J Exp Biol Advance Online Articles. First posted online on 25 April 2013 as doi:10.1242/jeb.082669
Access the most recent version at http://jeb.biologists.org/lookup/doi/10.1242/jeb.082669

Physiological adaptations to reproduction I. Experimentally increasing litter size enhances aspects of antioxidant defence but does not cause oxidative damage in mice

Key words: life history, reproduction, trade off, senescence, ageing

Michael Garratt[1*], Nicolas Pichaud[1,2], Edith D. Aloise King[1], Robert C Brooks[1]

[1]Evolution & Ecology Research Centre and School of Biological, Earth and Environmental Sciences, The University of New South Wales, Sydney, New South Wales 2052, Australia.

[2] School of Biotechnology and Biomolecular Sciences, The University of New South Wales, Sydney, New South Wales 2052, Australia.

*Author for correspondence (Michael.Garratt@unsw.edu.au)

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

Copyright (C) 2013. Published by The Company of Biologists Ltd

SUMMARY

Life history theory suggests that investment in reproduction can trade off against growth, longevity and both reproduction and performance later in life. One possible reason for this trade-off is that reproduction directly causes somatic damage. Oxidative stress, an overproduction of reactive oxygen species in relation to cellular defences, can correlate with reproductive investment and has been implicated as a pathway leading to senescence. This has led to the suggestion that this aspect of physiology could be an important mechanism underlying the trade-off between reproduction and lifespan. We manipulated female reproductive investment to test whether oxidative stress increases with reproduction in mice. Each female's pups were cross-fostered to produce litters of either two or eight, representing small and large levels of reproductive investment for wild mice. No differences were observed between reproductive groups at peak-lactation for several markers of oxidative stress in the heart and gastrocnemius muscle. Surprisingly, oxidative damage to proteins was lower in the livers of females with a litter size of eight than in females with two pups or non-reproductive control females. While protein oxidation decreased, activity levels of the antioxidant enzyme superoxide dismutase increased in the liver, suggesting this may be one pathway used to protect against oxidative stress. Our results highlight the need for caution when interpreting correlative relationships and suggest that oxidative stress does not increase with enhanced reproductive effort during lactation.

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

INTRODUCTION

Theories of ageing often predict that an organism's lifespan is heavily influenced by their investment in other life history traits earlier in life (Stearns, 1992). In particular, large investments in reproduction are expected to lead to faster ageing. This trade-off may occur on several different levels. From an evolutionary perspective, genes that facilitate investment in reproduction early in life may be selected for even if they confer deleterious effects later in life, as the force of natural selection diminishes with age (Williams, 1957). From a physiological perspective increased investment in reproduction by an individual may either reduce the resources available for protection against somatic damage (Kirkwood and Holliday, 1979) or directly damage the soma (Partridge et al., 2005; Harshman and Zera, 2007).

The physiological constraints that limit investment in both reproduction and lifespan are not well understood and stir ongoing debate. One aspect of physiology that has been implicated in ageing for 50 years is oxidative stress (Harman, 1956; Beckman and Ames, 1998), which occurs when there is a imbalance between the production of Reactive Oxygen Species (ROS) and an organism's capacity to mitigate their damaging effects (Monaghan et al., 2009). ROS are potentially damaging molecules, produced from a variety of sources in biological systems, although most notably the electron transport system during energy metabolism. To protect against the negative consequences of ROS organisms have evolved a variety of defence mechanisms that limit ROS production, reduce these molecules to less reactive forms and repair any damage incurred (Halliwell and Gutteridge, 1999). ROS can cause oxidative damage - reversible or irreversible damage to proteins, lipids and DNA, which can impair cellular function (Monaghan et al., 2009). Oxidative stress can further limit an organism's ability to respond to redox signals and regulate gene expression (Droge, 2002) and has therefore also been described as a disruption in redox signalling and control (Jones, 2006). Recent suggestions that oxidative stress could be an unavoidable consequence of reproduction (Costantini, 2008; Speakman, 2008; Dowling and Simmons, 2009; Monaghan et al., 2009) have led to further speculation that this aspect of physiology could mediate the trade-off between reproduction and lifespan (Dowling and Simmons, 2009; Monaghan et al., 2009).

Reproduction could cause oxidative stress via several non-mutually exclusive pathways. Investment in reproduction usually requires animals to increase energy

consumption (Gittleman and Thompson, 1988). As the majority of ROS are produced from the electron transport system during oxidative phosphorylation (Balaban et al., 2005), the increases in metabolic rate required to facilitate reproductive investment could produce greater levels of ROS (Alonso-Alvarez et al., 2004; Speakman, 2008). Further, investment in reproduction could limit the availability of resources required for the production or maintenance of defence mechanisms that protect against oxidative stress (Monaghan et al., 2009). Under this scenario, reproductive investment will require animals to reduce investment in antioxidant defence (or other protective mechanisms), therefore increasing their susceptibility to oxidative stress.

In spite of these predictions there is little direct experimental evidence that reproductive investment elevates oxidative stress. In birds, provisioning is expected to be the most demanding period of reproduction for parents and a number of authors have demonstrated changes in aspects of antioxidant defence during this period (Alonso-Alvarez et al., 2004; Wiersma et al., 2004; Christe et al., 2012). For example, Wiersma et al., (2004) demonstrated that zebra finches (*Taeniopygia guttata*) provisioning experimentally enlarged broods show a reduction in the activity of both superoxide dismutase (SOD) and glutathione peroxidise (GPx) in pectoral muscle when results are scaled to daily energy expenditure (DEE). In females, this change was principally due to an increase in DEE, while in males the decrease in SOD activity scaled to DEE was the result of a reduction in SOD activity itself. Another early study in zebra finches also demonstrated a sex-specific change in total antioxidant capacity in blood (Alonso-Alvarez et al., 2004). Males with enlarged broods showed a reduction in total antioxidant capacity when compared to males with reduced broods, while females in these two brood sizes did not differ for this marker. These results suggest, therefore, that some changes in oxidative balance are occurring with reproduction, although without measures of ROS production or oxidative damage it is difficult to ascertain whether oxidative stress is actually increasing with reproductive effort.

In mammals, females usually invest much more in parental care than males (Clutton-Brock, 1991) and lactation is the most energetically demanding period of a female's life. As a consequence, it has been predicted that oxidative stress could increase during this reproductive period (Speakman, 2008). Two studies in wild mammals have assessed the correlation between female reproductive effort and plasma Malondialdehyde (MDA), a marker of oxidative damage to lipids. In soay sheep (*Ovis aries*) no relationship was found between total reproductive effort and MDA (Nussey et al., 2009), while in eastern chipmunks

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

(*Tamias striatus*) MDA correlated positively with litter size in sexually mature females (Bergeron et al., 2011). Two other studies conducted in the laboratory on small rodents compared oxidative stress between females allowed to reproduce and those that were not. Surprisingly, Ołdakowski et al., (2012) found that MDA levels were lower in the skeletal muscle and kidneys of reproducing female bank voles (*Myodes glareolus*). Similarly Garratt et al., (2011) revealed a decrease in MDA levels as well as in several other markers of oxidative stress, in the livers of reproducing female house mice (*Mus musculus domesticus*). Garratt et al. (2011) did, however, report a positive correlation between protein oxidation and litter size in female house mice at peak lactation, hinting that oxidative stress may increase with reproductive effort during particular reproductive periods.

While this research has provided an interesting first insight into changes in oxidative stress with reproduction, the techniques used to test for a "cost of reproduction" in mammals have either been correlative (Nussey et al., 2009; Bergeron et al., 2011), or have consisted of indirect manipulations, i.e. manipulating the presence of a male rather than the level of reproductive effort itself. Such indirect manipulations have been criticised because the environmental change that causes the difference in reproductive investment (i.e. the presence of the male) may itself cause changes in the observed variable, in this case oxidative stress, rather than investment in reproduction itself (Lessells, 1991). By contrast, more direct manipulations, such as clutch size manipulation in birds or litter size manipulations in mammals, are much more likely to induce elevated investment in reproduction while allowing all or most confounding variables to be held constant.

In this study we manipulated female investment in reproduction by allowing some females to breed and keeping others as unmated controls. We then manipulated the reproductive females' investment in lactation by cross-fostering pups so that each female lactated for either two or eight pups, representing either a large or a small litter for wild-derived female mice (Berry, 1981). Such litter size manipulations have provided an insight into the energy expenditure of female mice during lactation (Hammond et al., 1994; Johnson et al., 2001). Furthermore, experimentally increasing the litter size of bank voles in the wild has revealed a survival cost of increased litter sizes, highlighting that these techniques can reveal broad scale trade-offs that occur between life history traits (Koivula et al., 2003). Females were allowed to reach day 14 of lactation, which is the point when energetic demands are at their peak (Johnson et al., 2001), and were then assessed for several markers of oxidative stress. As recently recommended (Selman et al., 2012), we used a multiple assay

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

approach to assess oxidative stress in several different tissues. We began by measuring two different markers of oxidative stress (protein thiols and the proportion of oxidised to total glutathione; Table 1) in three different tissues (liver, heart and gastrocnemius muscle). The liver and heart are metabolically active organs that, in mice at least, show an increase in mass during lactation, presumably to allow a greater processing of nutrients and removal of waste products (Hammond et al., 1994). The mass of the liver has also been found to be the most significant predictor of resting metabolic rate in at least one strain of mice. (Selman et al., 2001). Thus, it could be expected that these areas may be susceptible to oxidative stress during reproduction. Measurement of oxidative damage in the gastrocnemius muscle also allowed us to assess oxidative stress in a post-mitotic tissue that is not directly linked to lactation. As these two markers of oxidative stress were found to differ in the liver, we conducted a further examination of oxidative stress markers in this tissue (protein carbonyls and aconitase; table 1). We also assessed whether these changes were linked to any altered activity of endogenous antioxidants (total glutathione, superoxide dismutase, catalase; Table 1).

A negative relationship between clutch size and the ability of a chick's red blood cells to protect against a controlled free radical attack has previously been demonstrated in zebra finches (*Taeniopygia guttata*) (Alonso-Alvarez et al., 2006) and it has been suggested that these differences in resistance to oxidative stress could, at least in part, contribute to the reduced fitness of individuals raised in large broods. We also took this opportunity to explore the possibility of such a trade-off in a mammal by relating our measures of oxidative stress to the litter size into which each experimental female had been born.

In addition to manipulating reproductive investment, we fed females one of two different diets, which differed in presence of an antioxidant preservative (Oxystat, which contains the phenolic antioxidants butylated hydroxyanisole (BHA); 6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline (ethoxyquin); and 2,6-bis(1,1-dimethylethyl)-4-methylphenol (BHT); see methods). These antioxidants are used by the food industry to prevent rancidity over long-term storage (Halliwell and Gutteridge, 1999) . It has been suggested preservatives of this type can limit oxidative stress when added to rodent feeds at high levels (Jaeschke and Wendel, 1986; Malhotra et al., 2008). Thus we considered it prudent to ensure that if our study replicated the decreases in markers of oxidative stress reported previously in reproducing female rodents (Garratt et al., 2011; Ołdakowski et al., 2012), which it did, these effects were not caused by females increasing their food consumption during lactation and consuming more of these synthetic dietary additives.

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

MATERIALS AND METHODS

Subjects

Subjects (n = 60) were adult first generation captive bred female house mice derived from 30 wild mice caught at a chicken farm in the North-West of Sydney, Australia. Experimental females were weaned at 28 days old and remained with their female siblings until the beginning of the experiment. Males that were used to breed with females were housed singly after weaning at 28 days old. All animals were housed in cages (48 cm X 11.5 cm X 12 cm) lined with Shepherd's corn cob substrate and had shredded newspaper and tissue added for bedding. All animals had ad libitum access to water and to food (see below). Mice were maintained on a 12 hour reverse light cycle and experimental procedures were conducted under dim red light during the dark phase.

Animal Diets

Prior to the experiment animals were fed a maintenance rodent feed from Gordon's Speciality Stockfeeds (3930 Remembrance Drive, Yanderra, NSW 2574, Australia; see supplementary datasheet for complete listing of ingredients and supplementary Table 1 for a breakdown of constituents), which contained no added vitamin C and vitamin E at concentration of 40.192 iu per kg finished feed. This feed also contained oxystat, a premix that contains three different synthetic antioxidants (BHA: 8.32 ppm in finished feed; ethoxyquin: 5.49 ppm; BHT: 83.2 ppm) and a biological chelating agent (further information can be found on the distributors website: http://www.kiotechagil.com/products/oxistat/ accessed 1st February 2013; and in the supplementary information), at a finished feed rate of 832 ppm.

Two weeks prior to breeding, females were randomly allocated to one of two different versions of the maintenance rodent feed, both of which were manufactured by Gordon's Speciality Stockfeeds on the same day, using the same ingredients and treated in an identical manner. Feeds were delivered to the University of New South Wales and stored for three months prior to the experiment in an area of dry humidity, at room temperature as per the advice of the manufacturers of both Oxystat and the rodent feed. The first group of animals were fed the normal maintenance feed as outlined above (n = 30). The second group were fed an identical version of the maintenance feed, except it did not contain the Oxystat food

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

preservative (n = 30). Females on each diet were further randomly allocated to three different experimental groups: non-reproductive control (n = 10 for each diet), small litter (n = 10 for each diet) and large litter (n = 10 for each diet). Females in these different groups did not differ in age at the start of the experiment (normal food: controls = 112 ± 8 days old; large litter = 110 ± 10 days; small litter 110 ± 10 days; custom food: controls = 109 ± 8 days; large litter = 98 ± 7 days; small litter = 104 ± 10 days; difference between groups: $F_{2,53}$ = 0.26, P = 0.78; difference between diets: $F_{1,53}$ = 1.05, P = 0.31).

Breeding protocol

After two weeks on the experimental feeds, females in small litter and large litter groups were randomly allocated a male to breed with. Three days prior to breeding, females were given a small handful of bedding taken from the cage of the male they were to breed with and were housed adjacent to that male's cage for the next three days to allow familiarisation. Males and females were then housed in the same cage and allowed to breed for 18 days, after which the male was removed. If the female was not obviously pregnant after 18 days the male was allowed to remain with the female until she was pregnant. The time from pairing with a male until giving birth was not related to oxidative stress (Supplementary Table 2). After six weeks three females were still not pregnant and were removed from the experiment. Each control female was paired with a novel female after an identical familiarisation period with a male.

Within 24 hours of giving birth, the natural litter of each experimental female was removed and replaced with either a litter of two pups or eight pups. Females were randomly allocated to the two or eight pup treatments and there was no difference in the natural litter sizes that these two groups of females gave birth to (t_{31} = 1.51, P = 0.15). The cross-fostered pups were born within 24 hours of the natural litter and, on each occasion, the pups for each female's manipulated litter were derived from two different females (i.e. each female's litter was comprised of pups from two different females). Females were allowed to nurse these pups for 14 days and were then culled by cervical dislocation. Control females were culled in the same manner and over the same period as reproductive females. Four females failed to produce litters on days when there were other pups available to cross-foster and so were removed from the experiment. Final sample sizes for reproductive groups were as follows:

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

custom feed large litter = 9; custom feed small litter = 7; normal feed large litter = 9; normal feed small litter = 8.

Sample collection and biochemical assays

Immediately after being culled females were quickly dissected and organs flash frozen in liquid nitrogen and stored at -80^{o}C. Due to limitations in the number of samples that could be run concurrently for commercial assays (n = 6 from each group for protein carbonyls, superoxide dismutase), some randomly selected individuals from each group were omitted from these analyses.

Markers of oxidative stress

Total and oxidised glutathione were measured using the automated glutathione recycling assay (Anderson, 1996) modified for use on a plate reader (Vasilaki et al., 2006). Protein thiols were measured as described by Di Monte et al. (Dimonte et al., 1984) modified for use on a plate reader (Vasilaki et al., 2006). Protein carbonyls were measured using the ELISA kit of Biocell Corp (Aukland, New Zealand).

Enzymatic antioxidant capacity

Total superoxide dismutase activity was measured using the superoxide dismutase assay kit from Cayman Chemicals (Michigan, USA). Enzymatic activity of catalase was measured according to the method of Aebi (1984) modified for microplate. Briefly, homogenates were centrifuged at 13,000g for 3 min at 4°C. The resulting supernatant was incubated with 100mM of potassium phosphate complemented with 0.1% (v/v) Triton X-100 and 60mM of H_2O_2. The decrease in absorbance was measured at a wavelength of 240nm in UV-star® microplates (Greiner Bio-One, Germany) for 2 min (ε_{240}=43.6mL/cm/µmol).

Marker of ROS production

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

Aconitase is an enzyme of the tricarbloxylic acid cycle that is inactivated by superoxide, and therefore has been suggested to be a reliable marker for mitochondrial ROS production (Hausladen and Fridovich, 1994, 1996). Homogenates were transferred in 50mM Tris-HCl, 0.1% (v/v) Triton X-100, 0.6mM $MnCl_2$, 5mM sodium citrate, 0.2mM NADP, 0.4 U/mL isocitrate dehydrogenase, pH 7.4. Activity was measured by following the appearance of NADPH at 340nm (ε_{340}=6.22mL/cm/μmol) over 4 min.

Citrate synthase activity

Citrate synthase activity was measured in liver homogenates according to Pichaud et al. (Pichaud et al., 2010). Homogenates were transferred in 100 mM imidazole-HCl (pH 8), 0.1 mM 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), 0.1 mM acetyl CoA and 0.15 mM oxaloacetate. Activities were determined by following the increase in absorbance due to the reduction of DTNB at 412 nm.

Statistical analyses

Linear mixed effects models were used to test for differences between experimental groups and feed types in SPSS version 20. This allowed us to add the ID of each pair of wild caught parent mice used to breed experimental animals as a random effect to each model. Group and feed type were included as fixed effects and the litter size that experimental females were reared in was included as a covariate. Backwards selection was used to remove non-significant variables and interactions. For analyses of organ masses, log-transformed body mass was included as a covariate. Food consumption during lactation was analysed using a repeated measures General Linear Model (GLM), applying the greenhouse giesser correction to control for an effect of sphericity. Oxidative stress results were log-transformed where necessary to ensure normal distribution. Correlations between natural litter size, changes in pup number and oxidative stress were assessed using a Spearman's rank correlation test.

RESULTS

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

Female reproductive effort was manipulated in two ways: by allowing some females to get pregnant and keeping others as unmated controls, and by removing the natural litter of each female that reproduced and replacing it with pups birthed on the same day by different females. Females were either given two or eight pups, which resulted in females that were allocated a litter size of eight having an average of 2.2 more pups (±0.36) than the natural litter size they gave birth to and females allocated a litter size of two having their litter size reduced by an average of 4.5 pups (±0.26). Females allocated a litter size of eight therefore had a greatly increased reproductive burden when compared to females with a litter size of two ($t_{31} = 14.65$, $P < 0.001$).

Reproductive investment in wild-derived mice

Wild-derived females that were allocated a litter of eight pups consumed significantly more food over lactation than females allocated only two pups, and females from both of these reproductive treatments ate significantly more than non-reproductive control females (Fig. 1a; interaction between reproductive treatment and time: $F_{5.4,136} = 2.54$, $P = 0.030$; effect of reproductive treatment over the lactation period: $F_{2,34} = 45.10$, $P < 0.0001$; Tukey comparison between females with large litters and small litters, $P < 0.001$; females with large litters vs. controls, $P < 0.001$; females with small litters vs. controls, $P = 0.031$). The repeated measures ANOVA on food consumption over the lactation period revealed no differences between females allocated a rodent feed containing Oxystat, which is rich in various synthetic antioxidants, and those that were not ($F_{1,34} = 0.39$, $P = 0.54$), and no significant interactions involving these food types (food type x treatment = $F_{2,34} = 0.71$, $P = 0.49$; food type x time = $F_{2.7,136} = 0.09$, $P = 0.95$; food type x treatment x time = $F_{5.4,136} = 0.53$, $P = 0.76$).

Females in different reproductive groups also had different sized hearts (Fig. 1b; effect of group: $F_{2,49} = 3.45$, $P = 0.041$) and livers (Fig. 1c; effect of group: $F_{2,52} = 31.21$, $P < 0.001$) at peak lactation, indicating altered energy demands. Females with a litter size of eight had larger hearts (Tukey comparison: $P < 0.001$) and livers ($P < 0.001$) than females in the control group, while females with a litter size of two only had larger livers than controls ($P < 0.001$; difference in heart weight, $P = 0.62$). The diet females were allocated had no effect on the weights of either the liver ($F_{2,49} = 0.06$, $P = 0.81$) or heart ($F_{2,49} < 0.01$, $P = 0.99$).

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

Oxidative stress and litter size

Although females with a litter size of eight had greatly increased energetic demands, we found no evidence of increased oxidative stress in this treatment as indicated by protein thiol oxidation (Table 2). In the heart and gastrocnemius muscle, this marker of protein oxidation did not differ between females in different reproductive groups (Table 2). Surprisingly, in the liver, protein thiol groups were in higher abundance (indicating lower oxidative stress) in females with a larger litter when compared to those with a smaller litter (Table 2**;** Fig. 2a). To further ascertain the changes in protein oxidation that occur with reproductive investment in the liver we measured protein carbonyls, an irreversible marker of oxidative damage to proteins. This also indicated that females with large litters had decreased liver protein oxidation when compared to either females assigned a litter size of two and non-reproductive controls (Table 2; Fig. 2b)

As a further measure of oxidative stress we examined oxidation of glutathione, a marker of redox status and in some instances oxidative stress. Females in different reproductive groups did not differ in glutathione oxidation in either the heart or gastrocnemius muscle (Table 2). Glutathione oxidation did not differ significantly between groups in the liver, although there was a non-significant tendency for females with a litter size of two to have lower levels than females with large litters and non-reproductive controls (Table 2; Fig. 2c).

To further ascertain whether oxidative stress is related to reproductive effort, we tested whether oxidative stress was related to the difference between the size of litter a female bore and the size of litter she was experimentally assigned. Although the change in litter size ranged from a net increase in 5 pups to a net decrease by seven pups, the only significant relationship involved liver protein thiols, which indicated decreased protein oxidation in females with increased litter sizes (Table 3). We also did not observe any significant relationships between each female's natural litter size on the day of parturition and their level of oxidative stress at peak lactation, when either comparing all reproductive females together or analysing each reproductive group separately (Table 3).

We next tested whether the changes in markers of oxidative stress in the liver were related to the relatively large differences between the reproductive groups in liver mass. While liver mass was not a significant predictor of either protein carbonyls ($F_{1,28}$ = 0.23, P = 0.64) or oxidation of glutathione ($F_{1,44.1}$ = 0.60, P = 0.44), it was related to liver protein thiol

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

concentration ($F_{1,37}$ = 6.77, P = 0.013) - females with larger livers had a greater protein thiol concentration (indicating lower oxidation). However, the difference between reproductive groups for protein thiols remained significant ($F_{2,37}$ = 4.76, P = 0.015) after including liver size as a covariate, with reproductive females having lower levels of protein oxidation.

Antioxidant defence and litter size

Changes in markers of protein oxidation (i.e. protein thiols and carbonyls) and redox status (oxidation of glutathione) may be related to modulation of antioxidant defences. In accordance with a previous study in lactating mice, we observed no differences between females in different reproductive treatments in the total concentration of glutathione (Table 2; Fig. 2d), an important low-molecular-mass antioxidant. However, inspection of liver antioxidant enzymes revealed that the activity of superoxide dismutase was significantly different between reproductive treatments (Table 2; Fig. 2e). Females that were lactating for a large litter had a higher activity of SOD than non-reproductive females, while females that were lactating for a smaller litter had intermediate values that did not differ significantly from either high lactation females or non-reproductive controls (Fig. 2e). There was also a tendency for catalase activity to differ between females in different reproductive treatments, which was attributable to females with small litters having higher activity levels than females in the other two groups (Fig. 2f).

As a further proxy of oxidative stress we measured the activity of aconitase in the liver. This enzyme is very susceptible to deactivation by the superoxide radical and can be used as a marker of ROS levels (Gardner et al., 1995; Gardner, 1997). As a substantial proportion of aconitase is located in the mitochondria (Gardner et al., 1995), we also measured a marker of mitochondrial density, citrate synthase, and used this to correct aconitase activity for mitochondrial density (Pichaud et al., 2010). Interestingly, citrate synthase activity was also greater in the livers of reproductive females (both large and small litter treatments) than in non-reproductive control females (mean activity for females with large litters: 105.32 ± 9.9 units/mg protein; females with small litters: 90.56 ± 10.3 units/mg protein; non-reproductives: 66.95 ± 4.7 units/mg protein; effect of reproductive treatment: $F_{2,51}$ = 5.58, P = 0.007; comparison for females with enlarged litters vs. controls, P = 0.002; females with reduced litters vs. controls, P = 0.055; Table 2). This result indicates that mitochondrial density in the liver is increased at peak-lactation in wild-derived mice. When

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

aconitase activity was corrected by citrate synthase activity no differences were apparent between the females in different reproductive treatments ($F_{2,51}$ = 1.54, P = 0.23) for this marker of oxidative damage.

Birth litter size and oxidative stress

While we found little evidence of increased oxidative damage with manipulated litter size, we observed significant associations between the litter size that each female was born into (i.e. her number of siblings) and her protein oxidation in the liver (Table 1; Fig 3). Compared to females that were born into a small litter, females from large litters had lower levels of protein thiols and higher levels of protein carbonyls in the liver, which indicates increased oxidative stress. Thus, our results indicate that females born into large litters have higher oxidative stress in the liver that those females born into small litters.

DISCUSSION

We found little evidence that oxidative stress increases in female mice lactating for experimentally enlarged litters. Although there has been much discussion about whether oxidative stress could cause the trade-off between reproduction and lifespan (Costantini, 2008; Dowling and Simmons, 2009; Monaghan et al., 2009; Metcalfe and Alonso-Alvarez, 2010; Isaksson et al., 2011; Selman et al., 2012), experimental evidence supporting this theory is sparse and is limited to correlative studies or examination of antioxidant defences rather than oxidative stress or oxidative damage *per se*.

The most frequently suggested pathway linking oxidative stress with reproduction relates to the high levels of metabolism associated with reproductive investment. These metabolic costs have been well studied in small rodents: in lactating laboratory mice, metabolic rate can increase to over 400% of that observed in non-reproductive females (Hammond, 1997). In studies using mice more recently derived from the wild, metabolism is still much greater than controls (Cretegny and Genoud, 2006), although the difference is less pronounced. The metabolic costs of reproduction also increase with litter size (Speakman and McQueenie, 1996), although they have been found to plateau at large litter sizes in at least one strain of laboratory mouse (Johnson et al., 2001). Thus, females in this experiment with a

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

litter size of eight are likely to have experienced much greater metabolic rates than those assigned a litter size of two, - a prediction supported by the fact that females assigned eight pups consumed more food. Yet these increases in metabolism were not linked with an increase in markers of oxidative stress. Results from other studies in small rodents that have increased metabolic rate using different manipulations have also failed to find a straightforward relationship between metabolic rate and oxidative stress. For example, negligible effects were found on several different markers of oxidative damage and antioxidant protection in short-tailed filed voles (*Microtus agrestis*) that had higher metabolic rates over life due to cold exposure (Selman et al., 2008). Thus, although an increased metabolic rate could elevate ROS production and oxidative stress in some instances, the links between these factors are not straightforward (Barja, 2007).

One important factor influencing ROS production during metabolism is the level of uncoupling of mitochondria, with increased uncoupling leading to a reduction in the production of ROS (Brand et al., 2004). This uncoupling can be unregulated (basal leak of protons mainly attributed to mitochondrial anion carriers) or catalysed by uncoupling proteins (UCP) (Brand et al., 2004). As described in the accompanying paper (Pichaud et al., 2013), we did not find a significant effect of unregulated uncoupling (state 2') in the livers of lactating mice, suggesting that the basal proton leak in liver mitochondria is not significantly affected by lactation. UCP-2, the only uncoupling protein expressed in the liver, has been shown to be unchanged in the brown adipose tissue of lactating mice (Pedraza et al., 2001). The role of UCP-2 in the regulation of ROS production is still under debate (Brand and Esteves, 2005; Pecqueur et al., 2008; Collins et al., 2012). However, if UCP-2 can modulate ROS production in the livers of lactating mice, it is possible this could influence the levels of oxidative damage in this tissue during lactation.

Other metabolic and cellular changes may occur during lactation that could, theoretically, influence the production of ROS (see Pichaud et al., 2013). It has previously been suggested that a higher mitochondrial density (observed in reproductive females in our study) may be associated with greater ROS production (Keller et al., 2004; Magwere et al., 2006). This could occur because there are a greater number of mitochondria to produce ROS and, perhaps more importantly, each mitochondrion would be working at a lower rate, increasing proton motive force (Magwere et al., 2006). As ROS production is strongly dependent on proton motive force (Korshunov et al., 1997; Toime and Brand, 2010), this could cause greater ROS production with a higher mitochondrial density. Further

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

experimental studies are required to show direct links between mitochondrial density and ROS production. However, investigations of the cellular and metabolic changes that occur to mitochondria during lactation (in the accompanying study by Pichaud et al., 2013) suggest that adjustments in both mitochondrial density and function might lead to higher ROS production in reproductive females. This, in turn, could trigger up-regulation of antioxidant defences that detoxify the cell from ROS and thus decrease oxidative damage (Pichaud et al., 2013).

In this study we provide evidence that an up-regulation of one particular antioxidant, SOD, is associated with the reduction in markers of protein oxidation when both are examined in the liver. During peak-lactation SOD activity was much greater in females with large experimental litters compared to controls, and this activity might have helped to transform ROS to less reactive forms, thus limiting damage to cellular components. Past manipulations of SOD expression have shown that this enzyme protects against oxidative damage (Dalle-Donne et al., 2003; Perez et al., 2009), particularly in the liver of mice (Elchuri et al., 2005). Manipulating the expression of SOD or other aspects of antioxidant defence during lactation may help to reveal whether endogenous antioxidant defence protects against oxidative stress during lactation.

Females with large litters generally had lower levels of oxidative damage to proteins in the liver, although there was a tendency for this group to have a higher proportion of oxidised glutathione when compared to females with a litter size of two. While this result does not reach the threshold usually required for significance, it is worthy to note that the difference between reproductive treatments went in a different direction when compared to the other oxidative stress markers. In contrast to the markers of protein oxidation, the proportion of oxidized glutathione is not a marker of oxidative damage *per se* – but rather a marker of the redox status (Jones, 2006). Sometimes a more oxidised redox status can indicate increased oxidative stress and can correlate with oxidative damage. However, it must also be noted that a change to an oxidised redox status can, in some instances, be beneficial, eliciting transcriptional regulation that protects against oxidative stress (Klatt et al., 1999; Townsend et al., 2003). Further examination of markers of redox status may help to reveal the relevance of the trend we observed and, potentially, how animals adjust their physiology in relation to reproductive status.

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

Some authors have cautioned that particular synthetic phenolic antioxidants added to limit long-term oxidation of some food products may limit the occurrence of oxidative stress when provided in foods at high levels (Jaeschke and Wendel, 1986; Malhotra et al., 2008). Dramatic increases in food intake during lactation, such as those we documented, will also influence the gross intake of these antioxidants. As these antioxidants would be largely unavailable in natural diets, we tested whether their presence in rodent feeds was the cause of the reductions in markers of oxidative stress previously reported with lactation (Garratt et al., 2011; Ołdakowski et al., 2012). We found no differences in food consumption, oxidative damage or antioxidant defence between mice fed a standard rodent feed and those consuming a diet devoid of these antioxidants. Variation in food consumption and markers of oxidative balance was also not explained by an interaction between the diet an animal was consuming and their reproductive status. This indicates that the addition of these synthetic antioxidants to rodent chow is not responsible for the reduction in particular markers of oxidative stress observed in lactating females.

The food manipulation in our experiment was included as a precautionary measure, to ensure the suitability of our diet for experiments testing for changes in oxidative stress. However, other more biologically relevant dietary antioxidants, which are found in natural food sources, might influence oxidative stress during lactation. For example, Vitamins E has, in some studies, been shown to influence mammalian lifespan and levels of oxidative stress, although it is important to note that other studies have failed to find an effect on these parameters (Halliwell and Gutteridge, 1999; Keller et al., 2004; Banks et al., 2010). The diets fed to animals in our study had relatively low levels of vitamin E in relation to levels recommended by the American Society for Nutrition (Reeves et al., 1993), but enough to meet basic nutritional requirements. It is possible, however, that when animals reproduce in conditions where diets are deficient in vitamin E, or other dietary antioxidants, they may be more prone to oxidative stress. This is an area worthy of further investigation.

Our results highlight the need for caution when interpreting correlations between investment in life history traits and aspects of physiology. Correlations between oxidative damage and litter size have been reported two times in mammals (Bergeron et al., 2011; Garratt et al., 2011). However, these relationships may be generated from a number of different pathways and do not necessarily mean that oxidative stress increases with litter size. For example, females that produce large litters may have higher pre-existing levels of oxidative stress, perhaps if the level of reproductive investment is linked to metabolism, or if

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

514 the litter size a female produces is correlated with other aspects of physiology. Our
515 experimental study suggests that during their first lactation, females with larger litters do not
516 incur a cost of higher oxidative damage, at least in the tissues we examined. Our results
517 leave open the possibility, however, that if the costs of reproduction are exacted in oxidative
518 damage or oxidative stress they occur during or after later parities or in subsequent
519 generations.

520 We provide correlative evidence here that this latter possibility may be occurring.
521 Females that had the greatest number of siblings (i.e. were themselves derived from large
522 litters) had the greatest levels of protein oxidation in the liver, an effect that was consistent
523 across each experimental group. In birds, experimentally increasing brood size can have
524 negative effects on the antioxidant defences of chicks when they become adults (Alonso-
525 Alvarez et al., 2006). It has even been suggested that changes in this aspect of physiology
526 could, at least in part, be the cause of the reduced reproductive capacity of adult birds that
527 were reared in large broods (Alonso-Alvarez et al., 2006). It is possible that oxidative stress
528 may also be influenced by rearing environment and sibling competition in mammals,
529 although this remains to be tested by direct experimental studies and examination of markers
530 of oxidative stress in other tissues.

531

532 **ACKNOWLEDGEMENTS**

533 This study was approved by the UNSW Animal Ethics Committee (approval number:
534 11/45A). We thank Heather Try for assistance with animal maintenance.

535

536 **FUNDING**

537 This study was funded by an ARC Discovery Grant awarded to Robert C. Brooks

538

539 **REFERENCES**

540 **Aebi, H.** (1984). Catalase. In *Methods Enzymol.*, vol. 105, pp. 121-126. New York and London:
541 Academic Press.
542 **Alonso-Alvarez, C., Bertrand, S., Devevey, G., Prost, J., Faivre, B. and Sorci, G.** (2004). Increased
543 susceptibility to oxidative stress as a proximate cost of reproduction. *Ecol. Lett.* **7**, 363-368.

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

Alonso-Alvarez, C., Bertrand, S., Devevey, G., Prost, J., Faivre, B., Chastel, O. and Sorci, G. (2006). An experimental manipulation of life-history trajectories and resistance to oxidative stress. *Evolution* **60**, 1913-1924.
Anderson, M. E. (1996). Measurement of antioxidants: glutathione. In *Free radicals, a practical approach*, eds. N. A. Punchard and F. J. Kelly), pp. 213 - 226. Oxford, U.K.: Oxford University Press.
Balaban, R. S., Nemoto, S. and Finkel, T. (2005). Mitochondria, oxidants, and aging. *Cell* **120**, 483-495.
Banks, R., Speakman, J. R. and Selman, C. (2010). Vitamin E supplementation and mammalian lifespan. *Mol. Nutr. Food Res.* **54**, 719-725.
Barja, G. (2007). Mitochondrial oxygen consumption and reactive oxygen species production are independently modulated: Implications for aging studies. *Rejuvenation Research* **10**, 215-223.
Beckman, K. B. and Ames, B. N. (1998). The free radical theory of aging matures. *Physiol. Rev.* **78**, 547-581.
Bergeron, P., Careau, V., Humphries, M. M., Reale, D., Speakman, J. R. and Garant, D. (2011). The energetic and oxidative costs of reproduction in a free-ranging rodent. *Funct. Ecol.* **25**, 1063-1071.
Berry, R. J. (1981). Biology of the house mouse. New York: Academic Press Inc.
Brand, M. D. and Esteves, T. C. (2005). Physiological functions of the mitochondrial uncoupling proteins UCP2 and UCP3. *Cell Metab.* **2**, 85-93.
Brand, M. D., Affourtit, C., Esteves, T. C., Green, K., Lambert, A. J., Miwa, S., Pakay, J. L. and Parker, N. (2004). Mitochondrial superoxide: Production, biological effects, and activation of uncoupling proteins. *Free Radical Biol. Med.* **37**, 755-767.
Christe, P., Glaizot, O., Strepparava, N., Devevey, G. and Fumagalli, L. (2012). Twofold cost of reproduction: an increase in parental effort leads to higher malarial parasitaemia and to a decrease in resistance to oxidative stress. *Proceedings of the Royal Society B: Biological Sciences* **279**, 1142-1149.
Clutton-Brock, T. H. (1991). The evolution of parental care. New Jersey: Princeton University Press.
Collins, S., Pi, J. B. and Yehuda-Shnaidman, E. (2012). Uncoupling and reactive oxygen species (ROS) - A double-edged sword for beta-cell function? "Moderation in all things". *Best Pract. Res. Clin. Endoc. Metab.* **26**, 753-758.
Costantini, D. (2008). Oxidative stress in ecology and evolution: lessons from avian studies. *Ecol. Lett.* **11**, 1238-1251.
Cretegny, C. and Genoud, M. (2006). Rate of metabolism during lactation in small terrestrial mammals (Crocidura russula, Mus domesticus and Microtus arvalis). *Comp. Biochem. Physiol. A-Mol. Integr. Physiol.* **144**, 125-134.
Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A. and Colombo, R. (2003). Protein carbonyl groups as biomarkers of oxidative stress. *Clin. Chim. Acta* **329**, 23-38.
Dimonte, D., Ross, D., Bellomo, G., Eklow, L. and Orrenius, S. (1984). Alterations in intracellular thiol homeostasis during the metabolism of menadione by isolated rat hepatocytes *Arch Biochem Biophys* **235**, 334-342.
Dowling, D. K. and Simmons, L. W. (2009). Reactive oxygen species as universal constraints in life-history evolution. *Proc. R. Soc. B* **276**, 1737-1745.
Droge, W. (2002). Free radicals in the physiological control of cell function. *Physiol. Rev.* **82**, 47-95.
Elchuri, S., Oberley, T. D., Qi, W. B., Eisenstein, R. S., Roberts, L. J., Van Remmen, H., Jepstein, C. J. and Huang, T. T. (2005). CuZnSOD deficiency leads to persistent and widespread oxidative damage and hepatocarcinogenesis later in life. *Oncogene* **24**, 367-380.
Gardner, P. R. (1997). Superoxide-driven aconitase FE-S center cycling. *Biosci Rep* **17**, 33-42.
Gardner, P. R., Raineri, I., Epstein, L. B. and White, C. W. (1995). Superoxide radical and iron modulate aconitase activity in mammalian-cells. *J. Biol. Chem.* **270**, 13399-13405.
Garratt, M., Vasilaki, A., Stockley, P., McArdle, F., Jackson, M. and Hurst, J. L. (2011). Is oxidative stress a physiological cost of reproduction? An experimental test in house mice. *Proc. R. Soc. B* **278**, 1098-1106.

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

Gittleman, J. L. and Thompson, S. D. (1988). Energy Allocation in Mammalian Reproduction. *Am. Zool.* **28**, 863-875.
Halliwell, B. and Gutteridge, J. M. (1999). Free radicals in biology and medicine. Oxford, U.K.: Oxford University Press.
Hammond, K. A. (1997). Adaptation of the maternal intestine during lactation. *J Mammary Gland Biol Neoplasia* **2**, 243-252.
Hammond, K. A., Konarzewski, M., Torres, R. M. and Diamond, J. (1994). Metabolic Ceilings under a Combination of Peak Energy Demands. *Physiol. Zool.* **67**, 1479-1506.
Harman, D. (1956). Aging - a theory based on free-radical and radiation-chemistry *J Gerontol* **11**, 298-300.
Harshman, L. G. and Zera, A. J. (2007). The cost of reproduction: the devil in the details. *Trends Ecol. Evol.* **22**, 80-86.
Hausladen, A. and Fridovich, I. (1994). Superoxide and peroxynitrite inactivate aconitases, but nitric-oxide does not. *J. Biol. Chem.* **269**, 29405-29408.
Hausladen, A. and Fridovich, I. (1996). Measuring nitric oxide and superoxide: rate constants for aconitase reactivity. *Methods Enzymol.* **269**, 37-41.
Isaksson, C., Sheldon, B. and Uller, T. (2011). The Challenges of Integrating Oxidative Stress into Life-history Biology. *Bioscience* **61**, 194-202.
Jaeschke, H. and Wendel, A. (1986). Manipulation of mouse organ glutathione contents .2. time and dose-dependent induction of the glutathione conjugation system by phenolic antioxidants. *Toxicology* **39**, 59-70.
Johnson, M. S., Thomson, S. C. and Speakman, J. R. (2001). Limits to sustained energy intake I. Lactation in the laboratory mouse Mus musculus. *J. Exp. Biol.* **204**, 1925-1935.
Jones, D. P. (2006). Redefining oxidative stress. *Antioxidants & Redox Signaling* **8**, 1865-1879.
Keller, M., Sommer, A. M., Portner, H. O. and Abele, D. (2004). Seasonality of energetic functioning and production of reactive oxygen species by lugworm (Arenicola marina) mitochondria exposed to acute temperature changes. *J. Exp. Biol.* **207**, 2529-2538.
Kirkwood, T. B. L. and Holliday, R. (1979). Evolution of aging and longevity. *Proc. R. Soc. B* **205**, 531-546.
Klatt, P., Molina, E. P., De Lacoba, M. G., Padilla, C. A., Martinez-Galisteo, E., Barcena, J. A. and Lamas, S. (1999). Redox regulation of c-Jun DNA binding by reversible S-glutathiolation. *FASEB J.* **13**, 1481-1490.
Koivula, M., Koskela, E., Mappes, T. and Oksanen, T. A. (2003). Cost of reproduction in the wild: Manipulation of reproductive effort in the bank vole. *Ecology* **84**, 398-405.
Korshunov, S. S., Skulachev, V. P. and Starkov, A. A. (1997). High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. *FEBS Lett.* **416**, 15-18.
Lessells, C. K. M. (1991). The evolution of life histories. In *Behavioural Ecology*, eds. J. R. Krebs and N. B. Davies). Oxford: Blackwell Scientific Publications.
Magwere, T., Goodall, S., Skepper, J., Mair, W., Brand, M. D. and Partridge, L. (2006). The effect of dietary restriction on mitochondrial protein density and flight muscle mitochondrial morphology in Drosophila. *J. Gerontol. Ser. A-Biol. Sci. Med. Sci.* **61**, 36-47.
Malhotra, J. D., Miao, H., Zhang, K., Wolfson, A., Pennathur, S., Pipe, S. W. and Kaufman, R. J. (2008). Antioxidants reduce endoplasmic reticulum stress and improve protein secretion. *Proc Natl Acad Sci U S A* **105**, 18525-18530.
Metcalfe, N. B. and Alonso-Alvarez, C. (2010). Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. *Funct. Ecol.* **24**, 984-996.
Monaghan, P., Metcalfe, N. B. and Torres, R. (2009). Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. *Ecol. Lett.* **12**, 75-92.
Nussey, D. H., Pemberton, J. M., Pilkington, J. G. and Blount, J. D. (2009). Life history correlates of oxidative damage in a free-living mammal population. *Funct. Ecol.* **23**, 809-817.

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

Ołdakowski, Ł., Piotrowska, Ż., Chrząścik, K. M., Sadowska, E. T., Koteja, P. and Taylor, J. R. E. (2012). Is reproduction costly? No increase of oxidative damage in breeding bank voles. *The Journal of Experimental Biology* **215**, 1799-1805.
Partridge, L., Gems, D. and Withers, D. J. (2005). Sex and death: What is the connection? *Cell* **120**, 461-472.
Pecqueur, C., Bui, T., Gelly, C., Hauchard, J., Barbot, C., Bouillaud, F., Ricquier, D., Miroux, B. and Thompson, C. B. (2008). Uncoupling protein-2 controls proliferation by promoting fatty acid oxidation and limiting glycolysis-derived pyruvate utilization. *FASEB J.* **22**, 9-18.
Pedraza, N., Solanes, G., Iglesias, R., Vazquez, M., Giralt, M. and Villarroya, F. (2001). Differential regulation of expression of genes encoding uncoupling proteins 2 and 3 in brown adipose tissue during lactation in mice. *Biochem. J.* **355**, 105-111.
Perez, V. I., Bokov, A., Van Remmen, H., Mele, J., Ran, Q. T., Ikeno, Y. and Richardson, A. (2009). Is the oxidative stress theory of aging dead? *Biochimica Et Biophysica Acta-General Subjects* **1790**, 1005-1014.
Pichaud, N., Chatelain, E. H., Ballard, J. W. O., Tanguay, R., Morrow, G. and Blier, P. U. (2010). Thermal sensitivity of mitochondrial metabolism in two distinct mitotypes of Drosophila simulans: evaluation of mitochondrial plasticity. *J. Exp. Biol.* **213**, 1665-1675.
Reeves, P. G., Nielsen, F. H. and Fahey, G. C. (1993). Ain-93 purified diets for laboratory rodents - final report of the american institute of nutrition ad hoc writing committee on the reformulation of the ain-76a rodent diet. *J. Nutr.* **123**, 1939-1951.
Selman, C., Blount, J. D., Nussey, D. H. and Speakman, J. R. (2012). Oxidative damage, ageing, and life-history evolution: where now? *Trends in ecology & evolution (Personal edition)*.
Selman, C., Lumsden, S., Bunger, L., Hill, W. G. and Speakman, J. R. (2001). Resting metabolic rate and morphology in mice (Mus musculus) selected for high and low food intake. *J. Exp. Biol.* **204**, 777-784.
Selman, C., McLaren, J. S., Collins, A. R., Duthie, G. G. and Speakman, J. R. (2008). The impact of experimentally elevated energy expenditure on oxidative stress and lifespan in the short-tailed field vole Microtus agrestis. *Proc. R. Soc. B* **275**, 1907-1916.
Speakman, J. R. (2008). The physiological costs of reproduction in small mammals. *Phil. Trans. R. Soc. B* **363**, 375-398.
Speakman, J. R. and McQueenie, J. (1996). Limits to sustained metabolic rate: The link between food intake, basal metabolic rate, and morphology in reproducing mice, Mus musculus. *Physiol. Zool.* **69**, 746-769.
Stearns, S. C. (1992). The evolution of life histories. Oxford: Oxford University Press.
Toime, L. J. and Brand, M. D. (2010). Uncoupling protein-3 lowers reactive oxygen species production in isolated mitochondria. *Free Radical Biol. Med.* **49**, 606-611.
Townsend, D. M., Tew, K. D. and Tapiero, H. (2003). The importance of glutathione in human disease. *Biomedicine & Pharmacotherapy* **57**, 145-155.
Vasilaki, A., Mansouri, A., Van Remmen, H., van der Meulen, J. H., Larkin, L., Richardson, A. G., McArdle, A., Faulkner, J. A. and Jackson, M. J. (2006). Free radical generation by skeletal muscle of adult and old mice: effect of contractile activity. *Aging Cell* **5**, 109-117.
Wiegand, G. and Remington, S. J. (1986). Citrate synthase - structure, control, and mechanism. *Annu Rev Biophys Biophys Chem* **15**, 97-117.
Wiersma, P., Selman, C., Speakman, J. R. and Verhulst, S. (2004). Birds sacrifice oxidative protection for reproduction. *Proc. R. Soc. B* **271**, S360-S363.
Williams, G. C. (1957). Pleiotropy, natural selection, and the evolution of senescence. *Evolution* **11** 398-411.
Zelko, I. N., Mariani, T. J. and Folz, R. J. (2002). Superoxide dismutase multigene family: A comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. *Free Radical Biol. Med.* **33**, 337-349.

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

696

697

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

698 # Tables:

699 Table 1.Markers of oxidative damage and antioxidant defence examined in this study.

Marker	What it assesses	References
Protein Thiols	Groups on proteins that are essential for stability and/or function, but are susceptable to oxidation. A reduction in these groups is indicative of protein oxidation.	(Halliwell and Gutteridge, 1999; Di Monte et al., 1984)
Protein Carbonyls	Product of ROS attack on amino-acid residues in proteins. An increase is indicative of increased protein oxidation.	(Halliwell and Gutteridge, 1999; Dalle-Donne et al., 2003)
Total Glutathione	Most abunant intracellular thiol with a vareity of antioxidant roles.	(Halliwell and Gutteridge, 1999; Townsend et al., 2003)
Proportion of Oxidised to Total Glutathione	Oxidised glutathione is the product of glutathione oxidation. This ratio is an indicator of oxidative stress and the redox status of a sample.	(Jones, 2006; Anderson, 1996)
Superoxide Dismutase	Accelerates the dismutation of the superoxide radical to hydrogen peroxide and oxygen. Has been described as "the first and most important line of antioxidant defence".	(Halliwell and Gutteridge, 1999; Zelko et al., 2002)
Catalase	Catalyse the decompositon of hydrogen peroxide to oxygen. This is an important antioxidant.	(Halliwell and Gutteridge, 1999)
Aconitase	Enzyme that is very susceptible to deactivation by the superoxide radical and can be used as a marker of ROS levels.	(Gardner et al., 1995; Gardner, 1997)
Citrate Synthase	Enzyme that calayses a step of the citric acid cycle. Activity levels are used as a marker of mitochodrial density.	(Wiegand and Remington, 1986; Pichaud et al., 2010)

700

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

Table 2. Effect of reproductive treatment, diet and the number of siblings the experimental animals they themselves had while they were reared on markers of oxidative stress and antioxidant defence.

	N	Reproductive treatment			Food			Reproductive treatment X food			Dam's number of siblings		
	(C,H,L)	df	F	P	df	F	P	df	F	P	df	F	P
Oxidative stress marker													
Protein thiols													
Liver	17,15,13	2,34	5.55	0.008	1,37	0.44	0.51	2,33	1.02	0.37	1,14	4.82	0.045
Heart	18,16,15	2,39	0.76	0.47	1,39	> 0.001	0.99	2,39	0.66	0.53	1,18	1.34	0.26
Muscle	18,17,14	2,35	0.48	0.62	1,38	0.47	0.5	2,33	1.64	0.21	1,11	> 0.001	0.97
Proportion of oxidised to total glutathione													
Liver	20,18,15	2,38	3.22	0.051	1,43	1.36	0.25	2,38	0.24	0.79	1,14	1.47	0.25
Heart	20,18,15	2,42	0.92	0.41	1,42	0.086	0.77	2,41	0.79	0.46	1,15	0.51	0.49
Muscle	17,17,15	2,36	0.06	0.94	1,42	2.23	0.14	2,35	4.19	0.023	1,21.7	1.02	0.32
Total glutathione													
Liver	18,15,13	2,30	0.07	0.94	1,37	0.08	0.79	2,28	0.48	0.62	1,9	0.72	0.42
Heart	20,18,15	2,40	1.63	0.21	1,44	0.001	0.97	2,40	0.31	0.74	1,19	0.37	0.55
Muscle	17,17,14	2,27	0.85	0.44	1,41	1.56	0.22	2,26	1.42	0.26	1,12	0.31	0.59
Additional liver markers													
Carbonyls	13,11,12	2,29	4.71	0.017	1,29	1.71	0.2	2,29	2.46	0.1	1,29	4.21	0.049
Superoxide dismutase	12,12,12	2,27	4.44	0.021	1,27	1.64	0.24	2,27	1.11	0.45	1,27	1.04	0.32
Catalase	18,17,14	2,37	2.87	0.069	1,41	0.16	0.69	2,36	0.04	0.96	1,16	1.13	0.3
Mitochodrial marker													
Citrate synthase	19,18,15	2,40	5.58	0.007	1,44	0.014	0.91	2,39	0.06	0.94	1,21	1.1	0.38
Aconitase/citrate synthase	19,18,15	2,45	1.45	0.25	1,45	0.62	0.44	2,45	0.89	0.42	1,45	1.68	0.2

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

Table 3. Relationships between oxidative stress and (1) the difference between natural and manipulated litter sizes and (2) each female's natural litter size.

	Increase in pup number			Natural litter size - all reproductives			Natural litter size - 2 pups			Natural litter size - 8 pups		
	n	r_s	P-value	n	r_s	P-value	n	r_s	P-value	n	r_s	P-value
Protein thiols												
Liver	28	0.43	0.024	28	-0.07	0.74	13	0.24	0.43	15	0.08	0.78
Heart	31	-0.01	0.95	31	0.21	0.25	15	-0.41	0.12	16	0.42	0.11
Muscle	31	-0.18	0.34	31	0.15	0.43	14	0.03	0.93	17	0.21	0.43
Proportion of oxidised to total glutathione												
Liver	33	0.28	0.12	33	-0.12	0.50	15	-0.45	0.10	18	-0.09	0.74
Heart	33	0.33	0.063	33	-0.25	0.16	15	-0.53	0.04	18	-0.01	0.99
Muscle	32	-0.02	0.90	32	0.07	0.70	15	0.31	0.26	17	0.05	0.84
Total glutathione												
Liver	28	0.04	0.82	28	-0.01	0.097	13	0.21	0.49	15	-0.09	0.76
Heart	33	-0.18	0.31	33	0.24	0.17	15	-0.12	0.67	18	0.49	0.038
Muscle	31	0.01	0.96	31	0.22	0.25	14	0.03	0.92	17	0.43	0.084
Additional liver markers												
Carbonyls	23	-0.33	0.12	23	-0.02	0.94	12	-0.46	0.13	11	-0.39	0.24
Superoxide dismutase	24	0.3	0.16	28	-0.33	0.12	12	0.22	0.5	12	-0.29	0.37
Catalase	31	-0.21	0.27	31	-0.01	0.97	14	0.17	0.56	17	-0.3	0.24
Mitochondrial marker												
Citrate synthase	33	0.19	0.30	33	-0.06	0.74	15	0.25	0.38	18	-0.01	0.99
Aconitase/citrate synthase	33	0.15	0.39	33	0.08	0.65	15	-0.03	0.92	18	0.16	0.54

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

Figure legends

Figure 1. The impact of litter size manipulation on food consumption and organ weight. Females that had their litter size manipulated to eight pups ate much more food during lactation (a.), had heavier livers (b.) and heavier hearts (c.). Untransformed data is displayed with Means and SEMs.

Figure 2. Female reproductive investment and markers of oxidative stress and antioxidant defence in the liver. * denotes $P < 0.05$; ** denotes $P < 0.01$. Untransformed data is displayed with Means and SEMs.

Figure 3. Oxidative stress in the liver and the number of siblings that females were reared with. Females reared with a greater number of siblings had lower aconitase activity (a), a higher concentration of protein carbonyls (b) and a lower concentration of protein thiols (c), changes that indicate increased oxidative stress. Untransformed data is displayed with Means and SEMs.

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

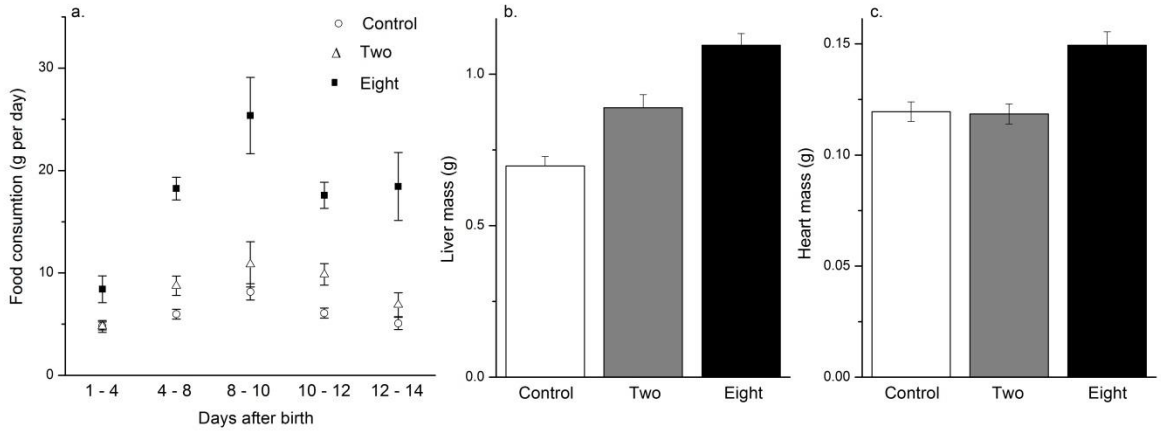

Figure 1.

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

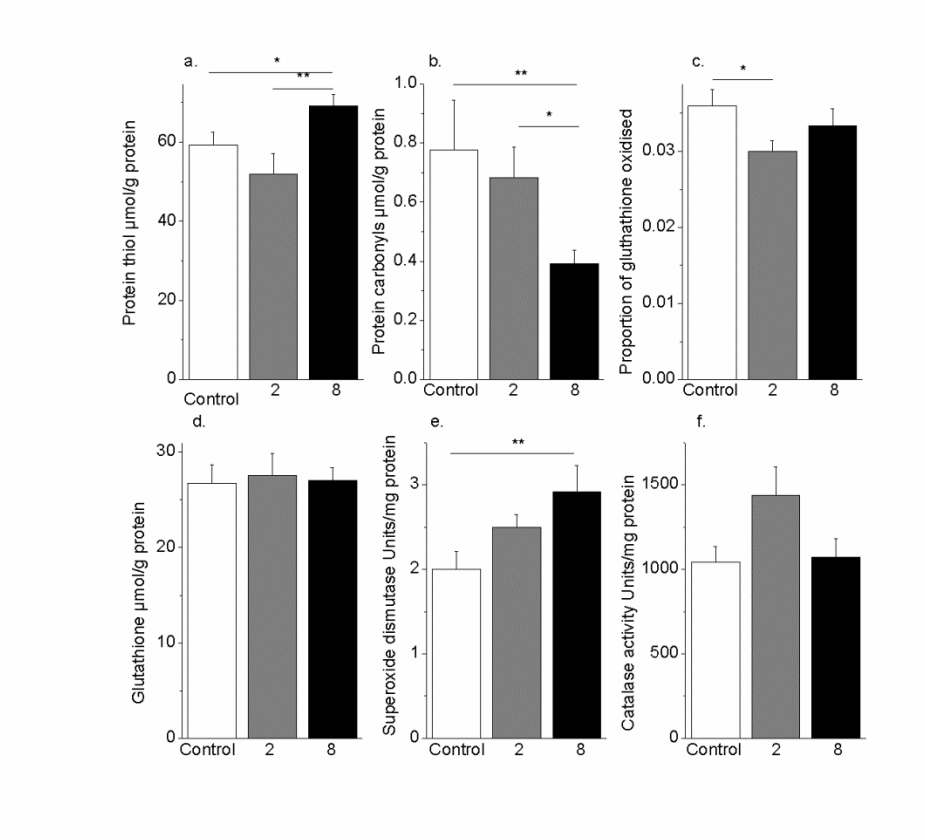

Figure 2.

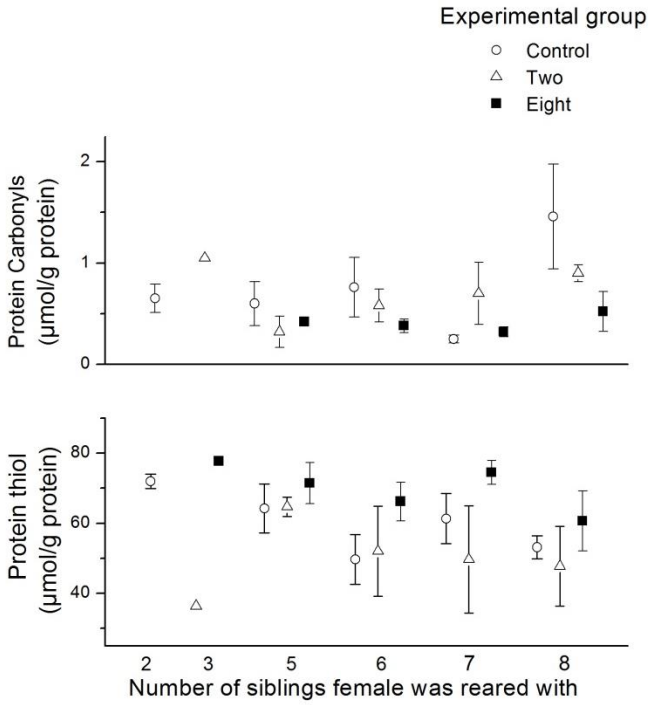

Figure 3.

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT