

RESEARCH ARTICLE

High activity before breeding improves reproductive performance by enhancing mitochondrial function and biogenesis

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ABSTRACT

Understanding of physiological responses of organisms is typically based on data collected during an isolated event. Although many fundamental insights have been gained from these studies, evaluating the response to a single event ignores the fact that each individual has experienced a unique set of events throughout its life that may have altered its physiology. The idea that prior experiences can influence subsequent performance is known as a carry-over effect. Carry-over effects may explain much of the variation in performance found among individuals. For example, high physical activity has been shown to improve mitochondrial respiratory function and biogenesis and reduce oxidative stress, and has been linked to improved health and longevity. In this study, we asked whether the bioenergetic differences between active and inactive individuals carry over to impact performance in a subsequent reproductive event and alter a female's reproductive outcome. Female mice that had access to a running wheel for a month before mating gave birth to a larger litter and weaned a heavier litter, indicating that high physical activity had a positive carry-over effect to reproduction. Mice that ran also displayed higher mitochondrial respiration and biogenesis with no changes in endogenous antioxidant enzymes. These results provide a mechanistic framework for how the conditions that animals experience before breeding can impact reproductive outcomes.

KEY WORDS: Carry-over effects, Running, Reproduction, Bioenergetics, Oxidative stress

INTRODUCTION

Identifying the sources of variation in fitness within a population is a central challenge in evolutionary ecology. It is well established that a portion of that variation is shaped by prior experiences. The pre- and post-natal environment, level of parental care, chronic stress and pathogen exposure at all life stages have been shown to alter the future performance of an individual (Naguib et al., 2006; Monaghan, 2008; Maestripieri and Mateo, 2009; Graham et al., 2011; Saino et al., 2012). Impacts that prior events have on subsequent performance are referred to as carry-over effects (Harrison et al., 2011; O'Connor et al., 2014). For many species, the environment experienced in the weeks and months before the onset of breeding is highly variable, and these environmental effects contribute to variance in the conditions of animals at the onset of reproduction. For females, individual condition determines the

relative amount of resources that can be partitioned to offspring development and self-maintenance.

Much of the research on carry-over effects in adults has focused on how extrinsic conditions impact the condition of females. Food quantity and quality on wintering grounds (Perryman et al., 2002; Cook et al., 2004; Sorensen et al., 2009; Inger et al., 2010; Skrip et al., 2016), stress prior to (Legagneux et al., 2012) and weather conditions during migration (Finch et al., 2014) have all been shown to alter subsequent reproductive success, as indicated by a change in number, condition and subsequent performance of the young that are produced. In many of these cases, variance in a female's reproductive success is correlated with variation in her body mass, a frequent indicator of variation in body fat. Although adipose stores can both permit and support subsequent reproduction, fuel availability alone does not determine the capacity to allocate energy to young, nor are residual fat stores the singular variable that determines a female's condition after reproduction has ended. The biochemical processes responsible for producing ATP are plastic. The capacity and relative efficiency of these processes may be improved or reduced in response to the intrinsic and extrinsic environment of the individual.

In relatively inactive models, such as humans and lab mice, running has been shown to elevate mitochondrial respiratory function (Ardies et al., 1987), reduce oxidative stress (Guers et al., 2016), and promote mitochondrial biogenesis (Meers et al., 2014). High physical activity (e.g. running) can exert effects on future reproduction, as intense, sustained exercise has been linked to inhibition of the reproductive axis (Warren and Perloth, 2001). In wild populations, relative level of activity will also vary among individuals. While high activity may be the norm for many species, bioenergetic differences are likely to occur between individuals or between seasons. Poor weather conditions may limit foraging time and whether a female must travel a relative long or relative short distance to mate may alter her bioenergetic capacity in the weeks and months before reproduction.

How much energy a mother allocates to reproduction is thought to be determined both by the size of the energy pool that she has available and how much of that pool she allocates to her own maintenance versus her offspring (Zera and Harshman, 2001; Zhang and Hood, 2016). By characterizing changes in mitochondria density, mitochondrial respiratory function, reactive oxygen species (ROS) emission, and markers of oxidative damage, we can deduce whether an increase in energy allocated to reproduction is associated with an increase in a female's capacity to produce ATP and thus, an increase in pool of energy that she has available. In addition, we can determine if an increase in energy allocated to reproduction is associated with a reduction in the allocation of energy to general tissue maintenance.

In this study, we asked whether the bioenergetics differences between active (with a running wheel) and inactive (no access to a wheel) individuals would carry over to reproduction. We predicted

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that mothers that ran before reproduction would allocate more resources to reproduction. In addition, we predicted that the muscle and liver of mothers that ran would display lower oxidative damage and higher mitochondrial respiratory function, effectively mitigating many of the potential costs of reproduction. We also expected that the impact of running on the mother's mitochondria would be greater in the liver and skeletal muscle than in the heart and brain because the liver and skeletal muscle have a high and variable metabolic demand (Rolfe and Brown, 1997) and because the body prioritizes consistent performance of the brain and heart over other organs (Zera and Harshman, 2001). Thus, mitochondrial performance in the liver and skeletal muscle should be more sensitive to the prior running event.

MATERIALS AND METHODS

This study was divided into two experiments. The goal of the first experiment was to confirm that our running protocol induced the reported beneficial adaptations to mitochondria in mice. The second experiment evaluated whether running before reproduction impacts a female's reproductive performance and their bioenergetic state after reproduction has ended.

Animal care

Adult outbred female ICR mice (obtained at 12 weeks of age) were used in both experiment 1 and 2 (Envigo, Huntingdon, UK). These experiments were conducted from May to July 2016. Animals were maintained on a 12 h:12 h light:dark cycle at a temperature of 24°C. All mice were housed individually in standard mouse boxes (29.12×19.05×12.7 cm). Food and water were provided *ad libitum* for the duration of the study. All animal procedures were approved by the Auburn University Institutional Animal Care and Use Committee (PRN #2016-2885).

Experiment 1

Twenty female ICR mice were randomly assigned to one of two experimental groups: no wheel ($n=10$) and wheel ($n=10$). Mice in the wheel group were given a saucer running wheel, which includes a Fast-Trac saucer that sits on top of an Igloo shelter (Bio-Serv, Flemington, NJ, USA). Under these conditions, running is voluntary and expected to mimic the time mice would spend running in the wild (Meijer and Robbers, 2014). No-wheel mice were housed with an Igloo without the attached saucer. Wheels (i.e. saucers) were kept in the boxes for 28 days and then removed. The activity of each mouse was recorded with a camera on three random nights. Mice with access to wheels were confirmed to be more active than mice without wheels and ran on average of 30 min 4 s per hour of each night recorded. Following removal of the wheels, all animals were given a 7-day period of rest to allow their tissues to return to a non-active state (Eston et al., 1996; Bruunsgaard et al., 1997). Then mice were euthanized and their tissues were dissected and evaluated as described below.

Experiment 2

Twenty additional female ICR mice were randomly assigned to one of two experimental groups: no wheel ($n=10$) and wheel ($n=10$). Mice were treated the same as in experiment 1 for 28 days with a 7-day resting period after voluntary running. Following the rest period, a male mouse was introduced into each female's cage. The male was removed when a vaginal plug was observed (7±4 days). One female from each group did not successfully reproduce and was removed from the study, resulting in $n=9$ for each reproductive group. Gestation lasted 17–20 days. Litter sizes were recorded and

adjusted to eight pups on the day of birth. Pups were weaned, weighed and euthanized 21 days postpartum. We summed the body masses of the pups reared by each female at weaning. Females were euthanized 7 days after weaning. This timing would have allowed the females' reproductive tissues to regress, permitting us to evaluate the consequences of reproduction independent of the ephemeral changes that are associated with reproduction (Zhang et al., 2017).

Tissue collection

Organs collected and methods of analysis were similar for each experiment. After euthanasia, the liver, hind leg muscles (including the tibialis anterior, soleus, gastrocnemius, quadriceps and hamstrings), heart and brain were removed and weighed. The left lateral and right medial lobes of the liver and the entire right leg muscle were used for mitochondrial isolation. The left leg muscle, remaining liver, brain and heart were flash-frozen in liquid nitrogen, and stored at -80°C for later analyses.

Mitochondrial measurements

Mitochondria were isolated following procedures outlined previously (Hyatt et al., 2016; Mowry et al., 2016, 2017). The fresh liver was minced and then homogenized in a Potter-Elvehjem PTFE pestle and glass tube. The resulting homogenate was centrifuged and the supernatant was then decanted through cheesecloth and centrifuged again. The resulting supernatant was discarded, and the mitochondria pellet was washed in liver isolation solution. This solution was again centrifuged, and the final mitochondria pellet was suspended in a mannitol–sucrose solution. The skeletal muscles were minced and then homogenized with a VITRUS polytron. Trypsin was added to aid the release of mitochondria from the myofibrils. The resulting homogenate was centrifuged, and the supernatant was then decanted through cheesecloth and centrifuged again. The mitochondria pellet was washed and finally resuspended in a mannitol–sucrose solution.

Mitochondrial respiration was determined polarographically (Oxytherm, Hansatech Instruments, Pentney, UK) following procedures outlined previously (Hyatt et al., 2016; Mowry et al., 2016, 2017). Respiration was measured using 2 mmol l⁻¹ pyruvate, 2 mmol l⁻¹ malate and 2 mmol l⁻¹ glutamate as a substrate. Maximal respiration (state 3) was defined as the rate of respiration in the presence of ADP and was initiated by adding 0.25 mmol l⁻¹ ADP to the chamber containing buffered mitochondria and respiratory substrates. State 4 respiration was measured after the phosphorylation of ADP was complete. The state 3 and 4 respirations were normalized to mitochondrial citrate synthase (CS). Respiratory control ratio (RCR) was calculated by dividing state 3 respiration by state 4 respiration.

The measurement of H₂O₂ emission in isolated mitochondria was conducted using Amplex Red (Thermo Fisher Scientific, Waltham, MA, USA) (Kavazis et al., 2009b; Hyatt et al., 2017). Formation of resorufin (Amplex Red oxidation) by H₂O₂ was measured at an excitation wavelength of 545 nm and an emission wavelength of 590 nm using a Synergy H1 Hybrid plate reader (BioTek, Winooski, VT, USA), at 37°C in a 96-well plate using succinate. Readings of resorufin formation were recorded every 5 min for 15 min, and a slope (rate of formation) was produced from these. The obtained slope was then converted into the rate of H₂O₂ production using a standard curve and was normalized to CS.

Enzymatic assays to determine electron transport chain complex activity in isolated mitochondria were performed as described previously (Spinazzi et al., 2012; Hyatt et al., 2017). Complex I

(NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (ubiquinol cytochrome *c* oxidoreductase) and complex IV (cytochrome *c* oxidoreductase) activities were determined and normalized to mitochondrial protein concentration.

The CS activity was measured in whole tissue homogenate and was used as a proxy for mitochondrial density (Spinazzi et al., 2012).

Western blot

Western blots were conducted on liver, skeletal muscle, heart and brain samples to analyze peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 α , a key regulator of mitochondrial biogenesis; GTX37356, GeneTex, Irvine, CA, USA); the antioxidants copper-zinc superoxide dismutase (CuZnSOD; GTX100554; GeneTex), manganese superoxide dismutase (MnSOD; GTX116093, GeneTex), glutathione peroxidase 1 (GPX-1; GTX116040, GeneTex) and catalase (GTX110704, GeneTex); a marker of lipid peroxidation [4-hydroxynonenal (4-HNE); ab46545, Abcam, Cambridge, MA, USA]; and a marker of protein oxidation (protein carbonyls; OxyBlot, s7150, EMD Millipore, Billerica, MA, USA). Each membrane was stained by Ponceau, and was used as the loading and transfer control. A chemiluminescent system was used to visualize marked proteins (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Images were taken and analyzed with the ChemiDoc-It Imaging System (UVP, LLC, Upland, CA, USA).

Statistics

A Grubbs' outlier test was used to identify statistical outliers, and each was removed. Unless otherwise noted in tables or figures, $n=10$ per group for experiment 1, and $n=9$ per group for experiment 2. All comparisons were completed with a two-sample *t*-test. All statistical analyses were performed with SigmaStat 3.5 (Systat Software, Inc., Point Richmond, CA, USA). Significance was established at $\alpha=0.05$.

RESULTS

Experiment 1: effect of running on mitochondria and oxidative stress

Experiment 1 was designed to test and confirm the effects of running on mitochondrial and ROS markers. The RCR of liver

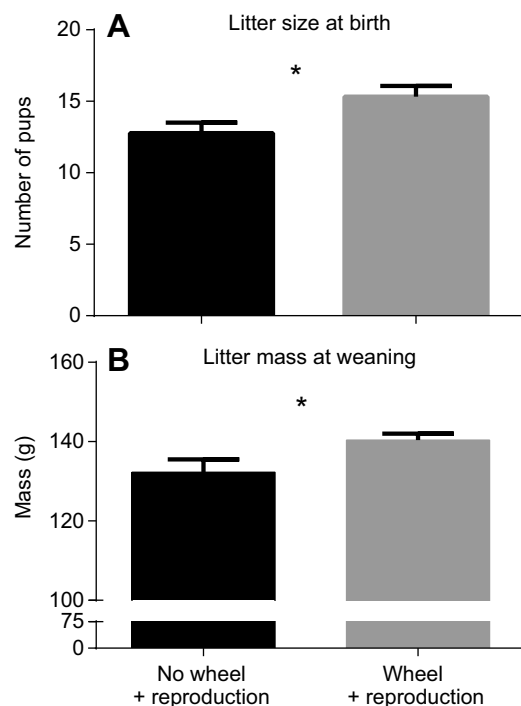


Fig. 1. Effect of prior running on reproductive output in mice. (A) Litter size at birth and (B) mass of weaned pups by each female. Data are presented as means \pm s.e.m., $n=9$ for each group. *Significant difference between treatments ($P<0.05$).

mitochondria did not differ significantly between mice that did or did not have access to a wheel ($t_{18}=0.95$, $P=0.36$; Fig. S1A). In contrast, RCR of muscle mitochondria was significantly higher after running ($t_{17}=2.13$, $P=0.048$; Fig. S1B). H_2O_2 emission by liver mitochondria did not vary between mice that did or did not have access to a wheel ($t_{18}=0.45$, $P=0.659$; Fig. S1C). H_2O_2 emission by muscle mitochondria displayed a trend suggesting that running may have contributed to lower H_2O_2 , but this was not significant ($t_{18}=1.83$, $P=0.084$; Fig. S1D).

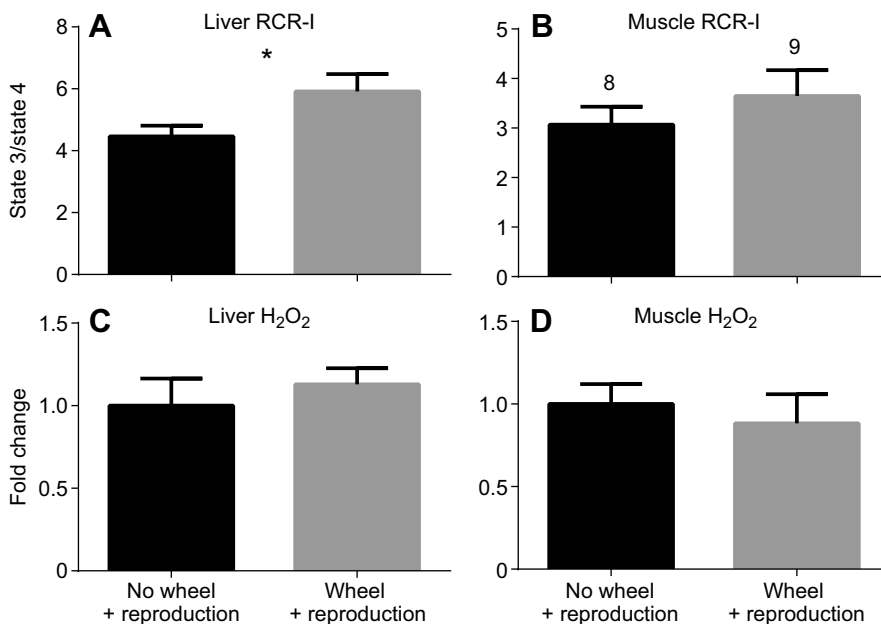


Fig. 2. Respiratory control ratio (RCR) and hydrogen peroxide emission by isolated mitochondria. (A) Liver RCR-I, (B) Muscle RCR-I, (C) Liver H_2O_2 and (D) Muscle H_2O_2 . Data are presented as means \pm s.e.m., $n=9$ for each group unless labeled above bars. *Significant difference between treatments ($P<0.05$).

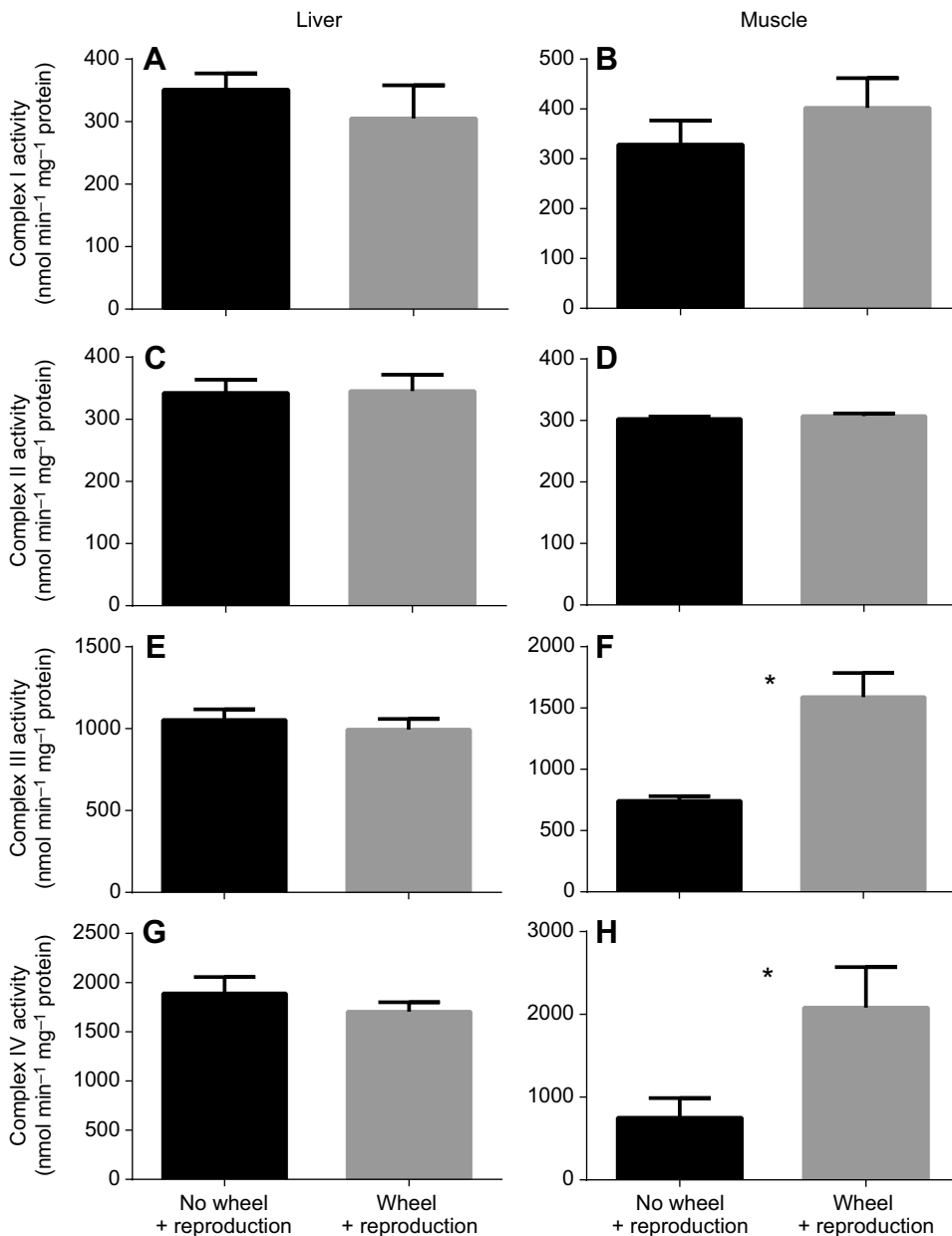


Fig. 3. Enzymatic activity of mitochondrial complexes isolated from liver and skeletal muscles. (A,B) Complex I, (C,D) complex II, (E,F) complex III and (G,H) complex IV. Data are presented as means \pm s.e.m., $n=9$ for each group. *Significant difference between treatments ($P<0.05$).

Running resulted in higher mitochondrial content, determined by quantifying CS activity, in the liver ($t_{18}=3.20$, $P=0.005$; Fig. S2A), muscle ($t_{18}=3.39$, $P=0.003$; Fig. S2B) and heart ($t_{16}=2.50$, $P=0.024$; Fig. S2C), but not the brain ($t_{17}=1.36$, $P=0.19$; Fig. S2D).

Liver, skeletal muscle, heart and brain protein levels of lipid peroxidation (4-HNE), protein oxidation (protein carbonyls) and antioxidants (catalase, GPX-1, CuZnSOD, MnSOD) did not differ between animals that had and had not run ($P>0.355$; Table S1).

Experiment 2: effect of prior running on reproductive output and maternal bioenergetic capacity

Females that ran before breeding gave birth to a larger litter than those females that did not have access to a wheel ($t_{16}=2.46$, $P=0.026$; Fig. 1A). In addition, the cumulative mass of the eight weaned pups was greater in those females that ran ($t_{16}=2.26$, $P=0.038$; Fig. 1B).

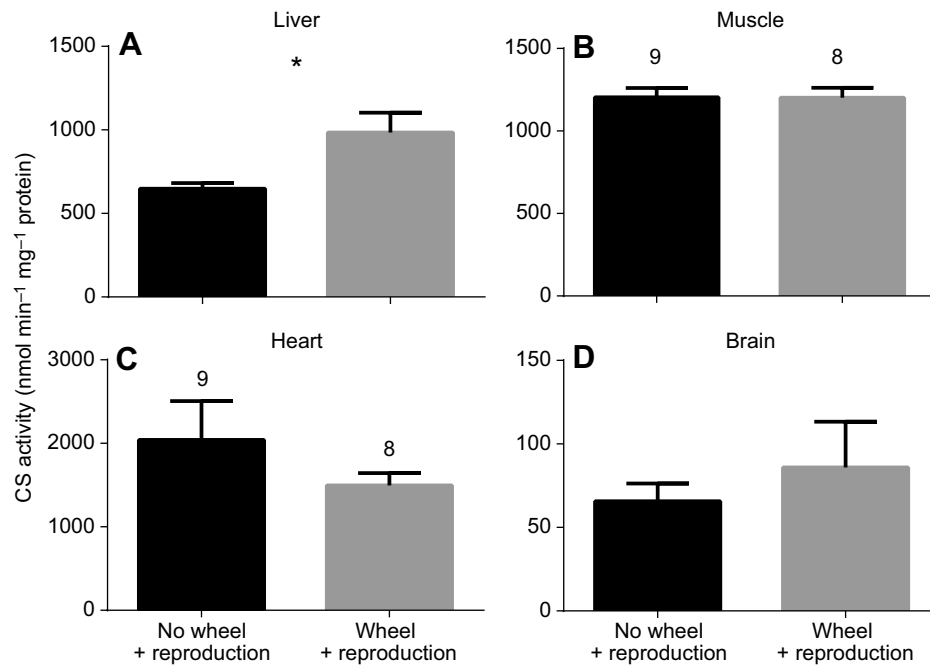
After reproduction ended, the mitochondria in the liver of females that had run before breeding displayed a higher RCR ($t_{16}=2.22$,

$P=0.041$; Fig. 2A). In contrast, muscle RCR did not vary ($t_{15}=0.89$, $P=0.388$; Fig. 2B). H_2O_2 emission of the liver and muscle did not vary between mice after reproduction ($P>0.505$; Fig. 2C,D).

The differences in RCR were not reflected in the enzymatic activity of the mitochondrial complexes of the liver ($P>0.13$; Fig. 3A,C,E,G), but muscle mitochondria displayed higher complex III ($t_{16}=3.56$, $P=0.003$; Fig. 3F) and IV ($t_{16}=2.35$, $P=0.031$; Fig. 3H) activities when mice ran before reproduction.

Mitochondrial content, which was estimated by CS activity, was greater in the liver of mice that ran before breeding ($t_{16}=2.74$, $P=0.015$; Fig. 4A), but not in the muscle, heart or brain ($P>0.05$; Fig. 4B–D). Such changes were consistent with PGC-1 α protein levels in liver ($t_{16}=3.21$, $P=0.005$; Fig. 5A).

Western blots indicated that liver 4-HNE was higher in the group with a wheel compared with the one without it ($t_{15}=3.00$, $P=0.009$; Table 1). Other markers of oxidative damage (i.e. protein carbonyls) and antioxidant levels in the liver, muscle, heart and brain did not vary between running and non-running mice (Table 1).



DISCUSSION

Reproduction is physiologically and energetically demanding, particularly for females. Any activities that alter a female's capacity to allocate energy to future reproduction have the potential to alter her overall reproductive performance (Festa-Bianchet et al., 1998; Cook et al., 2004; Vézina et al., 2012;

Dušek et al., 2017; Saino et al., 2017). In the present study, we showed that running prior to reproduction increased a female's litter size and the mass of the weaned pups. Running also resulted in various benefits to the bioenergetic capacity of the liver and skeletal muscle, and these findings are discussed below.

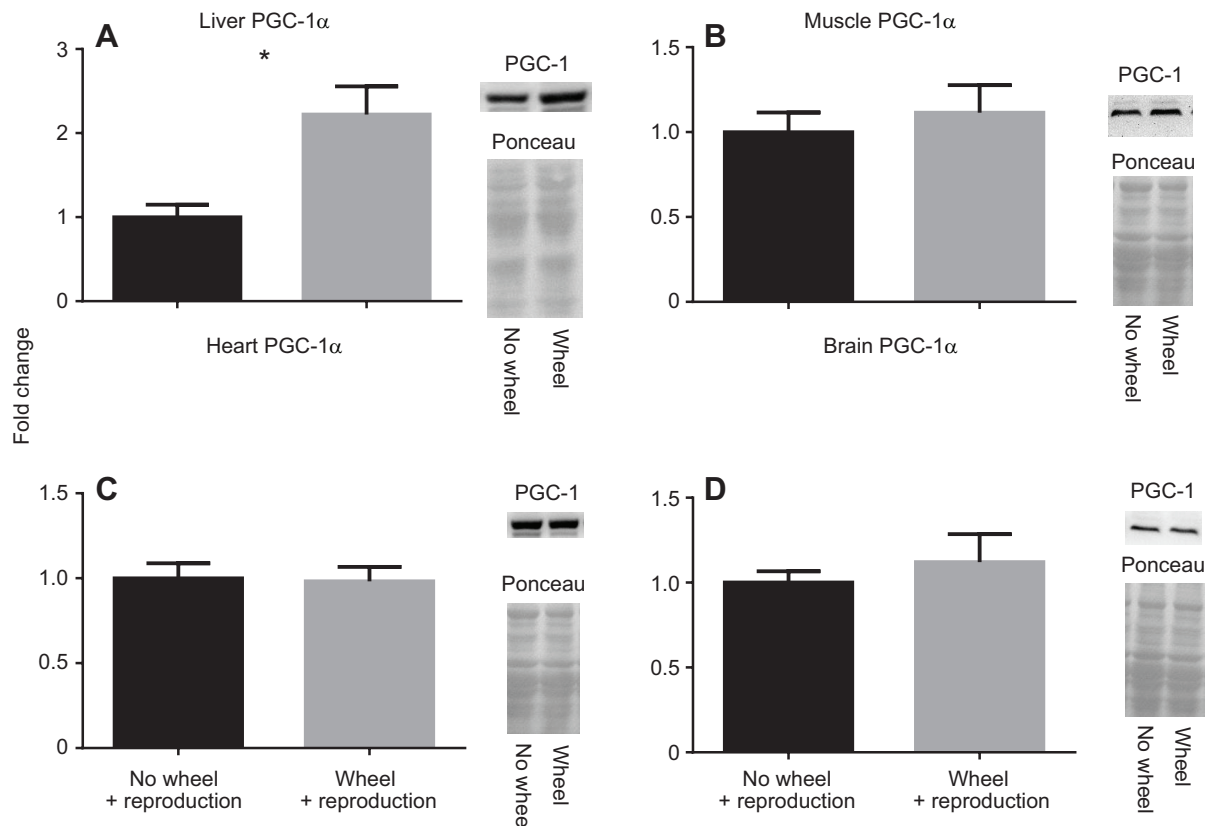


Fig. 5. Relative peroxisome proliferator-activated receptor γ coactivator 1 (PGC-1 α) expression in different tissues of mice. (A) Liver, (B) skeletal muscle, (C) heart and (D) brain. Data are presented as means \pm s.e.m., $n=9$ for each group. *Significant difference between treatments ($P<0.05$).

Table 1. Markers of oxidative damage (4-HNE and protein carbonyls) and antioxidants (CuZnSOD, MnSOD, catalase, GPX-1) in liver, skeletal muscle, heart and brain of mice

	4-HNE	Protein carbonyls	Catalase	GPX-1	CuZnSOD	MnSOD
Liver						
No wheel+reproduction	1.00±0.10^a (8)	1.00±0.09	1.00±0.20	1.00±0.13	1.00±0.17	1.00±0.09
Wheel+reproduction	1.10±0.15^b (9)	0.98±0.09	1.07±0.16	0.95±0.12	0.74±0.08	0.99±0.08
Skeletal muscle						
No wheel+reproduction	1.00±0.07	1.00±0.06	1.00±0.17	1.00±0.13	1.00±0.20	1.00±0.20
Wheel+reproduction	0.93±0.07	0.98±0.07	0.72±0.16	0.94±0.15	0.97±0.21	0.91±0.20
Heart						
No wheel+reproduction	1.00±0.12	1.00±0.81	1.00±0.17	1.00±0.16	1.00±0.21	1.00±0.11
Wheel+reproduction	0.97±0.15	0.99±0.80	0.72±0.16	1.24±0.14	1.08±0.30	1.09±0.16
Brain						
No wheel+reproduction	1.00±0.14	1.00±0.12	1.00±0.27	1.00±0.35	1.00±0.13	1.00±0.29
Wheel+reproduction	1.01±0.15	1.08±0.17	0.89±0.17	0.72±0.25	0.87±0.07	0.92±0.14

Values in bold are statistically significant, with letters indicating differences between groups (see Results).

Data were obtained by western blotting and are presented as means±s.e.m. The units for all variables are fold change relative to the no wheel+repro group. $n=9$ for each group or labeled in the column.

Our first experiment confirmed that our running protocol improved the bioenergetic capacity of female mice. Specifically, we found that skeletal muscle of mice that ran displayed improved mitochondrial RCR. Also, the liver, skeletal muscle and heart of the animals that ran had higher mitochondrial density. These observations are consistent with prior studies that reported that running has broad effects on mitochondrial respiratory function in the liver (Ardies et al., 1987; Gonçalves et al., 2014), skeletal muscle (Holloszy, 1967; Zoll et al., 2002; Votion et al., 2012) and heart (Kavazis et al., 2009a; Padrão et al., 2012).

In the second experiment, females were exposed to the same conditions prior to breeding, running or not, for 1 month, and the females that ran gave birth to a larger litter and allocated more resources to their young during lactation, as indicated by a heavier litter at weaning. The results of experiment 1 provide likely mechanisms for this difference in reproductive performance. For example, to support nutrient transfer to the young via the placenta and mammary glands, the liver increases glucose and lipid synthesis during reproduction (Zhang et al., 2017). Females that ran had increased and more efficient liver mitochondria, which has beneficial effects on energy and glucose metabolism (Rui, 2014). Beneficial effects of exercise on reproductive performance have also been shown in knockout and diseased mice, and mice artificially selected for high running (Girard et al., 2002; Irani et al., 2005; Vega et al., 2013), and it is probable that any condition that significantly increases or decreases activity before reproduction, such as time spent foraging, evading predators or moving between wintering and breeding grounds, could alter the performance of free-ranging and captive females (Meijer and Robbers, 2014). Not only was running associated with females having a higher capacity to allocate resources to reproduction, but running prior to reproduction also had benefits that persisted after the reproductive bout had ended. In skeletal muscle, an increase in enzymatic activity of complex III and complex IV provide support for the observed increase in mitochondrial respiratory performance (Larsen et al., 2012; Crane et al., 2013). These effects could benefit a female's future fitness, contributing to improved capacity in response to future stressors such as predator evasion or future reproduction (Kearney et al., 2012).

Another variable that could have played a role in the observed carry-over effects is emission of ROS (Sies, 1997). The negative effects of damage from ROS have been proposed to include future ability to compete, attract mates, allocate resources to reproduction, and support processes that combat senescence (Sohal and

Weindruch, 1996; von Schantz et al., 1999; Costantini, 2008; Monaghan et al., 2009; Metcalfe and Monaghan, 2013). Yet empirical evidence does not consistently show that an increase in ROS production is harmful (Speakman and Selman, 2011; Costantini, 2014, 2016; Speakman and Garratt, 2014; Blount et al., 2015; Mowry et al., 2016). In the present study, we showed no differences in ROS emission or oxidative damage in the tissues studied other than higher 4-HNE levels in liver of the females that ran and reproduced. Interestingly, these mice had higher mitochondrial density and enhanced reproductive output, which suggests that oxidative damage (as measured by one marker, 4-HNE) was insufficient to have had an immediate negative impact on mitochondrial function. We also did not observe any changes in antioxidant protein levels between groups. As Selman et al. (2002) noted, antioxidant protection and repair mechanisms are likely sufficient to enable an individual to cope with any changes in ROS production. These are important findings because it has been reported that the levels of oxidative damage or antioxidant protein levels alone are poor indicators of life history trade-offs (Speakman et al., 2015). Also, 4-HNE has been shown to play a role as a signaling molecule (Leonarduzzi et al., 2004) and the increased levels of PGC-1 α observed in the present study in the livers of females that ran before reproduction might have resulted from the signaling function of 4-HNE (Yoboue et al., 2014).

Conclusions

In this study, we found that a high level of activity before reproduction benefited females' reproductive performance and the condition of their mitochondria following the reproductive event. These results indicate that how prior conditions impact the reproductive performance of females is more nuanced than simply the effect of body fat. Prior conditions can also impact the density of mitochondria in tissue and their respiratory efficiency, which can determine how much females allocate to a reproductive event. In recent years, more studies have begun to focus on how carry-over effects may impact animals' life history traits. Both energetic and non-energetic mechanisms can underpin variation in life history traits – this study demonstrated the vital role of mitochondria in determining the interaction between two energy-demanding endeavors.

Acknowledgements

We thank the Hood lab undergraduate research assistants for help monitoring the mice, and Christine Kallenberg, Hayden Hyatt and Michael Roberts for technical support in the laboratory.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: Y.Z., A.B., W.R.H.; Methodology: Y.Z., A.N.K., W.R.H.; Validation: Y.Z., A.B.; Formal analysis: W.R.H.; Investigation: Y.Z., A.B., N.R.P.; Data curation: Y.Z., A.B., N.R.P., H.A.T.; Writing - original draft: Y.Z., A.N.K., W.R.H.; Writing - review & editing: Y.Z., A.N.K., W.R.H.; Supervision: A.N.K., W.R.H.; Project administration: Y.Z.; Funding acquisition: W.R.H.

Funding

This research was funded by National Science Foundation grant 1453784 and US National Institutes of Health grant R03 HD083654-01 to W.R.H. and A.N.K. This research was also funded by an Auburn University Cell and Molecular Biosciences Undergraduate Research Scholarship and an Undergraduate Research Grant-In-Aid from the Auburn University Department of Biological Sciences to A.B. Deposited in PMC for release after 12 months.

Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.177469.supplemental>

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