIONS IN METABOLISM AND MITOCHONDRIAL FUNCTION ARE EVIDENT 12 WEEKS FOLLOWING THE CESSATION OF LACTATION: EVIDENCE FOR THE RESET HYPOTHESIS

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ABSTRACT

Human epidemiological data show that breastfeeding reduces the prevalence of obesity, type II diabetes, hypertension, and several types of cancer compared to mothers that give birth but do not participate in lactation. The current study seeks to delineate the mechanisms involved in metabolism, mitochondrial function, and oxidative stress following lactation that may underlie a protective phenotype against disease. Ten-week old female Sprague-Dawley rats were divided into three groups (n = 8 per group): 1) rats that did not reproduce (NR), 2) rats that were allowed to mate and become pregnant but did not suckle their pups after giving birth (NL), and 3) rats that were allowed to mate and become pregnant and suckled their pups for 21 days before weaning (L). All animals were sacrificed at a time corresponding to 12 weeks following the weaning of pups. Our data show no statistical differences in body, skeletal muscle, or white adipose tissue mass between groups, but liver mass was lower in rats that lactated. Isolated liver mitochondrial respiration was higher in rats that lactated when using NADH-linked substrate and these rats had lower serum glucose concentration. Additionally, the lactation group exhibited changes in liver, skeletal muscle, and white adipose tissue peroxisome proliferator activated receptor delta (PPARδ) protein levels that may, in part, explain the observed lower serum glucose concentration. No statistical differences in markers of oxidative damage or antioxidant protein levels were detected between groups indicating that reproductive investment does not serve as a tradeoff for tissue maintenance. These novel animal findings provide evidence of differences in metabolic processes that persist 12 weeks following weaning, and provide mechanistic

insight on how lactation may confer a healthy phenotype later in life that is protective against metabolic disease in mothers that breastfed.

INTRODUCTION

Lactation imposes one of the most metabolically challenging events in a female's lifetime. Importantly, human epidemiological data have demonstrated an association between breastfeeding and a lower risk of obesity, type II diabetes, hypertension, and several types of cancer (6, 17, 31, 34). While a plethora of data exists on how metabolic perturbations such as exercise, fasting, and calorie restriction can promote a healthy phenotype (7, 15, 22, 44), very little research has been conducted on the mechanistic basis to explain how the metabolically tasking occurrence of lactation may confer health benefits to the mother.

During pregnancy, a female's body must undergo drastic alterations in metabolic processes that may predispose her to certain health disparities. With the onset of pregnancy, maternal insulin secretion increases in order to support glucose and amino acid transport to the fetus. However, a combination of increased insulin secretion, increases in circulating lipids, and increased visceral adiposity often leads to insulin resistance of maternal cells (26, 35). However, during breastfeeding metabolic alterations occur to shift resources toward milk synthesis and aide to revert insulin resistance. Indeed, breastfeeding results in improved glucose handling via a decrease in insulin production, improved insulin sensitivity, and a drop in β-cell proliferation. Conversely, lipid metabolism is decreased in metabolically active tissues and lipid stores are mobilized to facilitate lipid transport to the mammary gland for milk synthesis (14, 18). Thus, lactation aides in reducing postpartum adiposity; potentially reducing the risk of obesity (5). Given this evidence, Stuebe and Rich-Edwards proposed the reset

hypothesis which posits that lactation is a critical event in resetting metabolic processes that occur during pregnancy and reduce the prevalence of metabolic disease (43).

In this regard, liver, skeletal muscle, and white adipose tissue (WAT) contribute more than 50% of the metabolic rate in mammals (37), and thus modulation of the metabolic requirements of these tissues is important during the increased energy demands imposed by lactation. Indeed, Gutgessel and collaborators demonstrated reduced mRNA expression in skeletal muscle for genes involved in lipid oxidation, and Pichaud and collaborators showed reduced electron transport activity in liver during lactation (13, 33). Considering that dysregulation of mitochondrial function and metabolism have been demonstrated to play implicit roles in health disparities (e.g., obesity and type II diabetes) (25, 28), these would appear to be important markers for understanding how lactation may protect against disease. Furthermore, conditions of oxidative stress have been implicated in the development of these health disparities (1, 11), and thus may underpin a mechanism in which lactation may also play a role in reducing prevalence of disease. However, some researchers have posited that reproduction may actually be detrimental to health and impact longevity due to increases in oxidative stress (8, 9, 29). While energy requirements are increased during reproduction, and oxidants are produced as a by-product of ATP production in the mitochondria (42), evidence to support this claim is limiting.

Nonetheless, the persistence of mechanistic events that dictate changes in metabolism, mitochondrial function, and oxidative stress due to lactation or the absence of lactation following parturition have not been previously observed. Thus, the current study seeks to delineate the long-term differences that occur in markers of metabolism,

mitochondrial function, and oxidative stress due to the reproductive status. We hypothesized that markers of metabolism in animals that lactated would be reflective of a phenotype that is resistant against metabolic disease, having better functioning mitochondria, and be protected against oxidative damage compared to animals that gave birth but did not suckle their young.

METHODS

Animal husbandry.

All experimental procedures were approved by Auburn University's Institutional Animal Care and Use Committee. Ten-week old Sprague-Dawley rats were obtained from Harlan Laboratories, Inc. Animals (n = 8 per group) were acclimated with their diet and facility ten days prior to experimental start. Rats were housed under standard laborartory conditions (46 x 25 x 20 cm boxes, 12L:12D cycle, 22˚C, 50% RH), and given *ad libitum* access to food (Tekland Global Diet 2018) and water. Animals were randomly assigned to one of three treatment groups: 1) rats that did not reproduce (NR), 2) rats that were allowed to mate and become pregnant but did not suckle their pups after giving birth (NL), and 3) rats that were allowed to mate and become pregnant and suckled their pups after giving birth for 21 days (L). Female rats were paired with same-group counterpart, but animals in groups that underwent mating were separated during pregnancy and/or lactation. fThe NL animals had their pups removed within 12 hours of birth. The L animals had their litter size adjusted to 8 on the day of parturition. All animals were age-matched and sacrificed at a time that corresponded to 12 weeks following 21 days of lactation in L animals.

Whole animal metabolism.

Whole-animal metabolic measurements were completed in a Sable System Promethion Metabolic Screening System (Las Vegas, NV, USA) housed in Auburn University's Lab Animal Health Facility. Each rodent box was equipped with a flow-through respirometry system for continuously monitoring oxygen intake, carbon dioxide output, and an infrared grid that continuously monitored the animals' activities (19). This system provided data to monitor energy expenditure and respiratory quotient of each rat.

Blood collection and analysis.

Rats were fasted four hours prior to blood collection. Animals were anesthetized using isoflurane vapors and body mass was quickly recorded. The anesthetized animals were than decapitated, and blood was collected, allowed to clot on ice, and then centrifuged. Following centrifugation the serum was frozen at -80ºC for subsequent analyses. Serum glucose (STA-680, Cell Biolabs, San Diego, CA, USA) and non-esterified fatty acids (NEFA) (STA-618, Cell Biolabs) were quantified using the manufacturer's specifications.

Tissue collection and handling.

After the decapitation, the following tissues were excised and weighed: liver, triceps surae ('calf' muscle), retroperitoneal white adipose tissue (RetroP WAT) and perirenal white adipose tissue (PR WAT) pads. After the mass of each tissue was recorded, a sample from calf skeletal muscle and liver was used for mitochondrial isolation and the remainder of tissues were frozen in liquid nitrogen and stored at -80ºC for subsequent analyses.

Mitochondrial isolation.

Mitochondrial isolations for muscle were performed as previously described (16). Excised muscles (~750 mg) were trimmed to remove fat and connective tissues, weighed, and placed in 10 volumes of solution I (100 mM KCl, 40 mM Tris HCl, 10 mM Tris base, 1 mM $MgSO_4$, 0.1 mM EDTA, 0.2 mM ATP, and 2% (wt/vol) free fatty acid bovine serum albumin (BSA), pH 7.40). Muscles were minced with scissors and the mince was homogenized for 15 seconds with a polytron. Protease (Trypsin) was added (5 mg/g wet muscle), and the digested mince was mixed continually for 7 minutes. Digestion was terminated by the addition of an equal volume of solution I. The homogenate was centrifuged at 500 *g* for 10 minutes at 4ºC and the supernatant was rapidly decanted through a double layer of cheesecloth and centrifuged at 3,500 *g* for 10 minutes. The supernatant was discarded and the mitochondrial pellet was resuspended in solution I. The suspension was centrifuged at 3,500 *g* for 10 minutes. The supernatant was again discarded, and the pellet was resuspended in 10 volumes of solution II (similar to solution I, but without BSA). This resuspended pellet was subsequently centrifuged at 3,500 *g* for 10 minutes. The final mitochondrial pellet was suspended in 250 µl of a solution containing 220 mM mannitol, 70 mM sucrose, 10 mM Tris HCl, and 1 mM EGTA, pH 7.40. Mitochondria from liver were isolated as previously described (30). Briefly, liver (~750 mg) was weighed and placed in 10 volumes of solution III (250 mM sucrose, 5 mM HEPES, and 1 mM EGTA), minced with scissors and the mince was homogenized with a Potter-Elvehjem PTFE pestle and glass tube (2 passes). The homogenate was centrifuged at 500 g for 10 minutes at 4ºC. The supernatant was rapidly decanted through a double layer of cheesecloth and

centrifuged at 3,500 g for 10 minutes. The supernatant was discarded and the mitochondrial pellet was resuspended in solution III. The suspension was centrifuged at 3,500 g for 10 minutes. The final mitochondrial pellet was suspended in 250 µl of a solution containing (in mM) 220 mannitol, 70 sucrose, 10 Tris HCl, and 1 EGTA, pH 7.40.

Isolated mitochondrial oxidative phosphorylation.

Mitochondrial oxygen consumption was measured as described by Messer et al. (27). Briefly, mitochondrial oxygen consumption was measured polarographically in a respiration chamber (Hansatech Instruments, United Kingdom). Isolated mitochondria (20 µL) were incubated with 1 ml of respiration buffer adapted from Wanders et al. (47) (100 mM KCL, 50 mM MOPS, 10 mM KH_2PO_4, 20 mM glucose, 10 mM $MgCl_2$, 1 mM EGTA, and 0.2% fatty acid free BSA; pH = 7.0) at 37°C in a respiratory chamber with continuous stirring. For state 3 respiration, 2 mM pyruvate and 2 mM malate (complex I substrates) or 5 mM succinate (complex II substrate) was used in the presence of 0.25 mM ADP, and state 4 respiration was recorded following the phosphorylation of ADP as described by Estabrook (10). Respiratory control ratio (RCR) was calculated as state 3/state 4 oxygen consumption. Respiration values were expressed as a ratio to citrate synthase to compensate for mitochondrial enrichment in the samples

Mitochondrial oxidant emission.

Oxidant emission by mitochondria was determined using the oxidation of the fluorogenic indicator Amplex Red (Molecular Probes, Eugene, OR) in the presence of horseradish peroxidase (20). The assay was performed at 37°C in 96-well plates using succinate as the substrate. Specifically, this assay was developed based on the concept

that horseradish peroxidase catalyzes the hydrogen peroxide-dependent oxidation of nonfluorescent Amplex Red to fluorescent Resorufin Red. Resorufin Red formation was monitored at an excitation wavelength of 545 nm and an emission wavelength of 590 nm using a multiwell plate reader fluorometer (Synergy H1, BioTek, Winooski, VT, USA). We recorded the level of Resorufin Red formation, and hydrogen peroxide production was calculated with a standard curve.

Enzymatic assays for electron transport chain complex activity.

Complex I (NADH dehydrogenase) enzyme activity (EC 1.6.5.3) was measured as a function of the decrease in absorbance from NADH oxidation by decylubiquinone before and after rotenone addition (45). Complex II (succinate dehydrogenase) activity (EC 1.3.5.1) was measured as a function of the decrease in absorbance from 2,6-dichloroindophenol reduction (45). Complex III (ubiquinol cytochrome *c* oxidoreductase) activity (EC 1.10.2.2) was determined as a function of the increase in absorbance from cytochrome *c* reduction (45). Complex IV (cytochrome *c* oxidoreductase) activity was determined as a function of the decrease in absorbance from cytochrome *c* oxidation (45). Specificity of complex IV activity was determined by monitoring changes in absorbance in the presence of KCN (45). Citrate synthase (EC 4.1.3.7) was measured as a function of the increase in absorbance from 5,5'-dithiobis-2-nitrobenzoic acid reduction (45). Enzyme activities were expressed as a ratio to citrate synthase to compensate for mitochondrial enrichment in the cell samples.

Protein abundance.

The relative concentration of proteins was quantified by Western blot analysis (20). To accomplish this, tissue was homogenized 1:10 (wt/vol) in 5 mM Tris HCl (pH

7.5) and 5 mM EDTA (pH 8.0), and protease inhibitor cocktail (14224-396, VWR, Radnor, PA, USA) and was centrifuged at 1500 *g* for 10 minutes at 4°C. Protein content of the supernatant was quantified by the method of Bradford (4). Proteins were separated by polyacrylamide gel electrophoresis via 4-20% polyacrylamide gels (BioRad, Hercules, CA, USA). After electrophoresis, the proteins were transferred to PVDF membranes. Non-specific sites were blocked in phosphate-buffered saline (PBS) solution containing 0.1% Tween 20 and 5% non-fat milk. Membranes were then incubated overnight at 4ºC with primary antibodies purchased from GeneTex (Irvine, CA, USA) directed against peroxisome proliferator activated receptor alpha (PPARα, GTX101096, 1:1000), peroxisome proliferator activated receptor delta (PPARδ, GTX113250, 1:2000), peroxisome proliferator activated receptor gamme, coactivator 1 alpha (PGC-1α, GTX37356, 1:1000), superoxide dismutase 1 (SOD1, GTX100554 1:2000), superoxide dismutase 2 (SOD2, GTX116093, 1:2000), catalase (CAT, GTX110704, 1:2000), and glutathione peroxidase (GPX, GTX116040, 1:2000). Following incubation with primary antibodies, membranes were washed (five minutes x 3) with PBS-Tween and then incubated with secondary antibodies for one hour in room temperature. After washing (five minutes x 3), a chemiluminescent system was used to detect labeled proteins (GE Healthcare, Buckinghamshire, UK). Images of the membranes were captured and analyzed by using the ChemiDoc-It2 Imaging System (UVP, LLC, Upland, CA). Protein expression was normalized to Ponceau staining.

Assessment of indices of oxidative damage.

To determine the relative amount oxidative damage, we measured protein oxidation and lipid peroxidation. Lipid peroxidation was assessed by determining 4-

hydroxynoneal (4-HNE; *trans*-4-hydroxy-2-nonenal, $C_9H_{16}O_2$) expression via western blotting. Primary antibody for 4-HNE was purchased from Abcam (ab46545; 1:1000 dilution, Cambridge, MA, USA). Protein oxidation was measured by comparing relative expression of protein carbonyls using a commercially available kit (Oxy-Blot protein oxidation detection kit; Intergen, Purchase, NY, USA) via western blotting as described by the manufacturer's instructions.

Statistics.

Comparison between groups for each dependent variable were made by a one-way one-way analysis of variance (ANOVA), with a Tukey post hoc test being used to determine significance differences between groups when the ANOVA indicated statistical significance. However, in the case of state 4 succinate muscle, CAT liver, and 4-HNE muscle the Brown-Forsythe test was significant and thus the Kruskal-Wallis test was performed followed by the Dunn's for multiple comparisons post-hoc. Respiratory quotient and energy expenditure data were analyzed by a mixed model ANOVA. Data are presented as means ± SD, and significance was established at $p < 0.05$.

RESULTS

Body and tissue mass.

Lactation has been demonstrated to impact body composition following parturition (5). Liver mass was lower in L compared to NL ($p = 0.017$). However, no statistical changes were observed in body mass, combined mass of rear triceps surae calf muscle, RetroP WAT, or PR WAT following a 12-week period post lactation ($p > 0.05$) (Figure 1).

Serum glucose and NEFA concentrations.

Dysregulation of two important blood metabolites, glucose and NEFA, can be indicative of metabolic disease. Considering that regulation of glucose and NEFA must occur during lactation, we sought to determine if these effects persist 12-weeks following a 21 day lactation period. Serum glucose concentrations were lower in L compared to NR ($p = 0.046$) (Figure 1G). No statistical differences were detected in serum NEFA concentrations ($p > 0.05$) (Figure 1F).

Whole animal metabolism.

In order to observe if lactation resulted in changes in whole body metabolism, 24 hour energy expenditure (EE) and respiratory quotient (RQ) were assessed using metabolic chambers. RQ and EE data showed a significant time effect ($p < 0.001$), however no statistical differences were detected between groups ($p > 0.05$) (Figure 2A and 2B).

Mitochondrial function and oxidant emission.

Measurements of isolated mitochondrial respiration rates provide insight to the capacity to utilize substrate and produce ATP. State 3 and state 4 respiration was higher in liver mitochondria in L compared to NL ($p = 0.025$ and $p = 0.041$, respectively) when utilizing complex I substrate (pyruvate/malate (P/M)) (Figure 3A and 3B). State 3 respiration in skeletal muscle was lower in L compared to NR ($p = 0.004$) when utilizing the complex II substrate succinate (Figure 3J), and skeletal muscle RCR was lower in L compared to NL ($p = 0.045$) (Figure 3L). No statistical differences were detected in oxidant emission or enzymatic activity of mitochondrial complex activity in liver or skeletal muscle ($p > 0.05$) (Figure 3 and 4).

Markers of metabolism.

The PPAR superfamily is associated with the regulation of genes involved in oxidative metabolism. Specifically, PPARδ is associated with genes involved in lipid and glucose metabolism (24, 36). Liver PPARδ protein levels were higher in L compared to NR ($p = 0.024$) (Figure 5B). Skeletal muscle PPARδ protein levels were lower in L compared to NR and NL ($p = 0.005$ and $p = 0.039$, respectively) (Figure 5D). PPARδ protein expression in RetroP WAT was higher in NL and L compared to NR ($p = 0.04$ and $p = 0.009$, respectively) (Figure 5F).

Markers of oxidative stress.

The balance between oxidants and antioxidants is often referred to as oxidative stress. Endogenous antioxidant defense mechanisms exist to protect and detoxify oxidants. Specifically, SOD2 and SOD1 act to detoxify superoxide into the lesser harmful hydrogen peroxide. Hydrogen peroxide can be then further detoxified by CAT and GPX. No statistical differences were detected in liver (Figure 6), skeletal muscle (Figure 7) in any of the aforementioned oxidative stress markers. However, GPX protein levels were higher in RetroP WAT of NL compared to NR ($p = 0.040$), but no other differences were reported in either RetroP or PR WAT (Figure 8).

DISCUSSION

Epidemiological reports show that breastfeeding decreases the prevalence of several maternal health disparities late in life and Stuebe and Rich-Edwards proposed that lactation may reset a female's probability of metabolic disease (43). In the current study, we use a rat experimental model to observe the effects that lactation, as well as the effects of parturition without lactation, has on the mother. We report decreased

serum glucose concentration and differing PPARδ protein levels in liver, skeletal muscle, and WAT in animals that lactated. Furthermore, the capacity of mitochondrial respiration was increased in the liver of animals that lactated, while a decrease was observed in skeletal muscle mitochondrial respiration. Our novel findings highlight that metabolic changes persist in multiple tissues following lactation and may lend credence to the reset hypothesis. Further discussion of our results follows.

Lactation has lasting effects on metabolism.

Interestingly we did not observe any changes in body mass or body composition (muscle or WAT mass) following 12 weeks after the cessation of lactation. The impact of breastfeeding in human populations on postpartum mass in the years immediately following parturition are currently equivocal, with some populations showing a decrease while others demonstrate no changes (32). However, several reports have shown that prevalence of obesity is decreased later in life in women who breastfed compared to those that did not (31, 34, 40). Undoubtedly, variation within these findings are difficult to interpret due to differing experimental techniques and the impact of a lifetime of human behavior. Unlike human populations where reproduction often occurs in fully developed females, our study may be limited as the animals we used were still young and growing in size. Thus, perhaps the growth of these animals may have eclipsed changes in body mass and body composition that would have occurred due to the lactation period or that the effects of lactation are subtle enough that a longer duration of lactation is required for changes to be observed. Furthermore, it may also be that one reproductive bout is not enough to induce changes in body mass or body composition, as the aforementioned epidemiological studies report lifetime parturition and lactation

that includes multiple births and breastfeeding periods. However, we do report that changes in metabolic markers and mitochondrial function exist 12 weeks post-lactation.

Specifically, liver PPARδ protein levels were higher in animals that lactated. Previously, Sanderson and collaborators utilized a knockout model of PPARδ, and observed lower expression of genes relating to glucose metabolism in the liver and higher plasma glucose in a fasted state compared to wild type mice (39). In this regard, we demonstrate that rats that lactated had lower serum glucose concentrations, suggesting that these rats metabolize glucose at a faster rate. An increase in the capacity of the liver to metabolize glucose is further supported by our findings of increased state 3 and state 4 respiration in liver mitochondria when a complex I substrate (P/M) was used as the fuel. A higher state 3 mitochondrial respiration indicates a higher maximal capacity for ATP production, but also an increase of state 4 respiration may be indicative of an overall increase in energy utilization. State 4 mitochondrial respiration can be used as a proxy for leak respiration. Leak respiration has been linked to increases in energy expenditure due to its involvement in multiple roles such as thermogenesis and reducing oxidant production in the mitochondria (3). Thus, our novel findings of increased PPARδ expression in liver, increased mitochondrial respiration in liver, and decreased serum glucose provides evidence of mechanistic events in which lactation may be protective against the development of type II diabetes: supporting the lactation reset hypothesis. Furthermore, liver mass was decreased in animals that lactated. Should this be reflective of decreased hepatic fat content, this would indicate a reduced risk of developing type II diabetes, considering

hepatic fat content is associated with insulin resistance (38). However, our experimental design did not include measurements of hepatic fat and warrants further research.

We do not report any differences between groups in energy expenditure. However, measurements taken in the metabolic cages reflect whole body metabolism and do not provide information on relative metabolism of different tissue types. Indeed, our results would suggest a shift in tissue metabolism, as we also report that PPARδ protein levels were lower in skeletal muscle of animals that lactated. PPARδ regulates the expression of genes involved in lipid metabolism in skeletal muscle (23), therefore a decrease in PPARδ in skeletal muscle may indicate reduced lipid oxidation. Indeed, rats that lactated had a lower skeletal muscle state 3 mitochondrial respiration when a complex II substrate was utilized. Considering that beta oxidation results in a higher FADH/NADH ratio, this can be considered a decrease in the capacity to utilize substrate from oxidized lipid sources. A decrease in lipid metabolism in skeletal muscle and an increase in glucose metabolism in liver of rats that lactated would act to balance out our observed similarities of total energy expenditure between groups. Furthermore, PPARδ protein levels in RetroP WAT were higher in rats that reproduced and PPARδ plays a catabolic role in WAT via lipid oxidation (14, 36). Our findings of differing PPARδ protein levels in liver, skeletal muscle, and WAT indicates that a systemic signaling event drives many of the metabolic changes with lactation, however the specific upstream regulator of PPARδ in lactation is currently unknown and warrants further research.

Oxidative stress.

Reactive oxygen species (ROS) are capable of damaging cellular structures; but ROS are a naturally occurring phenomenon that can manifest during energy production

in the mitochondria. While ROS can also serve as important signaling stimuli, a balance must be maintained between oxidants and antioxidants in order to avoid cellular damage; this balance is referred to as oxidative stress. In this regard, the free radical theory of aging posits that ROS production over a lifetime leads to cumulative damage causal in cellular senescence. Furthermore, the soma theory of aging postulates that participation in reproduction detracts from available resources normally utilized for somatic maintenance (e.g. endogenous antioxidant defenses) (21). Given that a heightened energetic demand is required during reproduction and thus a greater opportunity for ROS production, researchers have posited that reproduction poses a life-tradeoff between longevity and reproduction (8, 9, 29). However, mechanistic evidence supporting increased oxidative stress due to reproduction remains equivocal (2, 41). On the contrary, several reports have actually observed decreased markers of oxidative stress in liver during lactation (12, 48). Indeed, other metabolic challenges such as exercise have actually shown to provide a hormetic response to oxidative stress (34). Nonetheless, our findings demonstrate no changes between groups in oxidative damage for skeletal muscle, liver, or WAT 12 weeks post lactation. Thus, it appears unlikely that reproduction would be detrimental to longevity due to alterations in oxidative stress. Importantly, oxidative stress is also implicated in many of the disease conditions that have been observed to be decreased in populations that breastfed, such as obesity, type II diabetes, and cancer (1, 11, 46). Thus, evidence of a hormetic response with lactation in oxidative stress would serve to support the lactation reset hypothesis.

Conclusions.

To our knowledge, we are the first to observe the prolonged effects of one bout of reproduction (with or without lactation) on whole animal, molecular, and cellular markers. While we did not observe alterations in body mass, skeletal muscle, or WAT, we report that liver mass was lower in animals that lactated 12 weeks following the weaning of pups. However, our study was limited in observations of body mass as the animals used were still growing after their pups had been weaned. We report novel findings of altered markers of metabolism and mitochondrial function in liver and skeletal muscle in animals that lactated. Specifically, serum glucose concentrations were lower in animals that lactated. Importantly, we report altered PPARδ levels in liver, skeletal muscle, and WAT. Furthermore, we observed that mitochondrial function is altered in liver and skeletal muscle 12 weeks following the cessation of lactation, such that respiration capacity is increased in liver and decreased in skeletal muscle of animals that lactated. Finally, we report that reproduction did not result in any lasting effects in oxidative damage or antioxidant levels, suggesting that oxidative damage does not play a role in the soma theory of aging. Our novel findings of altered metabolic and mitochondrial function following an extended recovery duration lends credence to the reset hypothesis. However, future research is needed to expound on our findings and to determine their effects on health parameters.

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Figure 1. Body mass, tissue mass, and serum metabolites for age-matched rats that did not reproduce (NR), and rats that did not (NL) or did (L) suckle their young for 21 days postpartum. A) Body mass, B) liver mass, C) calf muscle mass of both rear triceps surae, D) retroperitoneal (RetroP) white adipose tissue (WAT) mass, E) perirenal (PR) WAT mass, F) serum concentration of non-esterified fatty acids (NEFA), and G) serum concentration of glucose. Data shown are mean ± SD. * different from NR, and # different from NL ($p < 0.05$).

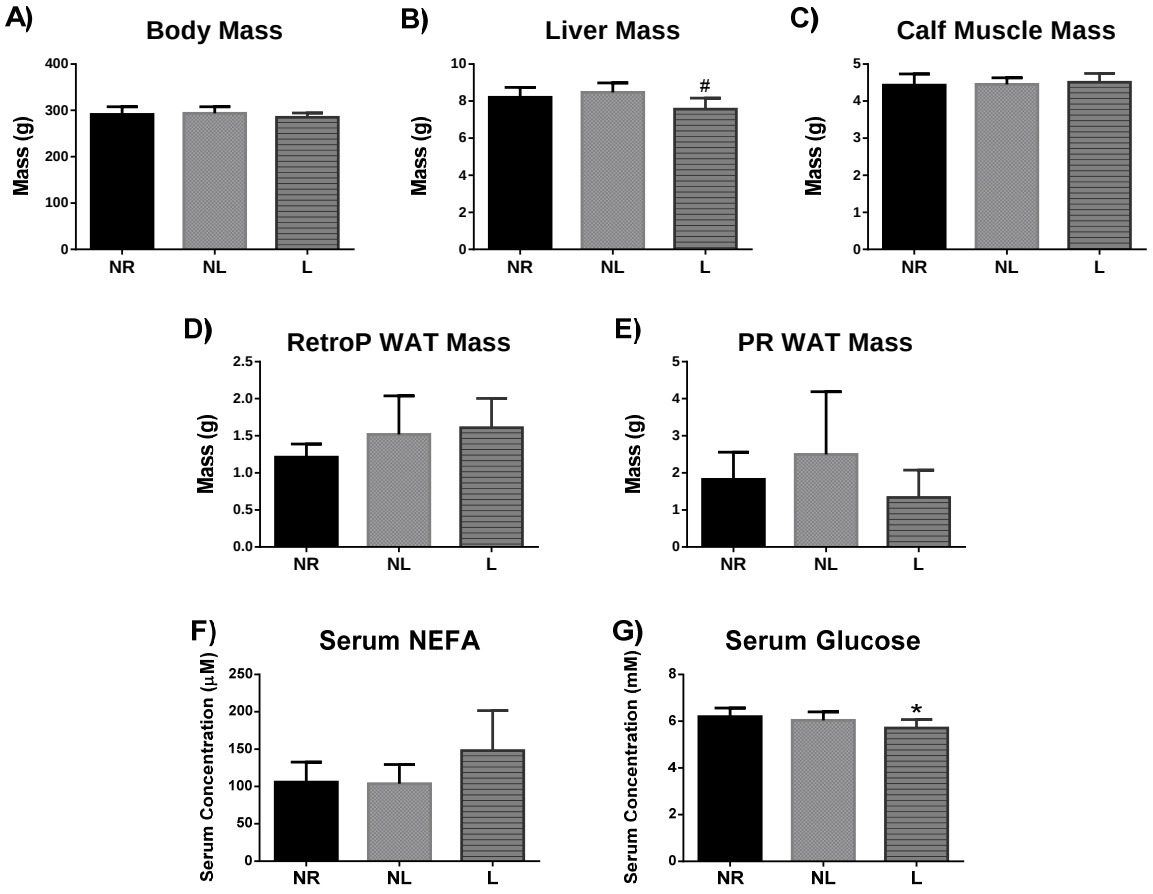
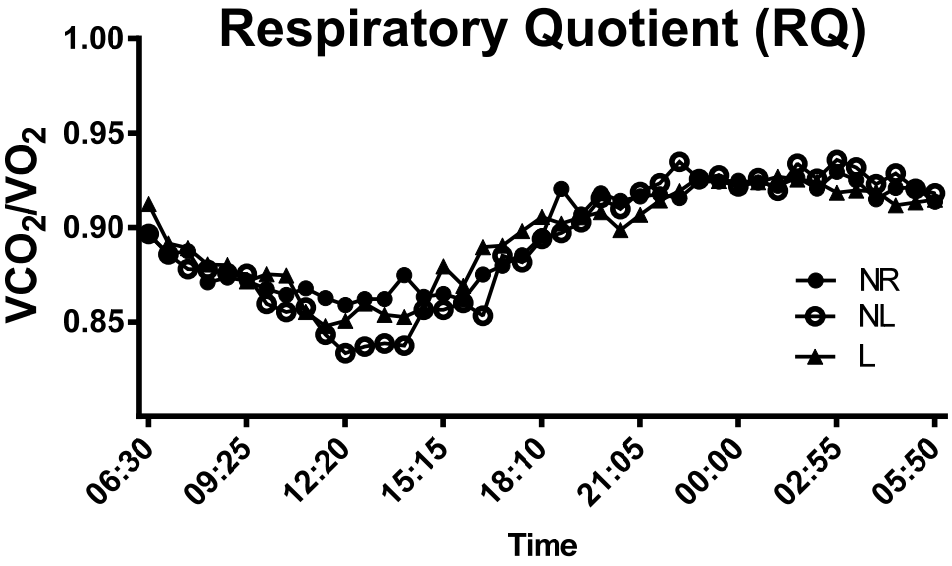

A)
Body Mass
Mass (g)
400
300
200
100
0
NR
NL
L
B)
Liver Mass
Mass (g)
10
8
6
4
2
0
#
NR
NL
L
C)
Calf Muscle Mass
Mass (g)
5
4
3
2
1
0
NR
NL
L
D)
RetroP WAT Mass
Mass (g)
2.5
2.0
1.5
1.0
0.5
0.0
NR
NL
L
E)
PR WAT Mass
Mass (g)
5
4
3
2
1
0
NR
NL
L
F)
Serum NEFA
Serum Concentration (μM)
250
200
150
100
50
0
NR
NL
L
G)
Serum Glucose
Serum Concentration (mM)
8
6
4
2
0
*
NR
NL
L

Figure 2. Whole animal metabolism for age-matched rats that did not reproduce (NR), and rats that did not (NL) or did (L) suckle their young for 21 days postpartum. A) Respiratory quotient and B) energy expenditure.

A)

Interaction; p = 0.951
Treatment; p = 0.968
Time; p < 0.001



B)

Interaction; p = 0.26
Treatment; p = 0.986
Time; p < 0.001

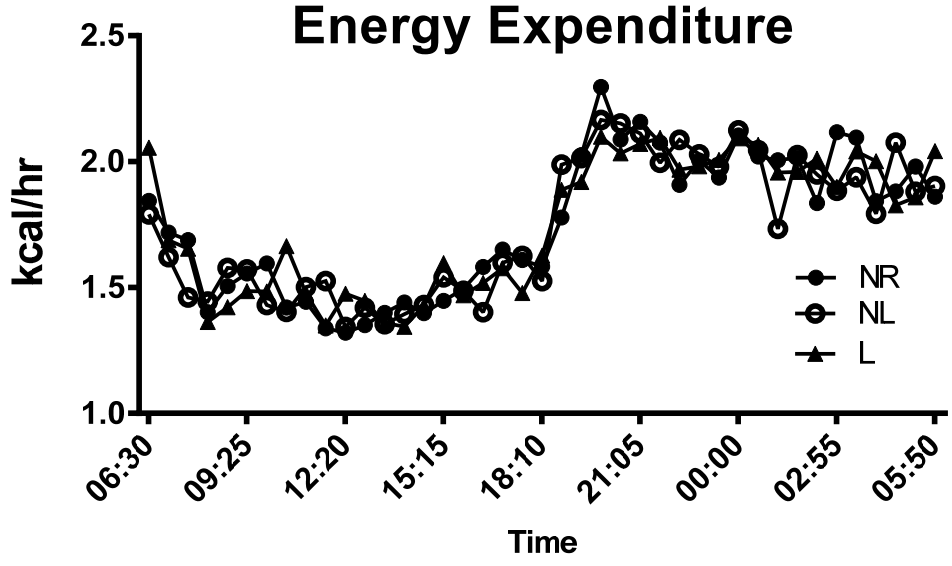


Figure 3. Respiration and oxidant emission from isolated mitochondria for age-matched rats that did not reproduce (NR), and rats that did not (NL) or did (L) suckle their young for 21 days postpartum. Data include liver A) state 3 respiration utilizing complex I substrates (pyruvate and malate; P/M), B) state 4 respiration utilizing P/M, C) respiratory control ratio (RCR) utilizing P/M), D) state 3 respiration utilizing complex II substrates (succinate; suc), E) state 4 respiration utilizing Suc, F) RCR utilizing suc. Data also include skeletal muscle G) state 3 respiration utilizing P/M, H) state 4 respiration utilizing P/M, I) RCR utilizing P/M, J) state 3 respiration utilizing suc, K) state 4 respiration utilizing suc, L) RCR utilizing suc. Finally M) oxidant emission from liver and N) oxidant emission from skeletal muscle are also presented. Oxygen consumption and hydrogen peroxide (H_2O_2) rates are normalized to citrate synthase (CS). Data shown are mean ± SD. * different from NR, and # different from NL ($p < 0.05$).

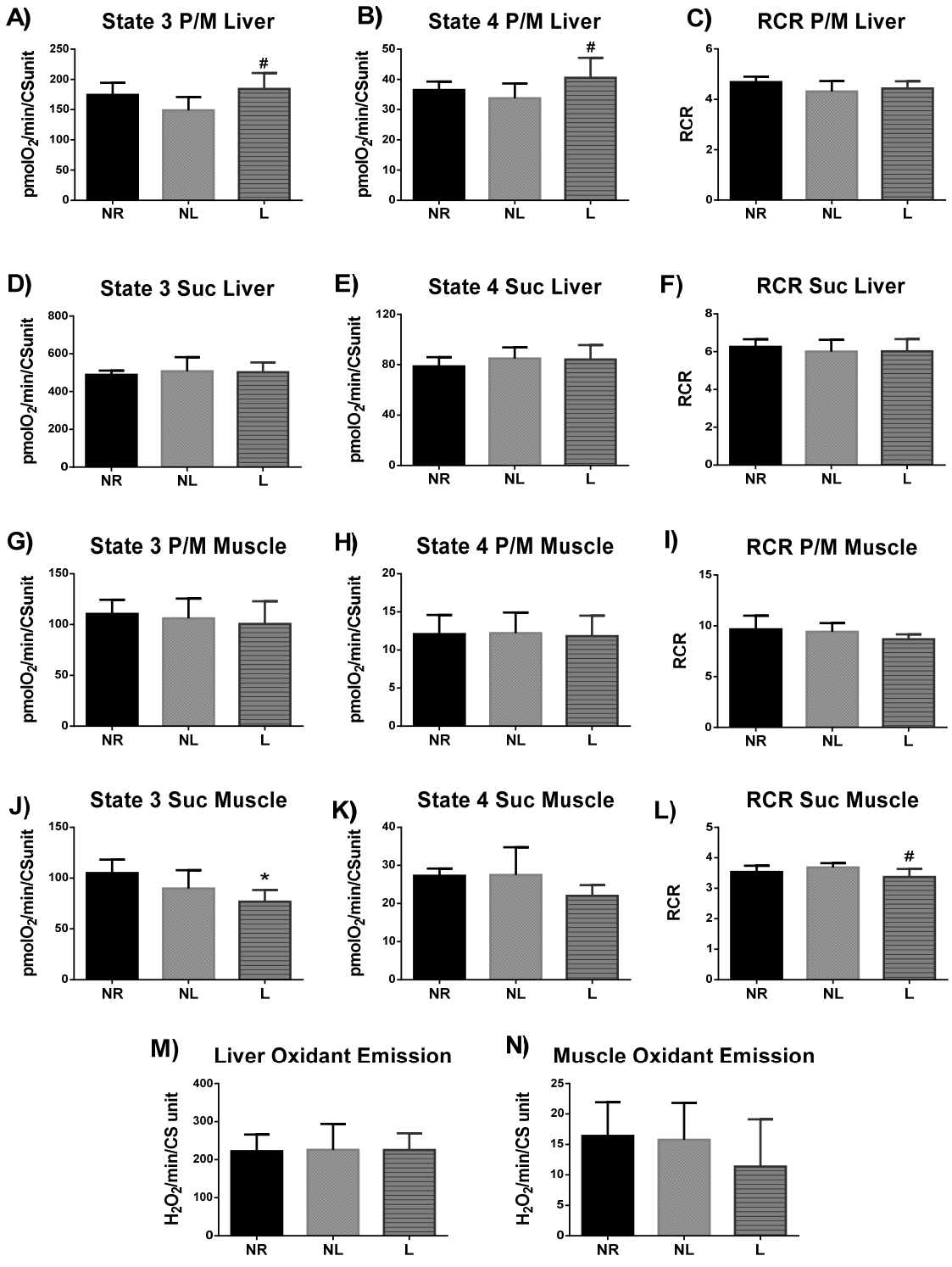

A)
State 3 P/M Liver
pmolO2/min/CSunit
250
200
150
100
50
0
#
NR
NL
L
B)
State 4 P/M Liver
pmolO2/min/CSunit
50
40
30
20
10
0
#
NR
NL
L
C)
RCR P/M Liver
RCR
6
4
2
0
NR
NL
L
D)
State 3 Suc Liver
pmolO2/min/CSunit
800
600
400
200
0
NR
NL
L
E)
State 4 Suc Liver
pmolO2/min/CSunit
120
80
40
0
NR
NL
L
F)
RCR Suc Liver
RCR
8
6
4
2
0
NR
NL
L
G)
State 3 P/M Muscle
pmolO2/min/CSunit
150
100
50
0
NR
NL
L
H)
State 4 P/M Muscle
pmolO2/min/CSunit
20
15
10
5
0
NR
NL
L
I)
RCR P/M Muscle
RCR
15
10
5
0
NR
NL
L
J)
State 3 Suc Muscle
pmolO2/min/CSunit
150
100
50
0
*
NR
NL
L
K)
State 4 Suc Muscle
pmolO2/min/CSunit
40
30
20
10
0
NR
NL
L
L)
RCR Suc Muscle
RCR
5
4
3
2
1
0
#
NR
NL
L
M)
Liver Oxidant Emission
H2O2/min/CS unit
400
300
200
100
0
NR
NL
L
N)
Muscle Oxidant Emission
H2O2/min/CS unit
25
20
15
10
5
0
NR
NL
L

Figure 4. Enzymatic complex activity of isolated mitochondria for age-matched rats that did not reproduce (NR), and rats that did not (NL) or did (L) suckle their young for 21 days postpartum. Data include A) complex I, B) complex II, C) complex III, and D) complex IV activity in the liver. In addition, data include E) complex I, F) complex II, G) complex III, and H) complex IV activity in skeletal muscle. Complex activity data are normalized to citrate synthase (CS). Data shown are mean ± SD. No statistical differences detected ($p > 0.05$).

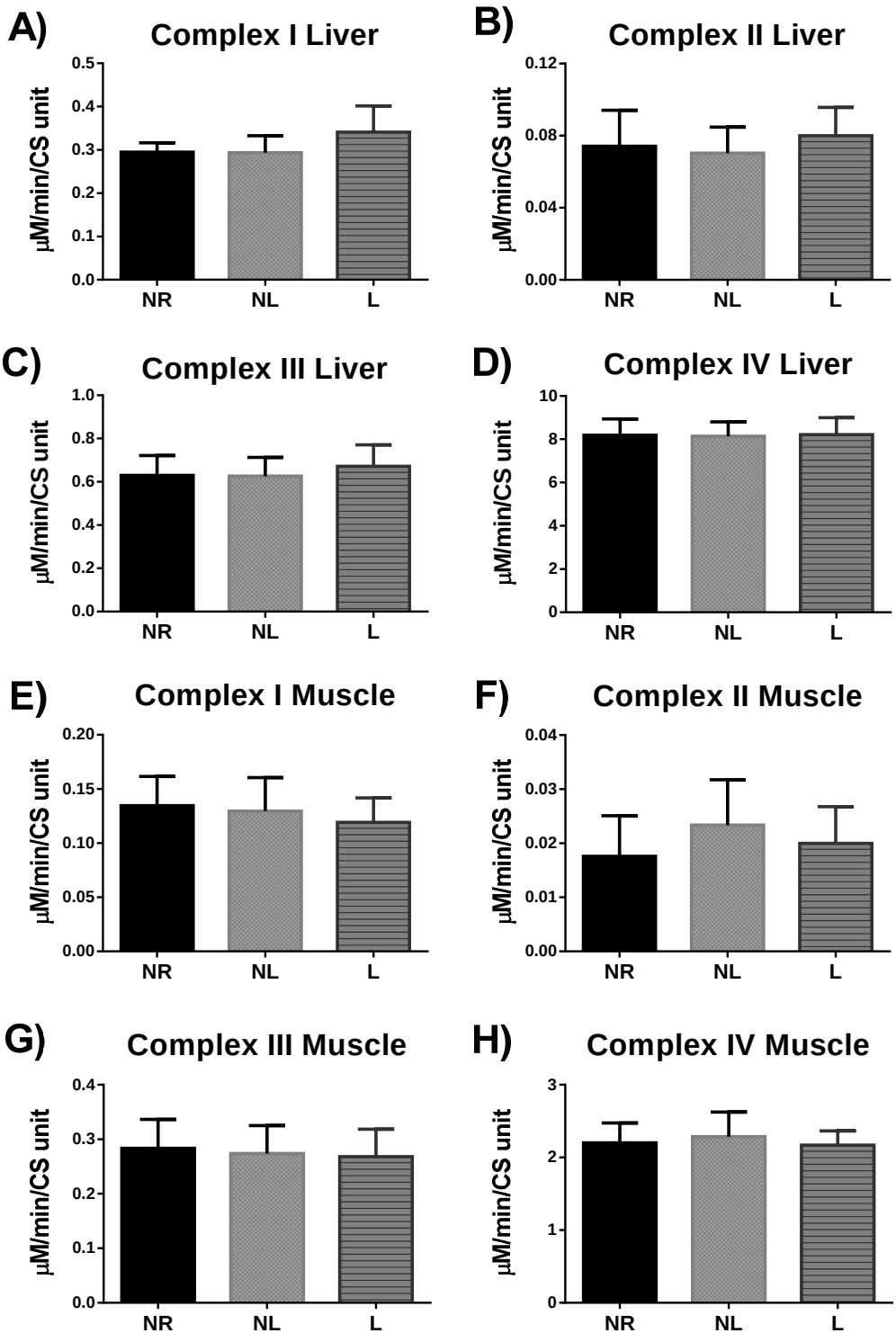

A)
Complex I Liver
µM/min/CS unit
0.5
0.4
0.3
0.2
0.1
0.0
NR
NL
L
B)
Complex II Liver
µM/min/CS unit
0.12
0.08
0.04
0.00
NR
NL
L
C)
Complex III Liver
µM/min/CS unit
1.0
0.8
0.6
0.4
0.2
0.0
NR
NL
L
D)
Complex IV Liver
µM/min/CS unit
10
8
6
4
2
0
NR
NL
L
E)
Complex I Muscle
µM/min/CS unit
0.20
0.15
0.10
0.05
0.00
NR
NL
L
F)
Complex II Muscle
µM/min/CS unit
0.04
0.03
0.02
0.01
0.00
NR
NL
L
G)
Complex III Muscle
µM/min/CS unit
0.4
0.3
0.2
0.1
0.0
NR
NL
L
H)
Complex IV Muscle
µM/min/CS unit
3
2
1
0
NR
NL
L

Figure 5. Markers of metabolism in liver, skeletal muscle, and Retroperitneal (RetroP) and perirenal (PR) white adipose tissue (WAT) for age-matched rats that did not reproduce (NR), and rats that did not (NL) or did (L) suckle their young for 21 days postpartum. Data include A) PGC-1α and B) PPARδ protein levels in liver. C) PGC-1α and D) PPARδ protein levels in skeletal muscle. E) PPARδ protein levels in WAT. Representative blots are shown under the graphs. Data shown are mean ± SD. * different from NR, and # different from NL ($p < 0.05$).

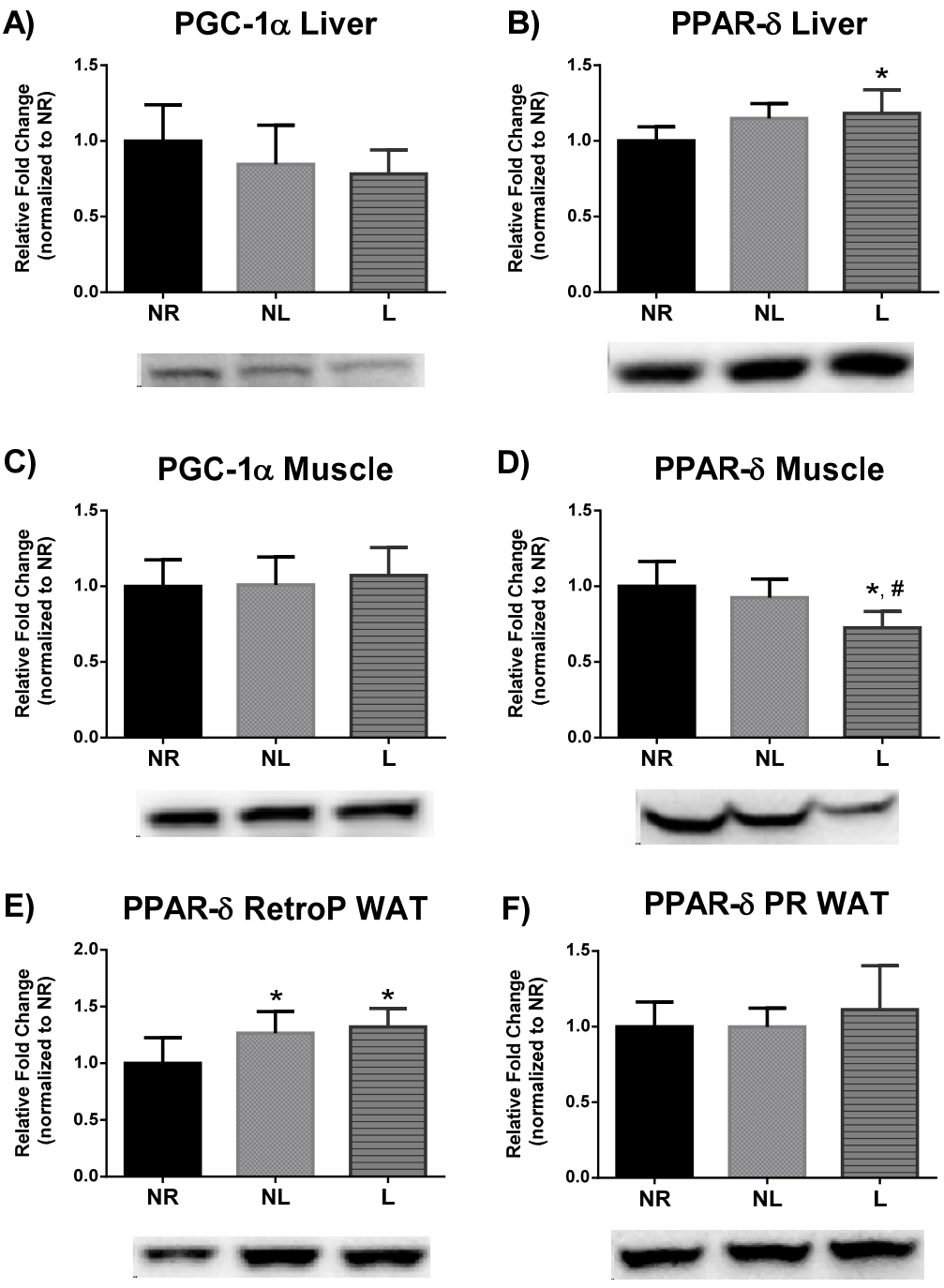
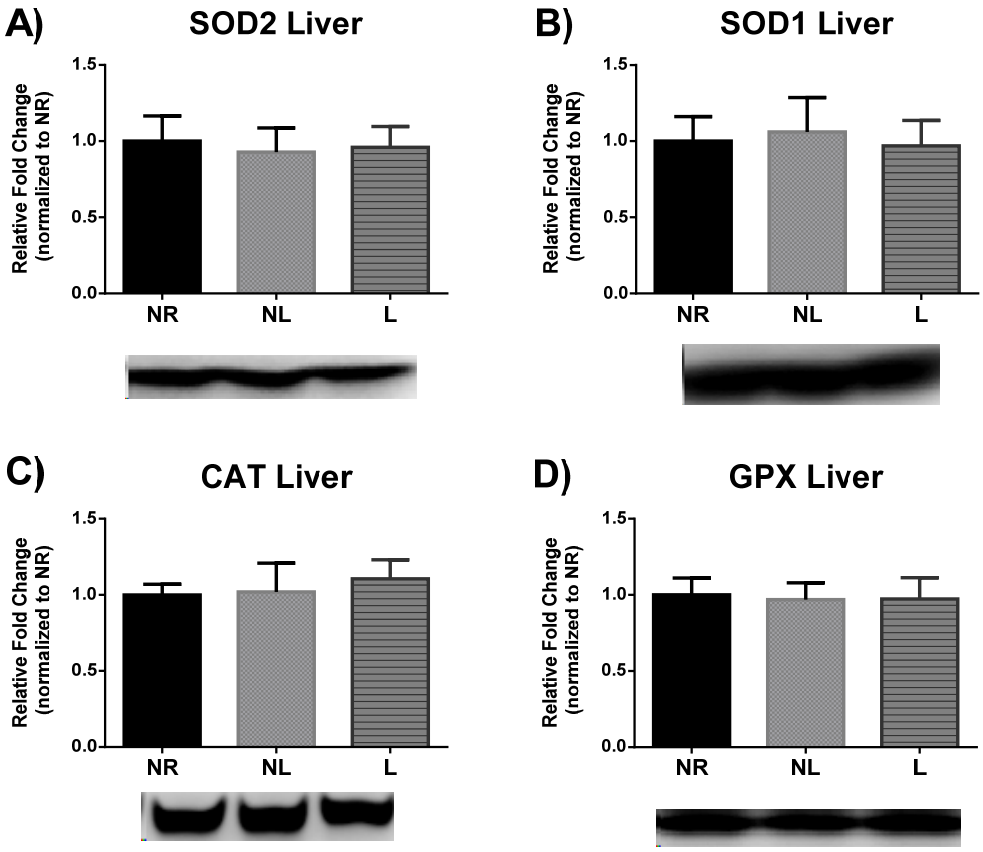

A)
PGC-1α Liver
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
NL
L
B)
PPAR-δ Liver
*
C)
PGC-1α Muscle
D)
PPAR-δ Muscle
*, #
E)
PPAR-δ RetroP WAT
2.0
F)
PPAR-δ PR WAT

Figure 6. Markers of oxidative stress in liver for age-matched rats that did not reproduce (NR), and rats that did not (NL) or did (L) suckle their young for 21 days postpartum. Data include protein level of the antioxidants A) SOD2, B) SOD1, C) CAT, and D) GPX. In addition, data include markers of oxidative damage including E) lipid peroxidation determined by 4-HNE, and F) protein carbonyls levels determined by using oxyblot. Representative blots are shown under the graphs (A-D) or to the right of the graphs (E and F). Data shown are mean ± SD. No significant differences detected ($p > 0.05$).


A)
SOD2 Liver
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
NL
L
B)
SOD1 Liver
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
NL
L
C)
CAT Liver
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
NL
L
D)
GPX Liver
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
NL
L

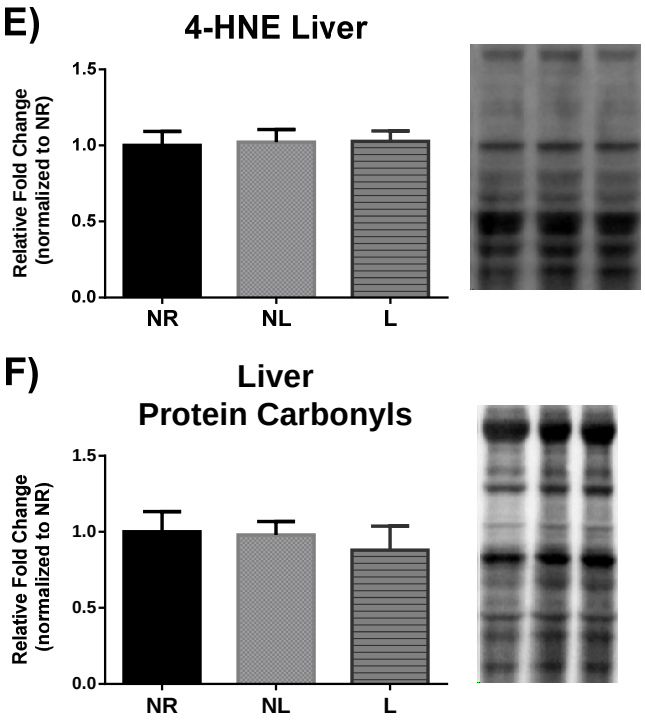
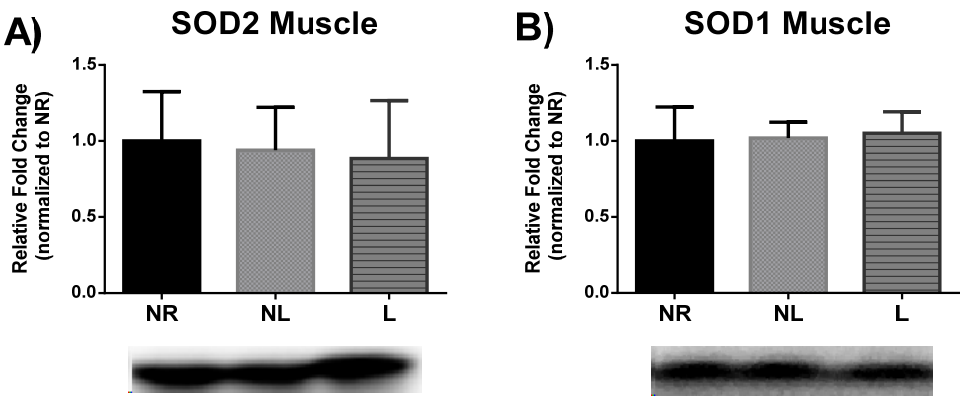
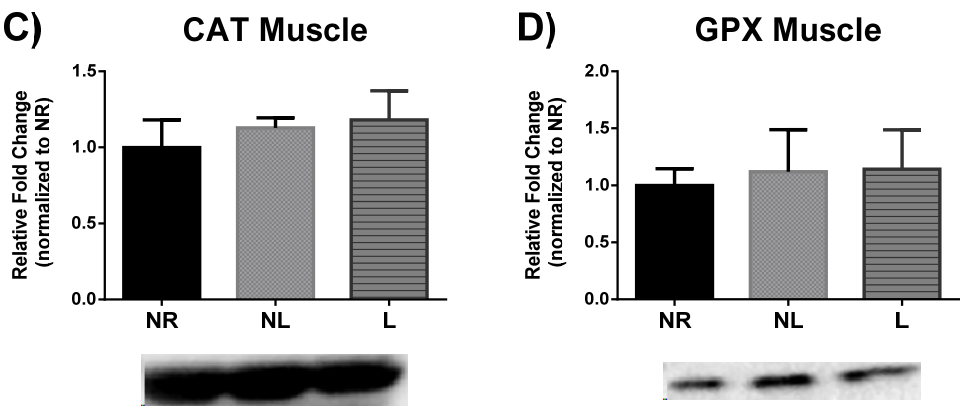
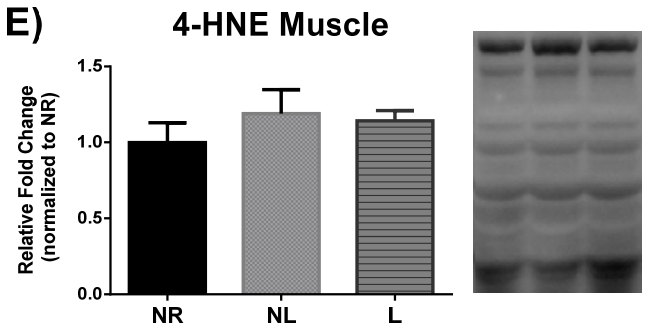

E)
4-HNE Liver
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
NL
L
F)
Liver
Protein Carbonyls
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
NL
L

Figure 7. Markers of oxidative stress in skeletal muscle for age-matched rats that did not reproduce (NR), and rats that did not (NL) or did (L) suckle their young for 21 days postpartum. Data include protein level of the antioxidants A) SOD2, B) SOD1, C) CAT, and D) GPX. In addition, data include markers of oxidative damage including E) lipid peroxidation determined by 4-HNE, and F) protein carbonyls levels determined by using oxyblot. Representative blots are shown under the graphs (A-D) or to the right of the graphs (E and F). Data shown are mean ± SD. No significant differences detected ($p > 0.05$).


A)
SOD2 Muscle
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
NL
L
B)
SOD1 Muscle
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
NL
L


C)
CAT Muscle
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
NL
L
D)
GPX Muscle
Relative Fold Change (normalized to NR)
2.0
1.5
1.0
0.5
0.0
NR
NL
L


E)
4-HNE Muscle
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
NL
L

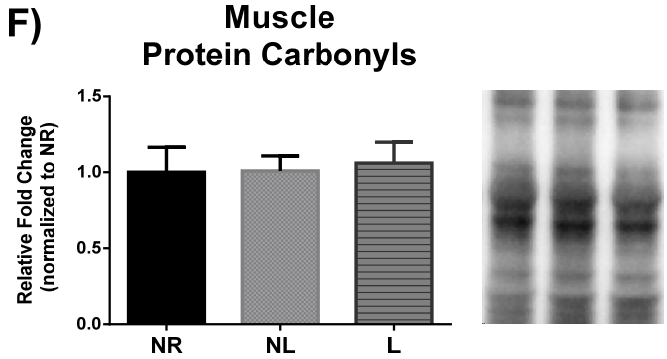
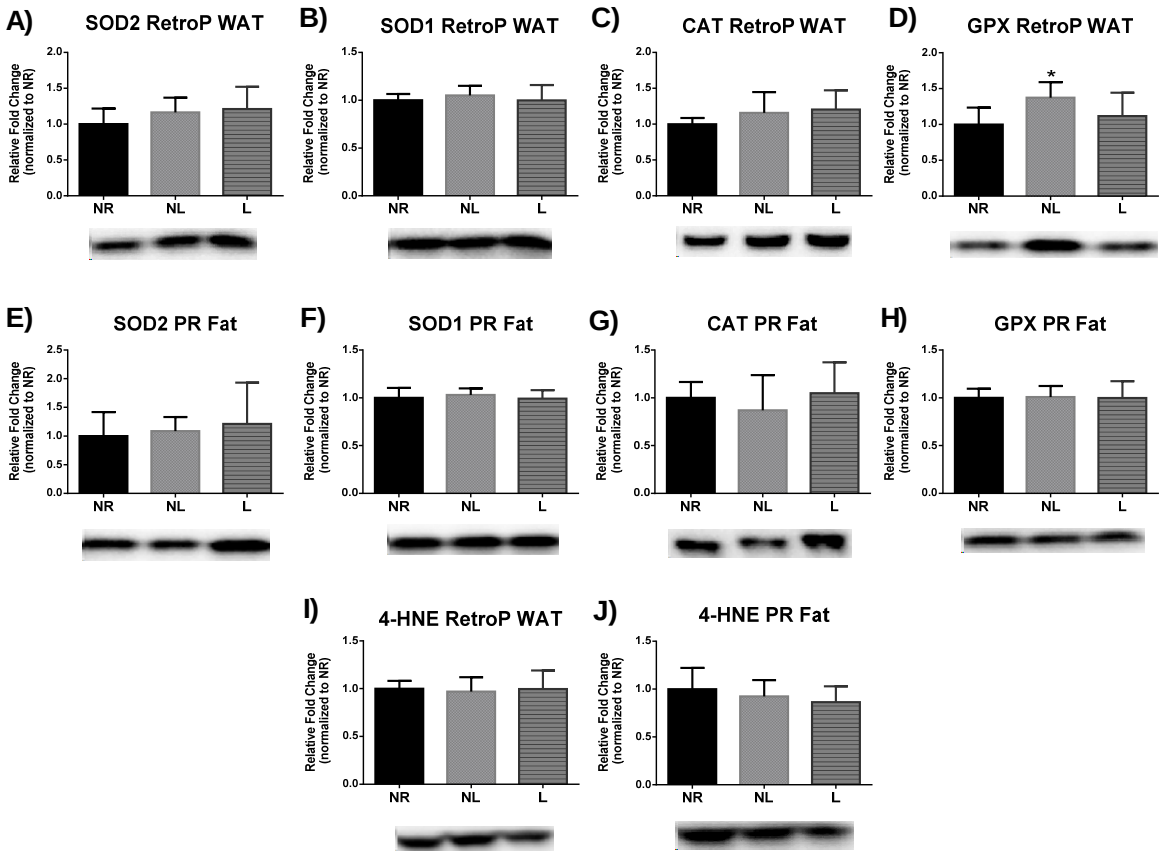

F)
Muscle
Protein Carbonyls
Relative Fold Change (normalized to NR)
1.5
1.0
0.5
0.0
NR
NL
L

Figure 8. Markers of oxidative stress in retroperitoneal white adipose tissue (RetroP WAT) and perirenal (PR) WAT for age-matched rats that did not reproduce (NR), and rats that did not (NL) or did (L) suckle their young for 21 days postpartum. Data include RetroP WAT and PR WAT protein level of the antioxidants SOD2, SOD1, CAT, and GPX (A-H). In addition, data include lipid peroxidation determined by 4-HNE in RetroP and PR WAT. Representative blots are shown under the graphs. Data shown are mean ± SD. * different from NR ($p < 0.05$).


A)
SOD2 RetroP WAT
B)
SOD1 RetroP WAT
C)
CAT RetroP WAT
D)
GPX RetroP WAT
E)
SOD2 PR Fat
F)
SOD1 PR Fat
G)
CAT PR Fat
H)
GPX PR Fat
I)
4-HNE RetroP WAT
J)
4-HNE PR Fat
Relative Fold Change (normalized to NR)
NR
NL
L
*