The persistent effects of stress during lactation on maternal and offspring mitochondria

by

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Abstract

An increase of circulating glucocorticoids in reproductive females may augment allocation of resources towards self-maintenance and away from offspring. Thus, elevated circulating glucocorticoids may negatively affect offspring development. However, maternal stress may induce preparative responses in offspring, such as higher tolerance to oxidative stress and more efficient mitochondria, which could positively impact survival. To test this hypothesis, corticosterone (CORT) was administered orally to female mice from days 7 to 21 after parturition. Thus, offspring were indirectly exposed to CORT through the mother's milk and aspects of their mitochondrial physiology were investigated at two timepoints, 5.5 weeks and 10 weeks of age. At both ages, mitochondria were isolated from liver and skeletal muscle to measure respiratory control ratio (RCR) and reactive oxygen species (ROS) production. Oxidative damage markers of protein oxidation and lipid peroxidation were quantified in each tissue. While effects on maternal mitochondrial physiology were limited, there were numerous effects of maternal stress on offspring. These include beneficial effects on mitochondrial respiration and lower ROS production in skeletal muscle tissue, as well as reduced oxidative damage markers in both tissues. Many of these effects were only revealed or often strengthened with offspring aging.

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List of Abbreviations

CORT	Corticosterone
VEH	Vehicle
HPA	Hypothalamus-Pituitary-Adrenal (axis)
RCR	Respiratory control ratio
ROS	Reactive oxygen species
CS	Citrate synthase (activity)
4HNE	4-hydroxynonenal

Introduction

In natural populations, animals are exposed to numerous stressors. The cumulative effects of these stressors can impact lifespan and evolutionary fitness, both through direct impact of the stress on the individual experiencing them (Kirkwood et al. 2000; Minois 2000) and the indirect impacts on offspring via maternal effects (Mousseau and Fox 1998; Sheriff and Love 2013). Whether stressors have modest or large, or whether they have positive or negative effects on reproductive animals and their young depends on their genotype, environment, age and reproductive status at exposure, and history of previous exposure (Crespi et al. 2013). In general, short-term or acute stressors often benefit health, while long-term or chronic stressors are commonly detrimental (Romero et al. 2015). These effects reflect hormesis, or the biphasic response to an environmental stressor, where mild doses stimulate and high doses inhibit these stressor responses (Costantini 2014)**.** For these reasons, environmental stressors appear to play an important role in shaping life history variables.

Researchers often view reproduction and environmental stressors to be associated with a cost (Williams 1966; Stearns 1992). The life history patterns that species evolve and individuals display are determined, at least in part, by resource availability and physiological constraints that prevent organisms from simultaneously maximizing energy allocation to multiple energetically demanding processes, such as reproduction and self-maintenance (Zera and Harshman 2001). It is generally assumed that when the demands of reproduction or cost of self-maintenance are high, these two variables will trade-off (Reznick 1985; Stearns 1989). A reduction in somatic maintenance could attenuate a female's lifespan and/or her capacity to allocate resources to future reproduction, whereas a reduction in resource allocation to her offspring can have lasting effects on offspring fitness (Williams 1957, 1966). Yet, several empirical studies have found that

reproduction can benefit a female's condition, which would benefit future reproduction and longevity and also play a role in the evolution of life history patterns (Cardoso et al. 2002; Olijnyk and Nelson 2013; Skibiel et al. 2013; Hood et al. 2019). Further, maternal effects that provide information about environmental stress from mother to offspring can be adaptive when the offspring's environment mirrors that of its mother (Love and Williams 2008; Sheriff and Love 2013) and when a brief stressor reduces both behavioral and neuroendocrine responses to stressors encountered later in life (Levine and Thoman 1969; Anisman et al. 1998; Catalani et al. 2000).

When presented with a stressor, non-reproductive females will commonly delay reproduction in favor of self-maintenance. Yet, when a stressor is presented during reproduction, females may alter somatic support, allocation to their young, and/or offspring phenotype via any of a number of maternal-offspring signaling mechanisms (Monaghan 2008; Brunton and Russell 2011; Macrì et al. 2011; Costantini 2014; Blount et al. 2016; Zhang and Hood 2016). In mammals, the signaling period of direct chemical communication between a mother and offspring is both long and complex, as bioactive compounds are transferred to the offspring via the placenta and in milk (Maestripieri and Mateo 2009; Rogowitz 2015). Thus, there is a high probability that a maternal stressor will influence the condition and performance of young well beyond birth.

The energetic demands of lactation, growth and development are particularly pronounced in small species (Oftedal 1984). In house mice, food intake increases nearly 3-5 times and liver mass roughly doubles to support the demands of milk synthesis (Hollister et al. 1987; Hammond and Diamond 1994; Johnson et al. 2001). Further, the body mass of pups increases 10-15 times during the first two months of life (Macarthur 1949). At the cellular level, these heightened

energetic demands are supported by mitochondria which are responsible for most of an animal's cellular energy (ATP) production. To support lactation, ATP production is amplified by increasing mitochondrial number via mitochondrial biogenesis and increasing mitochondrial efficiency by removing old or damaged mitochondria via autophagy (Benard et al. 2010; Youle and Van Der Bliek 2012). Experimental evidence has shown reproducing house mice to have higher mitochondrial density in the liver during lactation compared to non-reproductive controls (Pichaud et al. 2013), allowing them to meet the extreme energetic demands of reproduction. In rats, it has been shown that reduced nutrient transfer to young during fetal development associated with uteroplacental insufficiency not only retarded offspring growth rate but it also diminished the function of mitochondria in the pups' liver and skeletal muscle (Peterside et al. 2003; Selak et al. 2003).

While high functioning mitochondria are required to support reproduction, a decline in their performance is thought to be responsible for senescence (Yen et al. 1989; Ojaimi et al. 1999; Short et al. 2005). Oxidative damage caused by the release of reactive oxygen species (ROS) from the electron transport system has been hypothesized as both a key mechanism responsible for mitochondrial decline with senescence (Shigenaga et al. 1994; Cui et al. 2012) and an important mediator of the trade-off between reproduction and longevity (Dowling & Simmons, 2009; Monaghan et al, 2009). While support for both theories have waned (Selman et al. 2012; Kennedy et al. 2013; Itsara et al. 2014; Speakman and Garratt 2014; DeBalsi et al. 2017; Hood et al. 2018), it remains possible that the spike in ROS production occurring when animals are under prolonged stress (Manoli et al. 2007) could potentiate other processes that are responsible for senescence.

The goal of the present study was to evaluate the mechanisms responsible for the enhanced trade-off between reproduction and longevity when an animal is exposed to stressful conditions during reproduction. Glucocorticoid hormones are produced by the adrenal glands as a part of the hypothalamus-pituitary-adrenal (HPA) axis, the system responsible for the endocrine response to stressors. Corticosterone (CORT) represents the main glucocorticoid in rodents and is secreted as the end-product of HPA activation. Administration of CORT has been known to influence the generation of oxidative damage (Costantini et al. 2011). In this experiment, I manipulated plasma CORT levels by supplementing reproducing female mice with CORT orally. Non-invasive methods of glucocorticoid exposure and sampling, via fecal CORT metabolites, allowed us to confirm that voluntary oral treatment was primarily responsible for the spike in CORT levels experienced by the mother. I hypothesize that when CORT levels are artificially elevated during reproduction, their baseline mitochondrial physiology will be negatively impacted, and oxidative damage levels will be greater than unmanipulated reproducing females. These changes, in turn, have the potential to hasten senescence in reproductive females.

Stressful maternal conditions are also expected to impact offspring development. Species that give birth to altricial young, like rodents, are anticipated to complete most neuroendocrine development in the postnatal period (Darlington et al. 1999; Matthews 2002), and so lactation was targeted for our study's manipulation of maternal stress. Lactation requires substantial physiological investment beyond pregnancy for the continual development of offspring postnatally (Power and Schulkin 2013). I expect CORT transfer in milk to be the primary mechanism for these developmental effects, as maternal CORT treatment has been discovered in the stomach milk of neonatal rats, and shown to increase serum levels of CORT in offspring

post-treatment before returning to baseline immediately post-weaning (Brummelte et al. 2010). I predict that maternal stress, induced by an elevation in CORT, will positively influence mitochondrial physiology and reduce oxidative damage of young.

Methods

Animal Housing

Adult female wild-derived house mice (*Mus musculus*) were utilized in this investigation, which occurred from November 2018 to July 2019. Wild-derived mice were used because they have enhanced responsiveness to stressors compared to their laboratory counterparts (Harper 2008; Gaukler et al. 2015; Ruff et al. 2015; Abolins et al. 2017). All husbandry and experimental procedures were approved by the Auburn University Institutional Animal Care and Use Committee (PRN 2018-3447, 2018-3432). All animals were provided rodent chow (Teklad Global Diet 2019, Envigo, Indianapolis, IN) and water *ad libitum*. Animals were exposed to a natural light and dark cycle, as well as natural ambient temperatures and humidity.

Experimental Design

Twenty-six adult virgin female mice were divided into experimental (CORT; n=14) and vehicle (VEH; n=12) groups. All female mice were paired with a male until visible signs of pregnancy (enlarged nipples and increased girth - usually around 14 days of pregnancy), at which time the male was removed. Because wild-derived mice commonly cannibalize their first litter (Hood personal obs.), all females were bred twice and successfully weaned their second litter. All manipulations and data collection were based on the second litter. Litter size was adjusted to 5 pups on the day that females gave birth to their second litter as an attempt to standardize energy allocation to offspring. Experimental females were exposed to corticosterone (CORT) daily,

from days 7 to 21 post-partum during their second reproductive bout. CORT was provided to each female in peanut butter pellets (described below) promptly an hour before dark. To acclimate the females to the method of CORT delivery, peanut butter pellets without CORT (vehicle pellets) were offered to mice daily from birth to day 6 post-partum. Day of birth was identified as day 1. On days 7 to 21, the experimental animals received a peanut butter pellet with CORT and non-experimental animals continued to receive vehicle pellets. I targeted this time-period for CORT treatment because it spans peak lactation in the house mouse and avoids the period in early lactation when females facing a stressor are more likely to cannibalize their young. CORT treatment was terminated as the intestinal tract of the young changed to prepare for the switch to adult food in the third week of life and milk yield drops (Henning and Sims 1979; Knight et al. 1986). Pups were removed from the female's box when they were weaned at 28 days of age. Females were then returned to a non-reproductive state and sacrificed by decapitation 10 days after weaning of their second litter. This timing was selected to ensure that the changes in organ function supporting reproduction had returned to a condition optimized for self-maintenance thus, allowing us to quantify how elevated CORT during reproduction alters the baseline physiological condition of females that could subsequently reduce their longevity (Zhang and Hood 2016).

Offspring from the mothers' second reproductive bout were evaluated at two different ages: juvenile (5.5 weeks of age) and adulthood (10 weeks of age). At the time of sacrifice, one pup of each sex was collected if available, as maternal stress has been shown to have distinct sex effects in offspring (Liu et al. 2001; Slotten et al. 2006; Weintraub et al. 2010). Effects of indirect CORT exposure through the maternal milk supply were evaluated in juvenile offspring of both vehicle (n=12 females, 12 males) and experimental (n=13 males, 15 females) groups.

Effects were also evaluated in adult offspring of both vehicle (n=11 males, 12 females) and experimental (n=12 males, 14 females) groups to evaluate the persistence or delayed onset of these effects. Body mass (g) was measured in offspring on day 2, 12 and 28 post-partum, as well as at the time of euthanasia in both age groups.

Peanut butter pellet

The corticosterone-treated (CORT) peanut butter pellets with corticosterone mixed in while vehicle (VEH) mice were provided with peanut butter pellets made with peanut butter. To make the CORT pellets, 11 mg of CORT (≥98% purity; Enzo, Farmingdale, NY) was thoroughly mixed into 2.5 g of the organic peanut butter (Simple Truth, The Kroger Co., Cincinnati, Ohio). To ensure that the peanut butter and CORT were not contaminated, they were weighed in sterile, disposable weigh bouts and delivered with sterile, disposable spatulas. The peanut butter was warmed to 100C in a water bath. This temperature limit was used to prevent CORT degradation (Wotiz and Martin 1961). The vehicle batch of peanut butter pellets received no addition of CORT. This warm mixture was then dripped into a 4.5 x 4.5 x 5mm, 15-well pellet mold (Ted Pella, Inc., Redding, CA) and frozen at -80C to form the frozen pellet. Pellets were then carefully removed from the mold and stored at -20C until ready for consumption.

Fecal CORT Metabolites

To avoid an increase in CORT in both groups from the stress associated with blood sampling, glucocorticoids was quantified using feces. I confirmed that our experimental manipulation elevated maternal CORT by quantifying CORT metabolites in the feces of experimental and vehicle females. I also evaluated fecal CORT metabolites in adult offspring (10 weeks old). Feces were collected from reproductive females at three time points: before they were paired with a male for their second bout (baseline), after pups from that litter had aged 12 days (peak)

and after pups were weaned at 28 days of age (wean). Feces were only collected from adult offspring once on the day before sacrifice. For collection, each mouse was put into a clean box and then all fecal samples were collected from the box 24-hours later. Female offspring were housed together, so samples were difficult to differentiate between individuals. For this reason, all fecal analyses included the dam as a random factor. Mouse mothers commonly groom the feces of their young pups and increase coprophagy to offset nutrient demand while lactating (Soave and Brand 1991). Thus, maternal CORT exposure may be greater than the dose provided and circulating CORT may persist longer after treatment had ended because of additional exposure associated with coprophagy. Each fecal sample was then stored in -20C until ready for extraction.

DetectX corticosterone enzyme-linked immunofluorescent assay (ELISA; Arbor Assays, Ann Arbor, MI) was used for analyzing CORT steroid hormone levels in fecal extracts dried in an oven at 60C. For extraction, fecal samples were homogenized by hand using a disposable pestle in 15ml falcon tubes after adding 1ml of 80% ethanol for every 0.1 g of feces. Samples were then vortexed for 5 minutes and shaken vigorously for thirty minutes, while being quickly vortexed every 5 minutes in between. Samples were then centrifuged at 3.7 rpm for 10 minutes. The supernatant was collected and evaporated to dryness before reconstitution with 100 µl of ethanol, followed by at least 400 µl of assay buffer. The ethanol content should be well below <5% for this immunoassay and I accomplished this by using a 1:10 dilution of reconstituted sample with assay buffer. Samples were loaded into 96-well clear microtiter plates coated with donkey anti-sheep IgG and read at an optical density of 450nm with a Synergy H1 Hybrid plates reader (BioTek, Winooski, VT).

To confirm that fecal CORT was correlated with circulating CORT, a separate set of 14 female mice were evenly divided into vehicle (n=7) and experimental (n=7) groups. Experimental females were bred and given the exact same CORT treatment described above starting on day 7 post-partum. A blood sample of approximately 70-100 µl was collected from each mouse on day 12 post-partum from the facial vein (Hoff 2000). Once blood was obtained, each female was immediately returned to her pups in a clean box. Feces were then collected from the box the next morning, approximately 12 hours later. The CORT content of these blood and fecal samples were analyzed as described above, except plasma was extracted from blood samples through a single centrifugation event. Dissociation reagent (5 µl) from the ELISA kit was vortexed gently with plasma (5 µl) and incubated at room temperature for at least 5 minutes. Samples were then diluted 1:100 with 490 µl of assay buffer before plate loading. Our results suggested that plasma and fecal CORT are correlated with each other across all time points (Pearson correlation, r=0.55; Figure 1).

Mitochondrial isolation

Mitochondria were isolated from liver and skeletal muscle tissues of the experimental and vehicle females, as well as their offspring, following previously outlined procedures (Hood et al., 2018; Hyatt et al, 2017; Zhang et al, 2018). The largest lobe of the liver and skeletal muscle from the left hind limb were dissected immediately for live mitochondrial isolation. If either sample were too small for proper mitochondrial isolation, tissue was taken from the next largest lobe of the liver or skeletal muscle from the right hind limb. All remaining liver and skeletal muscle tissue were flash frozen in liquid nitrogen and stored in -80C for future analyses.

Immediately upon dissection, skeletal muscle tissue was trimmed to remove fat and connective tissues. Each tissue type was placed in their respective isolation buffers and quickly

minced with scissors. Liver and skeletal muscle tissue were homogenized in a Potter-Elvhjem PTFE pestle and glass tube, then centrifuged at 500xg for 10 minutes at 4C. The supernatant was filtered through a cheese cloth before another centrifugation round at 3500xg for 10 minutes. The resulting supernatant was discarded, while the mitochondrial pellet was washed with isolation buffer and centrifuged one last time at 3500xg for 10 minutes with liver tissue. For skeletal muscle tissue, the supernatant was again discarded, and the mitochondrial pellet was subsequently centrifuged at 3500xg for 10 minutes. The final mitochondrial pellets from liver and skeletal muscle tissue were then suspended in a mannitol-sucrose solution.

H_2O_2 Emission (ROS)

Mitochondrial H_2O_2 production was assessed in these tissues using AmplexRed Hydrogen Peroxide/Peroxidase assay kits (Thermofisher, Walthan, MA). Resorufin (oxidized AmplexRed) formation occurs with addition of hydrogen peroxide (H_2O_2). This assay was performed using succinate. Synergy H1 Hybrid plates reader (BioTek, Winooski, VT) excited at a wavelength of 544nm and measured an emission at 590nm at 37C in a 96-well black plate. The plate reader began reading 5 min after the last mitochondrial isolate sample was placed in the last well. Resulting values were normalized to mitochondrial protein concentration determined by Bradford assay (Bradford 1976).

Respiratory Control Ratio (RCR)

Mitochondrial isolates were maintained in a respirometer at 37C (Oxytherm, Hansatech Instruments, UK). Respiration rates were normalized to mitochondrial protein concentration using Bradford assay. Mitochondrial respiratory capacity was evaluated through respiratory control ratio (RCR), a measure of the ratio of maximum (state 3) to basal (state 4) respiration of isolated mitochondria from each tissue type. State 3 represents the maximal respiration rate

initiated by addition of 0.25 mM ADP substrate, while state 4 or basal respiration is measured when the phosphorylation of ADP is completed.

Mitochondrial density (CS Activity)

Citrate synthase (CS) activity of liver and skeletal tissues were measured as a proxy to mitochondrial density (Larsen et al. 2012; Spinazzi et al. 2012). Tissue homogenate from liver and skeletal muscle were used for this assay. Homogenization included a 1:10 (weight/volume) ratio of 5 mmol/L Tris HCL + 5mmol EDTA and protease inhibitor cocktail (VWR, Radnor, PA) and centrifugation at 1,500 g for 10 minutes in 4C. The supernatant was collected, and protein content was calculated using the Bradford assay. CS activity reactions were quantified as the increase in absorbance from 5,5'-di hiobis-2-nitrobenzoic acid reduction over a minute, reading values from the plate every 10 seconds.

Oxidative Damage Markers (4HNE & protein carbonyls)

Western blotting was used to quantify oxidative damage markers in each tissue, including adducts of lipid peroxidation (4-hydroxynonenal, 4HNE; Abcam, Cambridge, MA) and carbonyls of protein oxidation (Oxyblot; EMD Millipore, Billerica, MA). Sample preparation for western blot analysis involved homogenization of liver and skeletal muscle tissues. Whole tissue homogenates of each tissue were prepared with a 1:10 weight to volume ratio of 5.5 mmol/L Tris-HCL (pH 7.5), 5 mmol/L EDTA (pH 8.0) and protease inhibitor cocktail (VWR, Radnor, PA) before centrifugation at 1,500 g for 10 minutes in 4°C. With protein content information determined by Bradford assay, homogenates were further diluted to a concentration of 2ug/µl. Proteins from tissue homogenates were separated by electrophoresis with the use of 4% to 15% Criterion TGX precast gels (Bio-Rad, Hercules, CA). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes following separation and washed using Tris-buffered saline

solution with Tween (TBS-T; 5 min, 3x). Nonspecific site binding was blocked in gels measuring 4HNE with the use of Tris-buffered saline (TBS) solution containing 0.1% Tween-20 and 5% non-fat milk. Gels evaluating protein carbonyls using Oxyblot were blocked in a 1% Bovine Serum Albumin (BSA) with TBS-T solution. Membranes were incubated with either primary antibodies for 4HNE or protein carbonyls and then secondary antibodies after washing with TBS-T (5 min, 3x). Amersham ECL prime western blotting detection reagent was used to illuminate membranes and detect labeled proteins (GE Health- care, Buckinghamshire, UK). Western blot membranes were revealed with a ChemiDoc-It imaging system (Ultra-Violet Products Ltd., Cambridge, UK) and VisionWorks software (Analytik Jena AG, Jena, Germany), but they were analyzed with ImageJ software (National Institutes of Health, Bethesda, MD). All western blot membranes were analyzed separately by age class because they were randomized in this manner upon sample loading.

Statistical analyses

General linear models were used to evaluate the impact of CORT treatment on mitochondrial performance and oxidative damage variables in reproductive females. Females were age-matched prior to the investigation and age did not differ between treatment groups at termination of the study (t_{24}=-0.82, p=0.42) and averaged 341.8 days for all groups (VEH: 332 ±15.4 days, CORT: 350 ± 14.3 days). Date of death was included as a covariate to control for any seasonal effects in our model because, by chance, the vehicle group bred more quickly than the CORT-treated group (t_{24}=-3.34, p=0.003). The average date of death for females from the vehicle group (April 24 ± 6.19 days) was approximately a month earlier than CORT-treated females (May 23 ± 5.74 days). Body mass was also included as a covariate in all models because previous research

has shown condition-dependency in measured mitochondrial variables (Roussel et al. 2015; Mélanie et al. 2019; Park et al. 2020).

Linear random effects models were used to evaluate the impact of CORT treatment on mitochondrial performance and oxidative damage variables in offspring of different life stages. Maternal ID was included as a random factor for all offspring analyses to account for repeatability within a litter. Maternal age was not included as a covariate because there was no significant difference in age of mothers between treatment groups (t_{24}=-0.82, p=0.42), which averaged 341.8 days for both (VEH: 332 ±15.4 days, CORT: 350 ± 14.3 days). The offspring's age, however, is included as a covariate to separate emerging and persisting effects of indirect CORT exposure on offspring physiological development. Sex was also included as a covariate to tease apart sex-specific variation in responses to maternal CORT. Finally, date of death was included as a covariate in all models for controlling seasonal differences between treatment groups (see above). The analysis of fecal CORT metabolites in adult offspring also included body mass as a co-variate because it has been previously shown to be impacted by CORT treatment (Singleton and Garland 2019).

Results

Post-reproductive females

Reproductive females were evaluated for mitochondrial physiology and oxidative damage effects of CORT administration. 'Group' refers to treatment effects of either CORT-treated or vehicle mothers and their offspring. Females experienced high levels of CORT during treatment, indicated by a group×time interaction for fecal CORT metabolites ($F_{2,46}$=5.68, p=0.006; Table 1; Figure 2). Fecal CORT metabolites at peak lactation (day 12 post-partum) were significantly

higher in CORT-treated females compared to those in the vehicle group (t_{69}= 4.12, p= 0.001; Figure 2). There was also a significant increase in fecal CORT metabolites between baseline (before male pairing) and peak lactation (day 12 post-partum) in CORT-treated females (t_{46}=-4.21, p=0.002; Figure 2). There was no difference in fecal CORT metabolites at the time of weaning (day 28 post-partum) between CORT-treated or vehicle mothers (p>0.05; Figure 2). There was also no difference in fecal CORT metabolites between vehicle females at all time points (p>0.05; Figure 2). Given that fecal and circulating CORT were shown to be correlated, these findings suggest that the CORT treatment successfully elevated CORT levels in females, during the period of exposure.

I did not find an effect of CORT treatment on body mass of reproductive females at sacrifice (t_{24}=-1.43, p=0.168; Table 1), but body mass was still included as a co-variate in all models because our lab has previously shown that mitochondrial performance is condition-dependent (Park et al. 2020). However, the body mass of offspring whose mother was treated with CORT was lower at the time of weaning than offspring in the vehicle group (day 28 post-partum), when mothers that cannibalized pups (5 females cannibalized 1 pup; 1 female cannibalized 2 pups) or had a litter size smaller than 5 pups (2 females had 3 pups) were removed from the model (t_{16}=3.68, p=0.0025; Table 1; Figure 3). There was no difference in cannibalism rates between CORT treatment groups, as 3 mothers from each group cannibalized their young.

At the time of sacrifice, mitochondrial H_2O_2 emission (ROS) in skeletal muscle tissue was significantly reduced in CORT-treated females compared to those in the vehicle group (t_{24}=2.36, p=0.028; Table 1; Figure 4). There was no influence of CORT treatment on mitochondrial H_2O_2 emission (ROS) in liver tissue (p>0.05; Table 1). There was a trend,

however, towards lower state 3 respiration using complex I substrates in skeletal muscle mitochondria of CORT-treated reproducing females compared to vehicle females (t_{24}=1.70, p=0.10; Table 1; Figure 5). There was no effect of CORT treatment on any of the other mitochondrial respiratory indices (Complex 1: State 3, RCR; Complex 2: State 3, State 4, RCR), mitochondrial density (CS activity), or oxidative damage markers (4HNE; protein carbonyls) in either liver or skeletal muscle tissue (p>0.05; Table 1).

Offspring

Juvenile (5.5 weeks old) and adult (10 weeks old) offspring were evaluated for mitochondrial physiology and oxidative damage effects of postnatal maternal CORT. There was no effect of maternal CORT treatment on offspring body mass at sacrifice ($F_{1,24}$=1.43, p=0.243; Table 2). There were also no effects of maternal CORT treatment on offspring mitochondrial density in liver or skeletal muscle tissues (CS activity; p>0.05; Table 2). Feces were only collected in adult (10 weeks old) offspring. There was a significant group×sex interaction for fecal CORT metabolites of adult offspring ($F_{1,15}$=9.42, p=0.008; Table 2; Figure 6). Fecal CORT metabolites were significantly lower in female offspring of CORT-treated mothers compared to those of vehicle mothers (t_{38}=-2.85, p=0.036; Figure 6). However, fecal CORT metabolites did not differ between maternal CORT treatment groups in male offspring (p>0.05; Figure 6).

Isolated liver mitochondria were provided substrates for supporting state 4 respiration through complex I, and a significant group×age interaction was discovered ($F_{1,71}$=4.84, p=0.031; Table 2). This interaction seems to be driven by opposing directional effects in age classes, as juvenile offspring appear to have higher state 4 respiration with maternal CORT treatment while adult offspring of CORT-treated mothers had lower levels, but a post-doc analysis suggests that there were no significant differences between treatment groups or age classes (p>0.05). State 3

respiration in liver mitochondria using complex I substrates, however, did not differ between maternal CORT treatment groups (p>0.05). Respiration supported through complex II was shown to have a group×sex interaction in state 4 respiration of liver mitochondria ($F_{1,71}$=4.0, p=0.049; Table 2). This interaction seems to be driven by opposing directional effects in the sexes, as male offspring had reduced respiration with maternal CORT treatment while female offspring of CORT-treated mothers had higher levels, but a post-doc analysis suggests that there were no significant differences between treatment groups or sexes (p>0.05).

In skeletal muscle of offspring, isolated mitochondria provided substrates for supporting state 4 respiration through complex I showed a group×age interaction ($F_{1,67}$=17.6, p=0.0001; Table 2; Figure 7). This interaction appears to be driven by a significant increase of state 4 respiration using complex I substrates in accordance with aging in offspring of vehicle mothers ($t_{81.6}$=3.78, p=0.002; Figure 7). However, post-hoc analysis suggests there was no difference of state 4 respiration using complex I substrates in offspring of CORT-treated mothers (p>0.05; Fgiure 7). Isolated skeletal muscle mitochondria were also provided substrates for supporting respiration through complex II and there was a group×age interaction ($F_{1,67}$=9.10, p=0.0036; Table 2; Figure 7) in respiratory control ratio (RCR), the ratio of state 3 to state 4 respiration (state 3/state 4). This interaction was mediated through adult offspring, as RCR was significantly higher in offspring of CORT-treated mothers in this age class (t_{34}=2.82, p=0.038; Figure 7). There were also significant group×age interactions for both state 3 ($F_{1,67}$=4.0, p=0.050; Table 2) and state 4 ($F_{1,67}$=19.77, p<0.0001; Table 2; Figure 7) respiration using complex II substrates in skeletal muscle. The interaction in state 3 respiration using complex II substrates appears to be driven by opposing directional effects in age classes but again, there were no significant differences between treatment groups or age classes with the post-doc analysis (p>0.05). State 4

respiration using complex II substrates in skeletal muscle increased significantly with age in offspring of vehicle mothers (t_{84}=3.29, p=0.008; Figure 7) and adult offspring of CORT-treated mothers had significantly reduced state 4 respiration compared to those of vehicle mothers (t_{38}=-3.07, p=0.020; Figure 7).

There was a trend towards a group×sex interaction in the mitochondrial H_2O_2 (ROS) emissions of offspring in liver tissues ($F_{1,71}$=3.26, p=0.075; Table 2; Figure 8), driven primarily by higher levels in the male offspring of vehicle mothers compared to female offspring of vehicle mothers (t_{75}=-3.27 , p=0.009; Figure 8), but this pattern was lost with maternal CORT treatment. In skeletal muscle tissue, however, there was a significant group×age interaction for ROS emission of offspring ($F_{1,69}$=14.19, p=0.0003; Table 2; Figure 8). Overall, skeletal muscle ROS emission increased significantly with age in offspring of vehicle mothers (t_{89}=2.68, p=0.042; Figure 8). Maternal CORT caused deviations from this pattern, as there was no difference for offspring of CORT-treated mothers between the two age classes (p>0.05; Figure 8). Skeletal muscle ROS emission was significantly reduced in the adult offspring of CORT-treated mothers compared to those of vehicle mothers (t_{41}=-3.19, p=0.014; Figure 8). Interestingly, there was no difference in ROS emissions of skeletal muscle between maternal CORT treatment groups in juvenile offspring (p>0.05; Figure 8).

All western blot membranes were analyzed separately by age class because they were randomized in this manner upon sample loading. In juvenile offspring, there was a trend towards a group×sex interaction for markers of protein oxidation (protein carbonyls) in skeletal muscle tissue ($F_{1,22}$=3.63, p=0.07; Table 2; Figure 9). This interaction appears to be driven by opposing directional patterns of the sexes but there were no significant differences between treatment groups or sexes in the post-hoc analysis (p>0.05; Figure 9). Protein carbonyls were significantly

lower in the skeletal muscle of the adult offspring of CORT-treated mothers when compared to those of vehicle mothers ($F_{1,23}$=4.74, p=0.04; Table 2; Figure 9). Similar trends, albeit not significant, were found for protein carbonyls in the liver tissue of the adult offspring of CORT-treated mothers ($F_{1,23}$=2.76, p=0.11; Table 2; Figure 9) and markers of lipid peroxidation (4HNE adducts) in the liver tissue of both adult ($F_{1,22}$=3.23, p=0.08; Table 2; Figure 9) and juvenile ($F_{1,22}$=2.49, p=0.13; Table 2; Figure 9) offspring of CORT-treated mothers. There was no influence of maternal CORT treatment on protein carbonyls of liver tissue in juvenile offspring (p>0.05; Table 2). There was also no influence of maternal CORT treatment on 4HNE adducts in the skeletal muscle tissues of both adult and juvenile offspring (p>0.05; Table 2).

Discussion

When a mother experiences elevated CORT during reproduction, it can have lasting impacts on her condition and the condition of her offspring. The goal of this investigation was to determine if the effect of a 2-week elevation in CORT impacts the performance of mitochondria in the liver and skeletal muscle of mother's or their young. I targeted mitochondrial physiology when reproductive tissues had regressed to examine effects on basal conditions, which may be implicated in future reproduction and survival of the reproductive female. This was accomplished through the utilization of non-invasive CORT administration and sampling methods, which allowed us to limit exogenous stress exposure. Further, I evaluated the impact of maternal CORT on offspring after weaning (juvenile) and as offspring reach reproductive age (adult). These are time periods that will capture offspring at times that are critical to fitness, during initial independence and as young have the potential to breed themselves. Based on differences in litter mass at weaning, I found that maternal CORT exposure appeared to reduce

energy allocation to young and had lasting impacts on mitochondrial ROS emissions, respiration, and oxidative damage of offspring. However, the only persistent impact on mothers 17 days after the treatment ended, or 10 days after pup weaning, was a significant reduction in skeletal muscle H_2O_2 emissions (ROS) of females exposed to CORT, as well as a trend towards lower state 3 respiration using complex I substrates in this tissue.

Our results suggest that reproductive females are well-equipped to minimize the cost of elevated CORT during reproduction to themselves. I chose the current CORT dosage to fall near levels that have been previously shown to influence energy metabolism in the mitochondria of rodent livers (Jani et al. 1991). The correlation between fecal CORT and plasma CORT validate that that treatment should have increased circulating levels of CORT. Stress exposure is often associated with a decrease in body mass (Iio et al. 2011; Jeong et al. 2013), however there was no influence of exogenous CORT on reproductive females' body mass at sacrifice in this study which suggests minimal effects of CORT treatment on resource allocation to self-maintenance in CORT-treated mothers. While the effect of treatment had no impact on maternal mass, it appears that mothers may have transferred less milk energy to their offspring based on lower litter mass at weaning. After peak lactation, pups begin supplementing their dietary intake with adult food, so it remains possible that this effect was in part or entirely based on differences in rodent chow consumption by pups. It is also possible that effects may be due to differences in maternal care, as chronic social stress has been shown to attenuate latency to nursing, leading to reduced growth in offspring (Nephew and Bridges 2010).

Because stress during reproduction has the potential to shorten maternal lifespan (Kirkwood et al. 2000), I evaluated the lasting effects of maternal CORT on respiratory performance of mitochondria, ROS production and oxidative damage markers 17 days after

CORT treatment ended. Previous research has shown beneficial changes to liver mitochondrial respiration with reproduction, but no change in CS activity of this tissue (Hood et al. 2019). It appears that when females have elevated CORT, there was also no change in CS activity consistent with just reproductive females. Reproduction under stressful conditions also did not positively impact mitochondrial respiration measures in mothers, however there was a trend towards lower state 3 respiration using complex I substrates in skeletal muscle tissue.

Skeletal muscle tissue cells are post-mitotic and particularly susceptible to oxidative damage because they have a slow turnover rate and are no longer able to proliferate (Crane et al. 2013), potentially explaining the significant reduction in ROS with maternal CORT treatment as this tissue may be prioritized for protection from oxidative damage associated with treatment. As CORT administration has been previously shown to result in overproduction of ROS in skeletal muscle cells (Mitsui et al. 2002; Oshima et al. 2004), it is likely that a reduction of mitochondrial ROS post-CORT exposure was used as a compensation mechanism to protect mitochondria in this tissue against damage induced by CORT treatment (Brand 2000). Indeed, there were no measurable persistent effects of CORT treatment on oxidative damage markers. Although it is known from past studies that glucocorticoid exposure can result in oxidative damage (Costantini et al. 2011), there is also strong evidence that mice are able to recover from an oxidative challenge (Zhang et al. 2018).

Treatment levels of CORT may not have been sufficient for revealing persistent effects on baseline mitochondrial physiology in reproductive females. Studying the impacts of stress on rodent mothers is challenging as increasing maternal CORT can increase the probability that mothers will cannibalize their own young (Maguire and Mody 2016). While prior research has shown high doses of CORT to negatively influence muscle mitochondria number and function

post-treatment (Duclos et al. 2001), it appears that the mitochondria were able to recover from any changes experienced with CORT elevation during reproduction. This response is hinted by lower ROS emissions in skeletal muscle mitochondria of CORT-exposed mothers.

In contrast, it appears that maternal CORT elevation during reproduction has the largest impact on young. While the young of CORT-treated mothers were smaller at weaning, there was no influence of treatment on body mass of juvenile or adult offspring at sacrifice. This result suggests catch-up growth patterns, which has been shown to reduce lifespan (Metcalfe and Monaghan 2001). While the negative effects of catch-up growth are often attributed to oxidative damage, an accumulation of oxidative damage was not described herein. Our results are supported by studies in wild red squirrels (*Tamias hudsonicus*) that show a comparable elevation of maternal glucocorticoids lead to the production of offspring with lower birth weight and higher growth rate without inducing long-term oxidative costs that could influence survival of young (Dantzer et al. 2020).

Several effects of maternal stress on mitochondrial function were revealed with age in offspring. State 4 respiration using complex I substrates in skeletal muscle tissue increased with age in offspring of vehicle mothers, but this increase was not observed in offspring of CORT-treated mothers. In the same tissue, the increase of complex II RCR (state 3/state 4) is not revealed until the offspring of CORT-treated mothers reach adulthood. State 4 respiration using complex II substrates in skeletal muscle tissue increases with age in offspring of vehicle mothers, but offspring of CORT-treated mothers maintained lower levels into adulthood. Lower state 4 respiration allows mitochondria to efficiently consume little oxygen while maintaining the capacity to achieve maximal respiration, or state 3 (Brand 2000; Speakman et al. 2004; Brand and Nicholls 2011). Given that this effect was both observed with complex I and complex II

substrates, this effect should improve the efficiency of carbohydrate, protein, and fatty acid utilization while the mitochondria are idling. Paired with increased RCR using complex II substrates, the relative capacity for fatty acid metabolism in skeletal muscle would be improved during adulthood in the young of mothers exposed to stress, and so nutrients would become available for other tissues. Energy efficiency of animals whose mother experienced stress are expected to increase with RCR as it reflects the electron transport system's capacity to synthesize ATP and decrease ROS production, which could even slow processes of aging (Brand 2000; Speakman et al. 2004; Brand and Nicholls 2011).

Mitochondrial condition may be improved with reduced ROS emissions from skeletal muscle mitochondria and both liver and skeletal muscle displayed signs of lower oxidative damage in offspring of CORT-treated mothers. This is consistent with previous research in our lab which revealed that increased ROS emissions following an oxidative event quickly increased ROS and oxidative damage markers but then dropped below controls several days post-exposure (Zhang et al. 2018). These results are reflective of mitohormetic patterns, where a stressor initially causes damage that ultimately leads to improved mitochondrial function. Skeletal muscle ROS emissions were shown to increase with age in offspring of vehicle mothers, but this is not the case in adult offspring of CORT-treated mothers as they maintain ROS emission levels reflective of weanling offspring. Male offspring of vehicle mothers showed a trend towards higher ROS emissions from liver mitochondria than females, but this pattern is lost with administration of maternal CORT. Oxidative damage markers in offspring were also influenced by maternal CORT exposure. Liver 4HNE adducts, a product of lipid peroxidation, displayed a trend towards lower levels in juvenile offspring of CORT-treated mothers than the offspring of vehicle mothers and continued to be lower than vehicle offspring as they reached adulthood.

Lipid peroxidation has widespread effects and may lead to more oxidative damage in both DNA and proteins (Hulbert et al. 2007). Protein carbonyls, the product of protein oxidation, in the skeletal muscle displayed a trend of sex differences in juvenile offspring that disappeared with aging, as it appears that protein oxidation markers dropped later in males than in females.

Independent of treatment, additional sex-specific effects on offspring were observed. Female offspring maintained significantly higher fecal CORT metabolites than male offspring in both treatment groups, although the effect size was lessened with maternal CORT treatment. Male offspring of vehicle mothers showed a trend towards higher ROS emissions in liver mitochondria than females, which was lost with maternal CORT treatment. In skeletal muscle tissue, I observed a trend of sex-specific patterns in protein oxidative markers of juvenile offspring too, as reductions were observed in male offspring of CORT-treated mothers while female offspring of these mothers had higher levels. Again, these differences were not significant. Other studies have also shown sex-specific differences in how animals respond to stress (Liu et al. 2001; Slotten et al. 2006; Weintraub et al. 2010; Bale and Epperson 2015). Our sample size or maternal treatment of CORT may not have been sufficient to tease apart some of these sex differences in mitochondrial respiration, and so they warrant further investigation.

Conclusions

Short-lived species are at an increased risk of extrinsic mortality and expected to invest heavily into current reproduction at the cost of self-maintenance (Kirkwood and Austad 2000). Based on the present results within the context of this study, it is not clear that diminished mitochondrial performance is the mechanism responsible for hastened senescence of reproduction under stressful conditions. More interestingly, most maternal stress does appear to

have persisted positive effects on the metabolic capacity of young that could influence maternal fitness by mediating the bioenergetic capacity of offspring.

Tables

Table 1. Results of general linear models comparing mitochondrial physiology and oxidative damage of liver and skeletal muscle in corticosterone-treated (CORT) or vehicle (VEH) reproductive female mice. Fecal CORT metabolites results represent a linear mixed effect model with Time of fecal sample collection. Death date and mass were included as covariates and interactions between groups were evaluated. The p-values are given for all comparisons. Significant findings are reported in bold and marginal findings are italicized.

	VEH	**CORT**	**Statistics**				
	$\bar{x}$+se	**$\bar{x}$+se**	**num DF**	**den DF**	**Interact-ion term**	**F-value**	**p-value**
Fecal CORT metabolites* (µg/g)	B: 0.171±0.73 P: 0.0398±0.70 W: 0.106±0.70	B: 0.123±0.65 P: 3.96±0.65 W: 0.107±0.67	2	46	**Group ×Time**	5.68	**0.006**
	VEH	**CORT**					
	$\bar{x}$+se	**$\bar{x}$+se**	**num DF**	**den DF**	**Main effect**	**t-value**	**p-value**
Body mass (g)	20.5±0.61	21.7+0.56	1	24	Group	-1.43	0.17
D28 Litter mass (g)	68.1+1.65	59.3±1.45	1	16	**Group**	3.68	**0.0025**
LIVER:							
Citrate synthase (nM/min/mg protein)	1.95±0.13	1.77±0.12	1	24	Group	0.95	0.35
H_2O_2 emission (pmols/mg protein/min)	34.5±4.1	34.9±3.8	1	24	Group	-0.065	0.95
Complex I substrates							
State 3 respiration (nmole O^2/mg/ protein/min)	135±13.1	139±11.9	1	24	Group	0.981	0.83
State 4 respiration (nmole O^2/mg/ protein/min)	25.7±2.0	29.4±1.8	1	24	Group	-1.24	0.23
Respiratory Control Ratio (state 3/state 4)	5.23±0.36	4.86±0.33	1	24	Group	0.687	0.5
Complex II substrates							
State 3 respiration (nmole O^2/mg/ protein/min)	226±11.6	224±10.5	1	24	Group	0.792	0.38
State 4 respiration (nmole O^2/mg/ protein/min)	50.5±3.3	55.2±3.0	1	24	Group	-0.944	0.36
Respiratory Control Ratio (state 3/state 4)	4.53±0.127	4.18±0.24	1	24	Group	0.871	0.39

Oxidative damage markers							
4HNE adducts (fold-change)	0.993±0.035	0.996±0.032	1	24	Group	-0.059	0.95
Protein carbonyls (fold-change)	0.970±0.063	0.923±0.057	1	24	Group	0.494	0.63
SKELETAL MUSCLE:							
Citrate synthase (nM/min/mg protein)	1.61±0.18	1.70±0.16	1	24	Group	-0.354	0.73
H_2O_2 emission (pmols/mg protein/min)	361±54.6	168±49.7	1	24	**Group**	2.36	**0.028**
Complex I substrates							
State 3 respiration (nmole O^2/mg/ protein/min)	351±31.5	271±28.7	1	24	*Group*	1.70	*0.10*
State 4 respiration (nmole O^2/mg/ protein/min)	81.8±8.1	65.9±7.4	1	24	Group	1.26	0.22
Respiratory Control Ratio (state 3/state 4)	4.45±0.48	4.39±0.44	1	24	Group	0.08	0.94
Complex II substrates							
State 3 respiration (nmole O^2/mg/ protein/min)	449±39.9	383±36.3	1	24	Group	1.11	0.28
State 4 respiration (nmole O^2/mg/ protein/min)	167±17.1	148±15.5	1	24	Group	0.721	0.48
Respiratory Control Ratio (state 3/state 4)	2.70±0.13	2.67±0.12	1	24	Group	0.166	0.87
Oxidative damage markers							
4HNE adducts (fold-change)	0.984±0.032	0.995±0.029	1	24	Group	-0.261	0.83
Protein carbonyls (fold-change)	1.02±0.082	0.995±0.075	1	24	Group	0.218	0.83

*random factor = id; B = Baseline; P = Peak; W = Wean

Table 2. Results of linear mixed effect models comparing mitochondrial physiology and oxidative damage of liver and skeletal muscle in the offspring of corticosterone-treated (CORT) or vehicle (VEH) mice. Death date, sex, and age were included as covariates. Mass was included as a covariate for analyzing fecal CORT metabolites only. The p-values are given for all comparisons. Significant findings are reported in bold and marginal findings are italicized.

	VEH	CORT	Statistics				
	$\bar{x}$+se	$\bar{x}$+se	num DF	den DF	Main effect/Int-eraction	F-value	p-value
Body mass (g)	17.2±0.47	16.4±0.43	1	24	Group	1.43	0.24
Fecal CORT metabolites* (µg/g)	F: 0.135±0.009 M: 0.047±0.010	F: 0.098±0.009 M: 0.058±0.008	1	15	**Group ×Sex**	9.42	**0.008**
LIVER:							
Citrate synthase (nM/min/mg protein)	1.03±0.046	0.971±0.043	1	24	Group	0.704	0.410
H_2O_2 emission (pmols/mg protein/min)	F: 31.1±3.7 M: 42±3.71	F: 36.7±3.4 M: 39.3±3.6	1	71	*Group ×Sex*	3.26	*0.075*
Complex I substrates							
State 3 respiration (nmole O^2/mg/ protein/min)	182±46.9	169±43.7	1	24	Group	0.033	0.86
State 4 respiration (nmole O^2/mg/ protein/min)	J: 26.3±1.8 A: 29.8±1.5	J: 27.1±1.3 A: 25.5±1.8	1	71	**Group ×Age**	4.84	**0.03**
Respiratory Control Ratio (state 3/state 4)	5.28±0.31	5.76±0.28	1	24	Group	1.10	0.30
Complex II substrates							
State 3 respiration (nmole O^2/mg/ protein/min)	234±9.3	222±8.7	1	24	Group	0.792	0.38
State 4 respiration (nmole O^2/mg/ protein/min)	F: 52.2±3.2 M: 58.5±3.2	F: 54.2±2.9 M: 50.5±3.0	1	71	**Group ×Sex**	4.00	**0.05**
Respiratory Control Ratio (state 3/state 4)	4.30±0.17	4.31±0.17	1	24	Group	0.002	0.96
Oxidative damage markers							
Juvenile:							
4HNE adducts (fold-change)	1.05±0.033	0.97±0.032	1	22	Group	2.49	*0.13*
Protein carbonyls (fold-change)	1.04±0.056	0.946±0.051	1	23	Group	1.23	0.28

Adult:							
4HNE adducts (fold-change)	1.047±0.036	0.951±0.033	1	23	Group	3.23	*0.08*
Protein carbonyls (fold-change)	1.04±0.044	0.934±0.042	1	23	*Group*	2.76	*0.11*
SKELETAL MUSCLE:							
Citrate synthase (nM/min/mg protein)	0.138±0.13	1.53±0.12	1	24	Group	0.578	0.45
H_2O_2 emission (pmols/mg protein/min)	J: 247±47.9 A: 398±39.2	J: 290±34.4 A: 219±45.7	1	69	**Group ×Age**	14.2	**0.0003**
Complex I substrates							
State 3 respiration (nmole O^2/mg/ protein/min)	383±16.8	376±15.4	1	24	Group	0.072	0.79
State 4 respiration (nmole O^2/mg/ protein/min)	J: 67.1±8.1 A: 100.3±6.3	J: 83.1±5.7 A: 78.3±7.6	1	67	**Group ×Age**	17.6	**0.0001**
Respiratory Control Ratio (state 3/state 4)	4.81±0.30	4.96±0.28	1	24	Group	0.118	0.73
Complex II substrates							
State 3 respiration (nmole O^2/mg/ protein/min)	J: 450±32.1 A: 507±27.2	J: 486±23.5 A: 457±30.6	1	67	**Group ×Age**	4.002	**0.05**
State 4 respiration (nmole O^2/mg/ protein/min)	J: 156±14.7 A: 211±12.0	J: 180±10.5 A: 156±13.9	1	67	**Group ×Age**	19.8	**<0.0001**
Respiratory Control Ratio (state 3/state 4)	J: 2.72±0.24 A: 2.46±0.19	J: 2.77±0.17 A: 3.29±0.23	1	67	**Group ×Age**	9.10	**0.004**
Oxidative damage markers							
Juvenile:							
4HNE adducts (fold-change)	1.00±0.062	0.982±0.057	1	22	Group	0.039	0.85
Protein carbonyls (fold-change)	F: 1.05±0.061 M: 0.981+0.061	F: 0.973±0.042 M: 1.04+0.057	1	22	*Group ×Sex*	3.63	*0.07*
Adult:							
4HNE adducts (fold-change)	1.03±0.032	0.995±0.029	1	23	Group	0.624	0.44
Protein carbonyls (fold-change)	1.07±0.049	0.91±0.046	1	23	Group	4.74	**0.04**

For each model: parent = random factor; J = juvenile; A = adult; F = female; M = male; *Adults

Figures

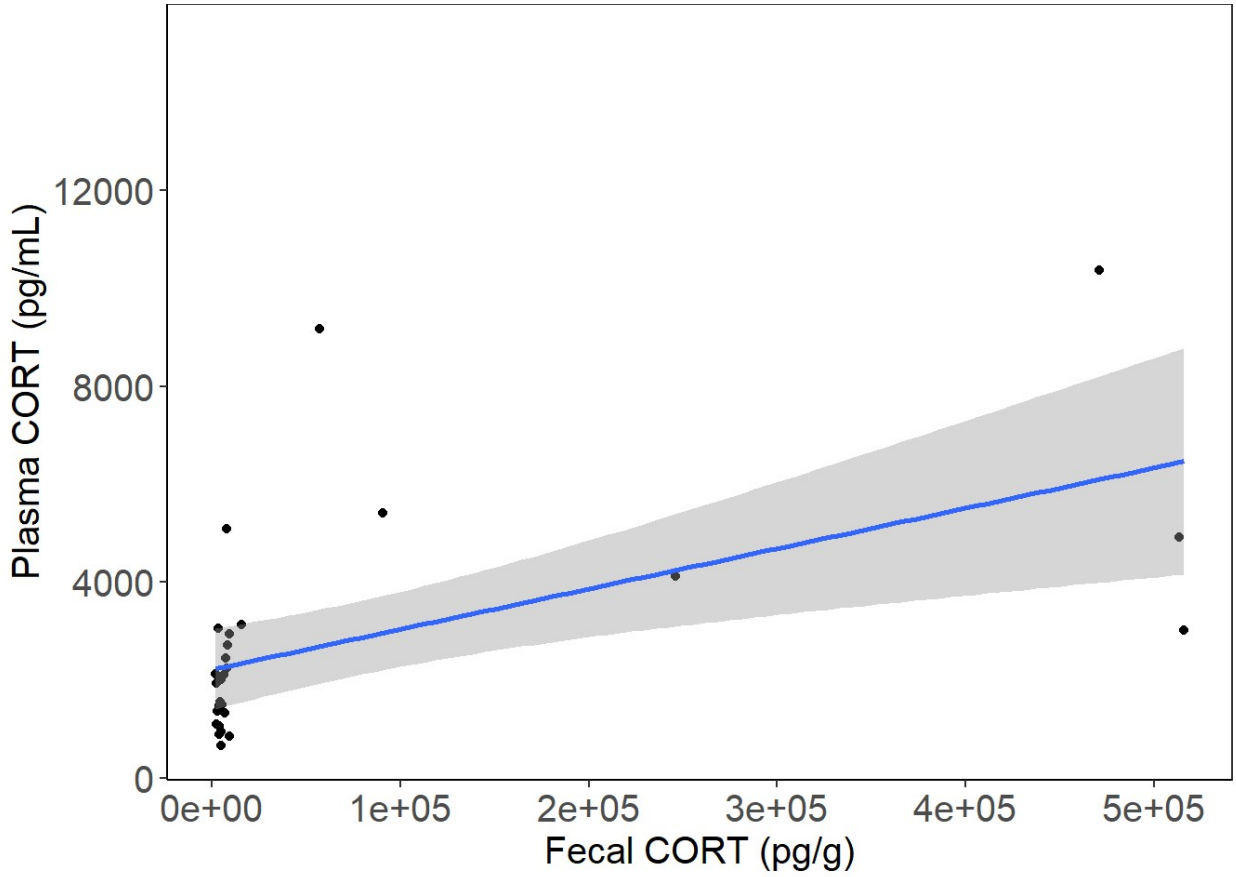

Figure 1. Correlation between corticosterone (CORT) metabolites (pg/g) found in both plasma and fecal samples of reproducing female mice, including baseline and peak lactation levels in all animals.

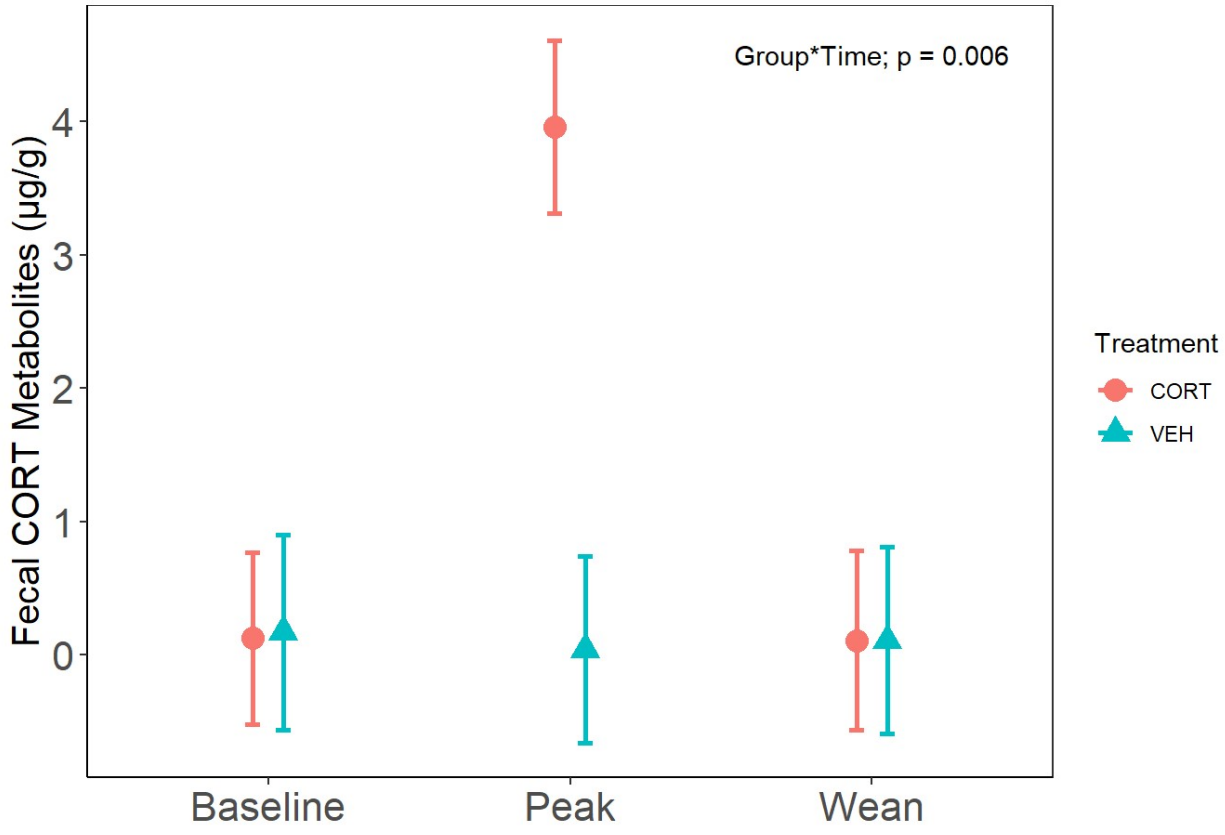

Figure 2. Fecal corticosterone metabolites (µg/gm fecal solid) in reproducing mice across baseline (before pairing with male), peak lactation (d12 post-partum), and wean (d28 post-partum) timepoints in corticosterone-treated (CORT) and vehicle (VEH) females.

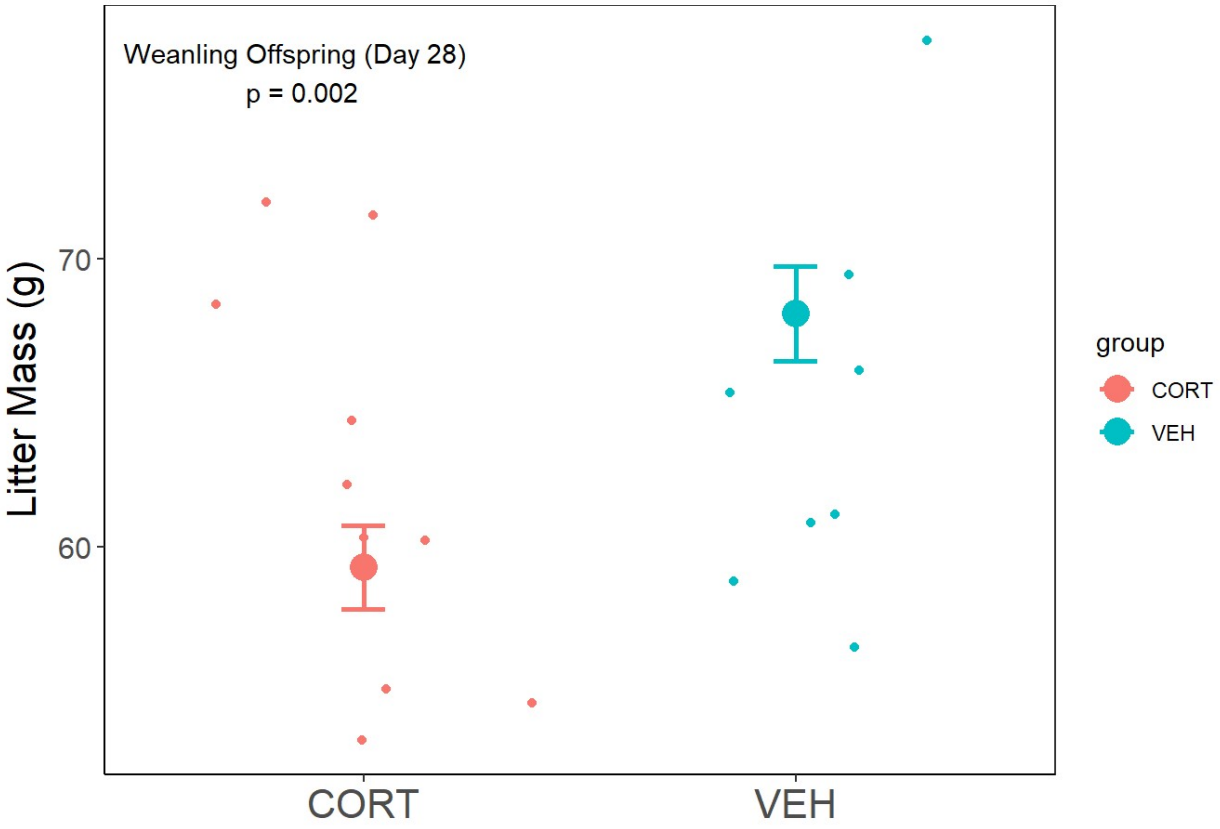

Figure 3. Cumulative litter mass (g) of offspring at weaning (day 28 post-partum) of corticosterone-treated (CORT) and vehicle (VEH) mothers that did not cannibalize or give birth to a litter size less than 5 pups.

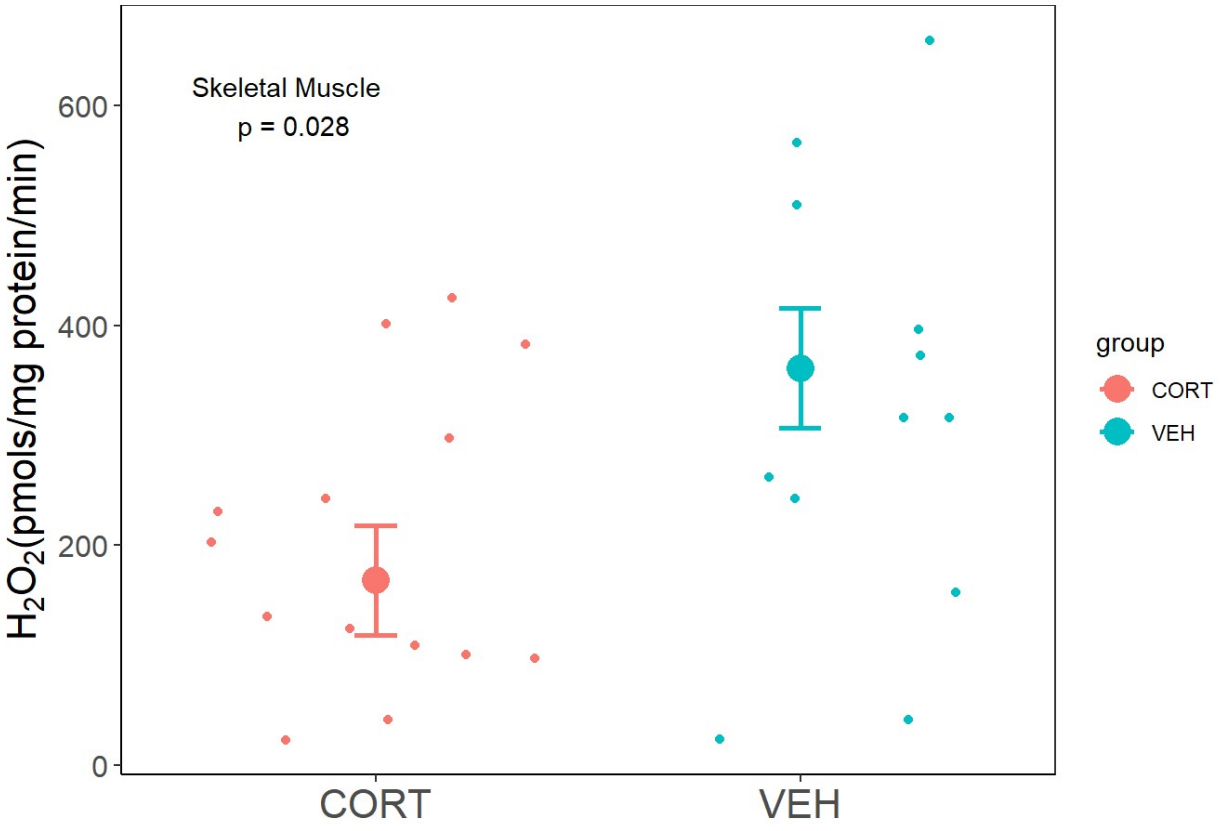

Figure 4. Mitochondrial H_2O_2 emission (pmols/mg protein/min) in skeletal muscle tissue of corticosterone-treated (CORT) and vehicle (VEH) reproducing female mice.

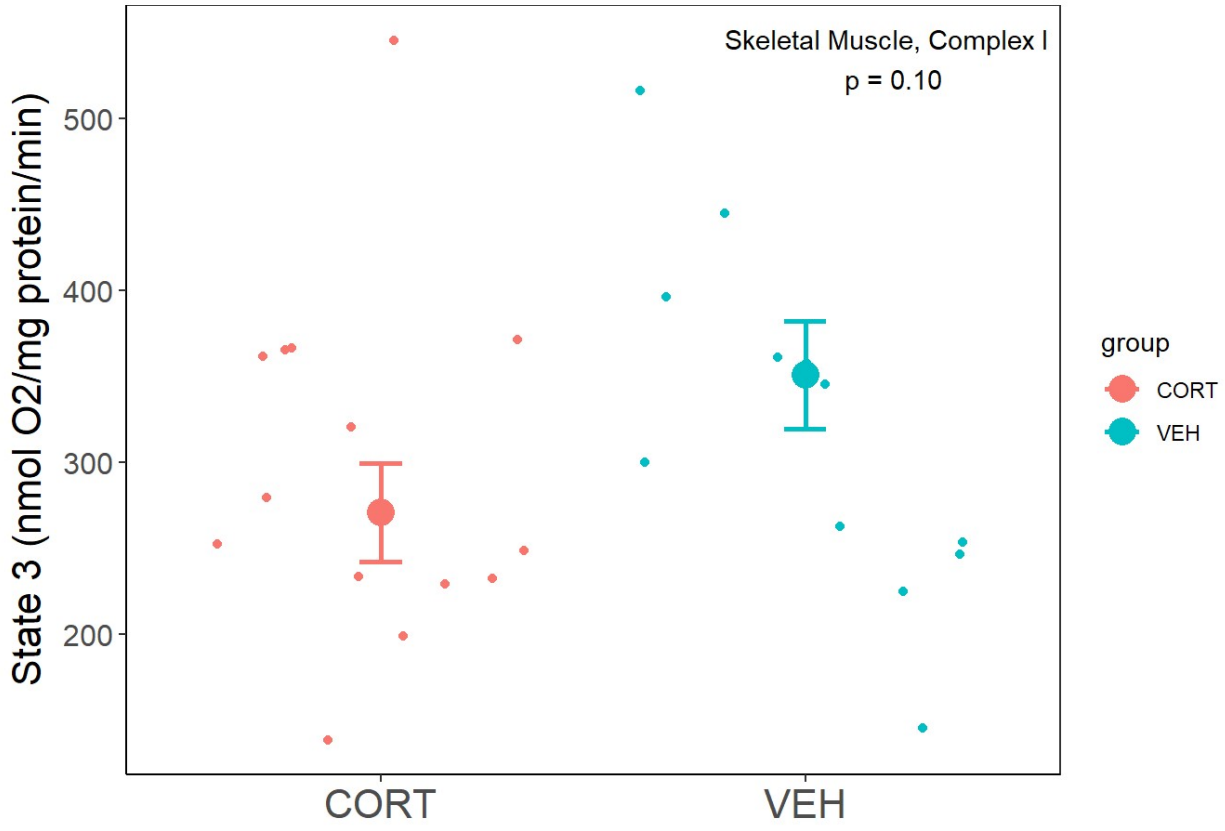

Figure 5. State 3 respiration (pmols/mg protein/min) using complex I substrates in skeletal muscle mitochondria of corticosterone-treated (CORT) and vehicle (VEH) reproducing female mice.

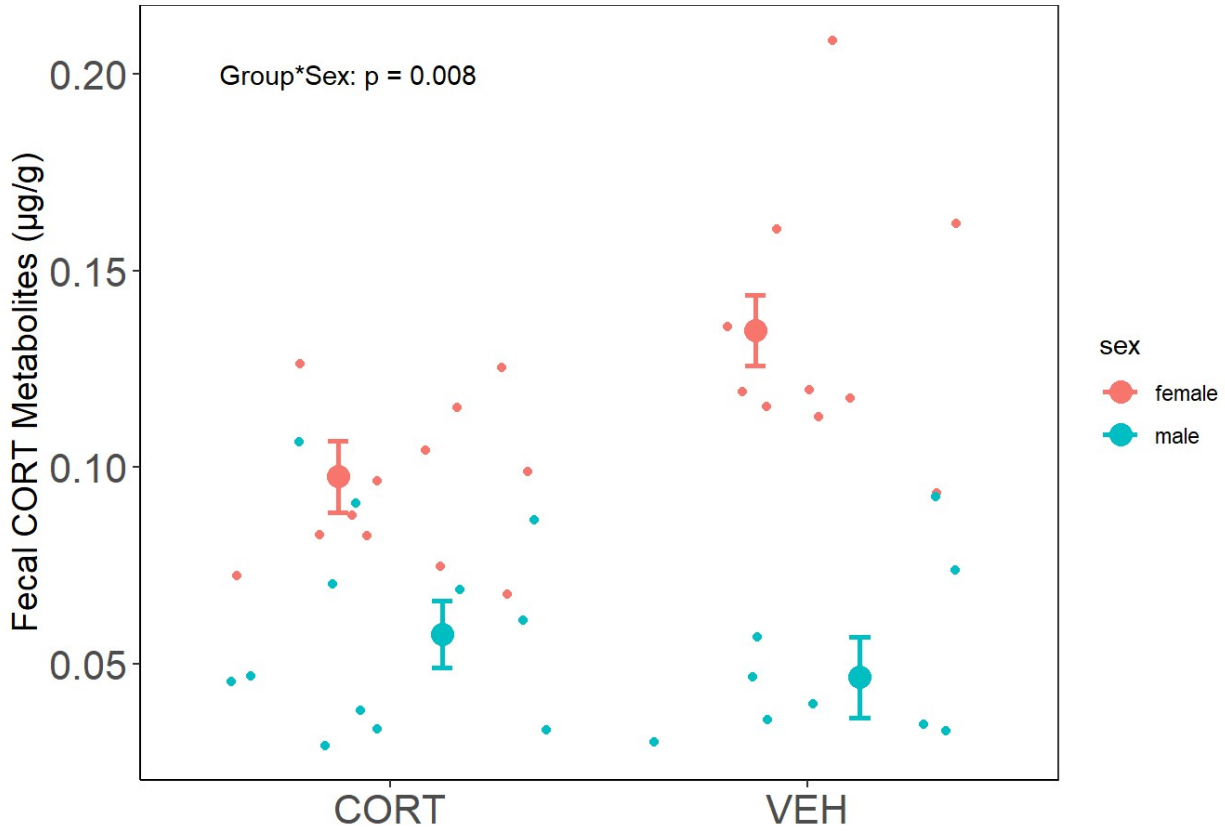

Figure 6. Fecal corticosterone metabolites (µg/gm fecal solid) in offspring of vehicle (VEH) and CORT-treated (CORT) reproductive mice.

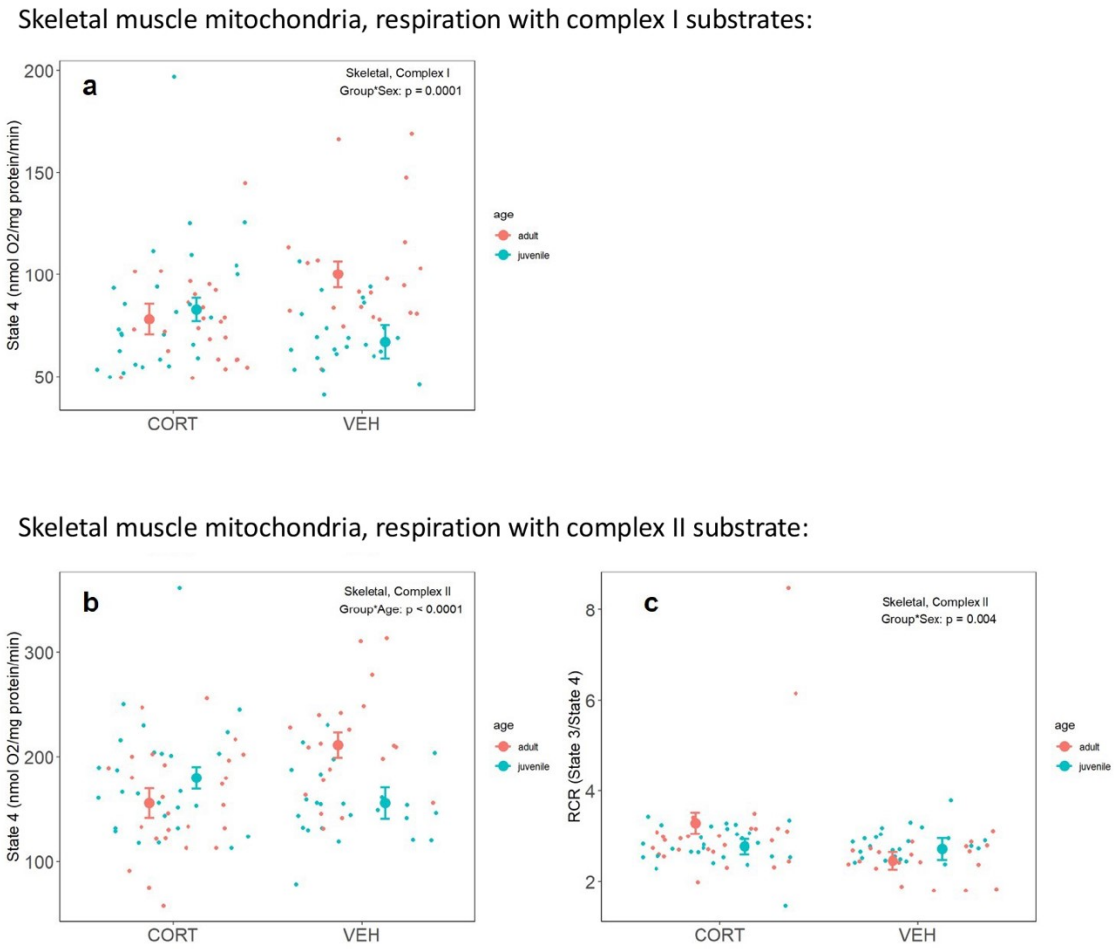

Figure 7. Significant interactions on mitochondrial respiration in offspring of corticosterone-treated mothers (CORT) and vehicle mothers (VEH). Data included **a** state 4 respiration using complex I substrates, **b** state 4 respiration using complex II substrates, and **c** respiratory control ratio (RCR) using complex II substrates in skeletal muscle tissues.

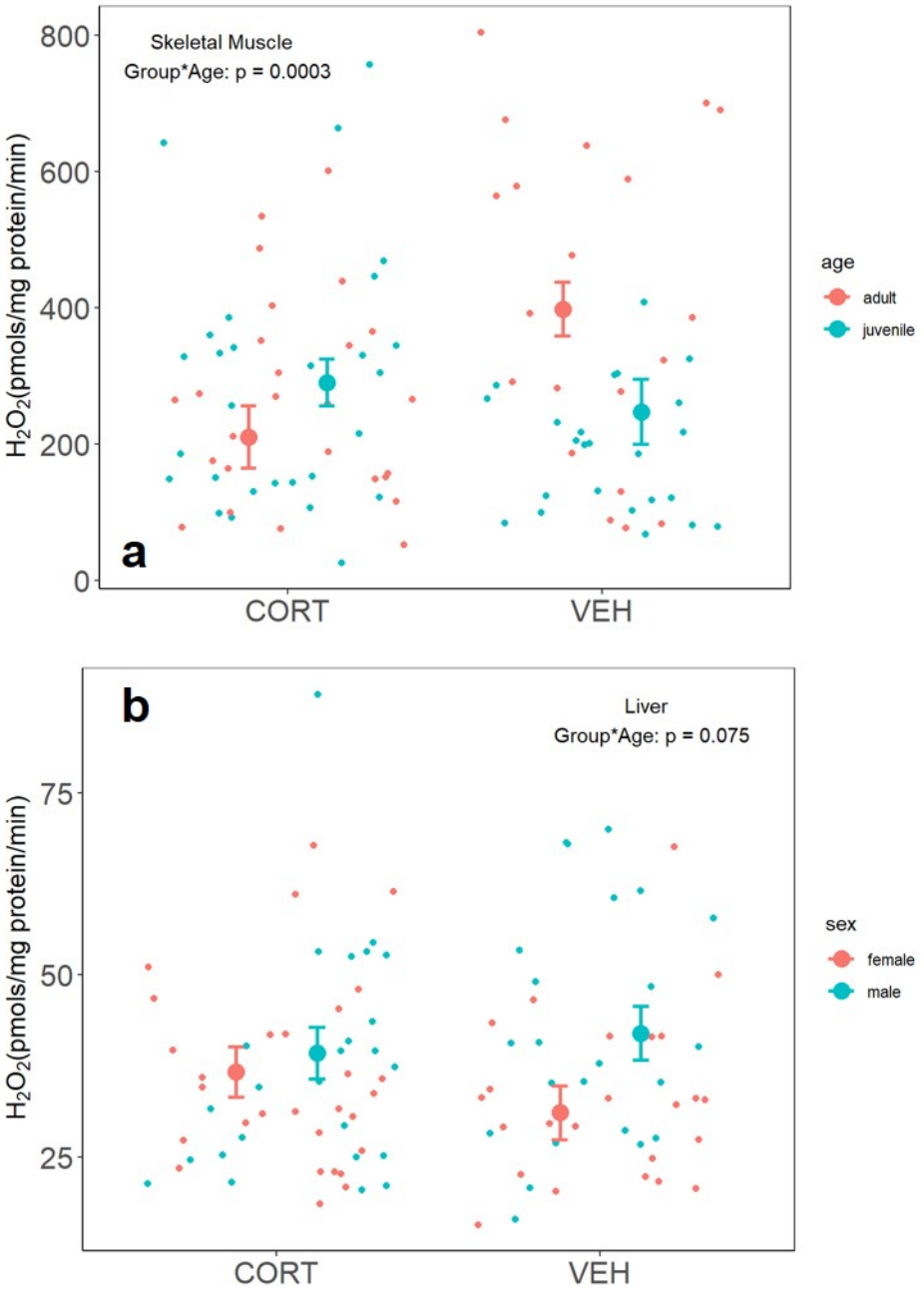

Figure 8. Significant and near significant effects in mitochondrial H_2O_2 emission (pmols/mg protein/min) of skeletal muscle tissue for the offspring of corticosterone-treated (CORT) and vehicle (VEH) reproductive mice.

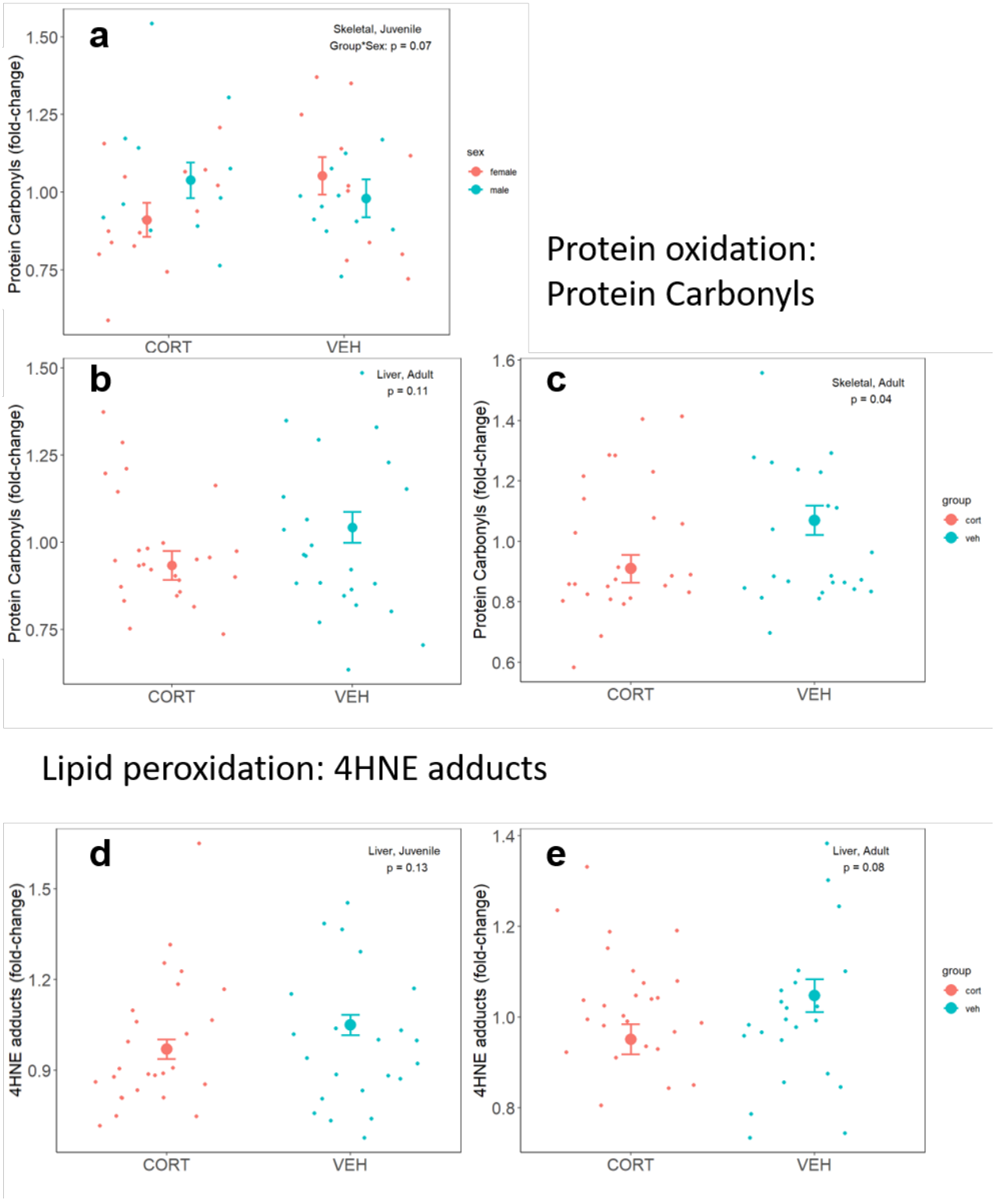

Figure 9. Significant and near significant effects on oxidative damage markers in offspring of corticosterone-treated mothers (CORT) and vehicle mothers (VEH). Data included **a** group×sex interaction in skeletal muscle protein carbonyls of juvenile offspring, **b** liver protein carbonyls of adult offspring, **c** skeletal muscle protein carbonyls of adult offspring, **d** liver 4HNE adducts of juvenile offspring, and **e** liver 4HNE adducts of adult offspring.

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