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Oxidative stress in response to natural and experimentally elevated reproductive effort is tissue dependent

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Summary

1. Oxidative stress is a potential proximal physiological cost of reproduction. Detecting this cost may be performed in several different ways – manipulating reproductive status, correlating natural variations in effort to oxidative stress or manipulating reproductive effort. Here, we manipulated reproductive status and studied oxidative stress due to natural and experimental variation in reproductive effort in Brandt's voles (*Lasiopodomys brandtii*), using a variety of markers and tissues.

2. We measured markers of oxidative stress in lactating (raising 6 to 8 offspring) and non-reproductive voles (Experiment I) and found that a marker of oxidative protection [serum total-superoxide dismutase (SOD) activity] was reduced, and a marker of oxidative damage (protein carbonyls) was increased, in the serum, in lactating compared with non-reproductive voles. However, protein carbonyls in the liver were lower in lactating compared with non-reproductive voles, consistent with increased liver SOD activity. Lipid damage [malonaldehyde (MDA)] in both serum and liver was unrelated to reproductive status.

3. We compared these markers of oxidative stress between natural large ($n \geq 9$) and small litter sizes ($n \leq 5$; Experiment II), and between manipulated large (11–13) and small litter sizes (2–3; Experiment III) and found that liver MDA and SOD activity was higher in voles with natural large compared with natural small litter sizes, but there were no differences in other markers. There was no effect of litter size on all measures when it was experimentally manipulated.

4. The effects of reproductive status on oxidative stress were critically dependent on the exact markers and tissues used. The effects of natural variation in reproductive effort suggested that there might be an oxidative stress cost associated with large litter sizes, but this effect was not replicated in the experimentally manipulated litters.

Key-words: Brandt's vole (*Lasiopodomys brandtii*), lactation, life-history trade-offs, litter size, oxidative damage

Introduction

Fundamental trade-offs in life history are generally presumed to be a consequence of partitioning limited resources among various physiological functions (Stearns 1992; Speakman 2008). Reproduction, particularly lactation, is the most energetically demanding period of a mammal's life (Millar 1977; Loudon & Racey 1987; Piersma & Van Gils 2011). Recently, oxidative stress has been suggested as a possible physiological cost of reproduction that could limit investment in other life-history components (Costantini 2008; Dowling & Simmons 2009; Monaghan, Metcalfe & Torres 2009; Selman *et al*. 2012).

Oxidative stress occurs when the rate of production of reactive oxygen species (ROS) exceeds the capacity of the antioxidant defence and repair mechanisms (Finkel &

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Holbrook 2000; Monaghan, Metcalfe & Torres 2009; Metcalfe & Alonso-Álvarez 2010). ROS are physiological by-products of normal metabolic processes; their unstable and very reactive nature can cause damaging effects on many biomolecules (e.g. DNA, proteins and lipids) unless quenched by enzymatic and non-enzymatic antioxidants (Balaban, Nemoto & Finkel 2005; Dowling & Simmons 2009). Oxidative stress has been implicated as a proximate mechanism responsible for the natural ageing process as well as a variety of disease states (Finkel & Holbrook 2000; Selman *et al.* 2012).

During reproduction, metabolic rate is increased (Speakman 2008), which potentially could cause increased ROS production and result in oxidative stress (Alonso-Álvarez *et al.* 2004; Speakman 2008). However, ROS are not simply generated in direct proportion to oxygen consumption (Speakman *et al.* 2004), and further, animals can potentially up-regulate a variety of antioxidant defences in response to increased ROS production, repairing oxidative damage and limiting its subsequent impact (Monaghan, Metcalfe & Torres 2009). Consequently, the association between reproductive effort and oxidative stress is potentially complex and not necessarily straightforward.

Recently, both field and laboratory studies have tried to explore the association between reproductive effort and oxidative stress. Some results in free-ranging animals including mammals and birds have indicated that oxidative stress was positively linked to reproductive effort (Bergeron *et al.* 2011; Christe *et al.* 2011; Heiss & Schoech 2012; Fletcher *et al.* 2013), but others have failed to find any association (Nussey *et al.* 2009; Markó *et al.* 2011). Although in the case of the study by Nussey *et al.* (Nussey *et al.* 2009), this might be because plasma samples were collected after reproduction was completed. Oxidative damage assays in plasma may reflect the recent oxidative state because turnover of plasma constituents is high. However, in contrast to these studies, some studies of lactating female mammals in captivity have indicated that oxidative stress may actually be decreased during reproduction. For example, two measures of oxidative damage (MDA and protein thiols) in the liver were reduced during lactation in captive house mice (Garratt *et al.* 2011). Similarly, lipid oxidative damage was lower (kidney and muscle) and protein oxidative damage unaltered (kidney, muscle and heart) in breeding female bank voles (*Myodes glaeolus*) relative to non-breeding females (Oldakowski *et al.* 2012). The contrast between field and laboratory studies of lactating mammals is striking and may have two contributing causes. First, in the laboratory, reproducing mammals have ready access to food and do not generally have other simultaneous pressures like thermoregulatory demands (but see (Hammond & Diamond 1992; Johnson & Speakman 2001) or immune challenges to cope with (but see Kristan & Hammond 2000). These demands in the wild may exacerbate the demands of reproduction making oxidative stress more evident. Alternatively, it is notable that measurements taken in field studies have focused on using serum as the preferred sample source for measurement, while laboratory studies have addressed the levels of damage primarily in tissues.

Most of these previous studies in mammals have focused on the relation between natural variation in reproductive effort and oxidative stress. However, it is well-established that natural and experimental variations in reproductive effort are not expected to covary in the same way with measures of body condition (Remick 1992). Females, for example, may adjust their investment in reproduction so as to limit their exposure to oxidative damage.

Consequently, experimental manipulation of reproductive investment (e.g. litter size manipulation) may be necessary to reveal whether oxidative damage and protection are altered in relation to reproductive effort (Metcalfe & Monaghan 2013). Indeed, several studies of birds have found that an experimental increase in reproductive effort (manipulations of brood size) is associated with a decrease in activity of antioxidants or resistance to oxidative stress (Alonso-Álvarez *et al.* 2004; Alonso-Alvarez *et al.* 2006; Bertrand *et al.* 2006; Christe *et al.* 2011). Changes in antioxidant status do not necessarily indicate oxidative stress (Wiersma *et al.* 2004; Monaghan, Metcalfe & Torres 2009). Surprisingly, only a single previous study has examined whether oxidative damage and protection are altered with experimentally manipulated reproductive effort in small mammals (Metcalfe & Monaghan 2013). A manipulation of litter size in wild-derived house mice revealed no effect of reproductive level on protein oxidation in the heart and gastrocnemius muscle and decreased damage in the livers of mice with experimentally increased levels of reproductive effort (Garratt *et al.* 2013).

Previous data have indicated that reproduction, particularly lactation, in Brandt's voles (*Lasiopodomys brandtii*) is physiologically costly, and food intake and metabolic rate were increased in lactating compared with non-reproductive voles, especially in the voles with naturally large or enlarged litter sizes (Zhang, Li & Wang 2008; Wu *et al.* 2009; Xu, Yang & Wang 2012). These data indicated that the energetic costs of maternal maintenance increased with the extent of energetic investment of lactation. These higher requirements for energy for reproduction might force a reduction in investment in somatic protection and hence lead to increased oxidative damage. In this study, we studied the variation in oxidative stress due to natural and manipulated variation in reproductive effort in Brandt's voles. Moreover, to examine whether oxidative stress is a cost of reproduction in lactating Brandt's voles, we measured a number of markers of oxidative stress (including oxidative damage and antioxidant activity in both the liver and serum) in lactating (for voles raising natural litter sizes between 6 and 8 offspring) and non-reproductive voles (Experiment I). To examine whether oxidative stress was altered with overall reproductive effort in lactating Brandt's voles, we also compared these markers of oxidative stress between voles with natural large ($n \geq 9$) and natural small litter sizes ($n \leq 5$; Experiment

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II). Finally, we measured the same parameters in voles with manipulated large (11–13) and manipulated small litter sizes (2–3; Experiment III). We predicted, based on life-history theory, that lactation would be associated with elevated oxidative stress, and lactating voles with large litter size would experience greater oxidative stress than those voles with small litter size. We anticipated that while these associations might be unclear with natural variations in reproductive investment (experiments I and II), they should be significant in experimentally manipulated litters (Experiment III) if oxidative stress is a proximate cost of reproduction in this species.

Materials and methods

ANIMALS AND HOUSING CONDITIONS

Brandt's voles inhabit mainly the grasslands of Inner Mongolia of China, Mongolia and the Baikal region of Russia. In the wild, 90% of female voles commenced breeding in April and the reproductive season lasted until August (Zhang & Wang 1998). During this period, one female could potentially raise 1–2 litters, and the natural litter size varies from 2 to 13. Thus, it is an ideal model to explore the effect of natural variations in reproductive effort on oxidative stress markers. Moreover, the females are tolerant of large manipulations in litter size allowing the study of the effect of manipulated reproductive effort on oxidative stress markers (see Metcalfe & Monaghan 2013 for rationale of this approach). Finally, it has been suggested that studies of domesticated rodents like mice may be less than ideal for the study of oxidative stress because of their long history of domesticated breeding which may have favoured animals resistant to stress (Speakman & Garratt, in press); hence, studies of recently captive animals may provide a better test of the oxidative stress hypothesis.

One hundred and eighteen virgin adult female Brandt's voles, weighing 40–55 g and aged 120–150 days old, were used in this study. They were the offspring of voles from our laboratory colony. The colony was established in 1999 and is regularly supplemented with additional wild individuals to maintain genetic diversity. Voles were kept individually in plastic cages (30 × 15 × 20 cm) under a 16-h: 8 h light/dark cycle and room temperature (21 ± 1 °C). Commercial standard rabbit pellets (Beijing KeAo Feed Co., Beijing, China) and water were provided *ad libitum*. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences (Permit Number: IOZ11012).

EXPERIMENTAL PROCEDURES

One hundred and eleven randomly selected voles were paired with males for 1 day and then were immediately separated from the males. Of these, only 48 became pregnant. The animals that did not become pregnant took no further part in this study. Another 7 virgin females were randomly selected and used as non-reproductive controls, and were not paired with males. The voles were checked twice a day to determine the day of parturition (day 0 of lactation). Animals were sacrificed on day 18 of lactation (peak lactation). Each vole was euthanized by CO_2 asphyxiation between 0900 and 1100 h, and trunk blood was collected which was allowed to clot for 30 min at 4 °C and centrifuged at 4 °C for 30 min at 1500 ***g***. Serum was collected and stored in sealable polypropylene micro-centrifuge tubes at −80 °C until oxidative damage and antioxidant activity assays were performed 6 months later. The livers were obtained and cut into small pieces and washed the residual blood with ice-cold saline and then were snap-frozen in liquid nitrogen and stored at −80 °C until assay. During assay, the livers were homogenized with ice-cold saline (1 g tissue per 9 mL saline) on ice. The homogenate was centrifuged for 10 min at 1500 ***g*** at 4 °C. The supernatants were used for assay.

Experiment I

In the first experiment, we examined whether oxidative stress is the consequence of reproduction in lactating voles compared with non-reproductive voles (N, n = 7). Voles whose natural litter size was 6–8 (most common litter size of this species) were defined as the lactating group (L_0, n = 7).

Experiment II

The second experiment explored the effects of naturally large and naturally small litter sizes, which were presumed to reflect different reproductive effort. Lactating females whose natural litter size was more than 8 (mean = 10·6) or less than 6 (mean = 3·4) were selected and defined as the large group (L, n = 9) or the small group (S, n = 10), respectively.

Experiment III

To further test the relationship between reproductive effort and oxidative stress, in this experiment, we manipulated litter size to examine the effect of increased or decreased reproductive effort on the oxidative stress. Animals whose original litter size at birth was 6–8 were used in this experiment. We manipulated litter size by adding or removing pups on the day of parturition. Litters with same parturition date were mixed together and assigned randomly to females. By adding or removing 5 pups, we assigned pups randomly to three treatment groups: E, enlarged group (initial litter size 6–8, with 5 pups added, n = 7, mean = 11·9); C, control group (with the initial litter size unchanged, n = 7, mean = 6·7); R, reduced group (initial litter size 6–8, with 5 pups removed, n = 8, mean = 1·9). Maternal voles readily accepted foreign pups, and the survival of their offspring did not differ between mothers raising their own pups (experiment II) and cross-fostered pups (experiment III).

REPRODUCTIVE PERFORMANCE

Initial (day of parturition) and final (day 18 of lactation) litter size and litter mass were recorded.

LIPID PEROXIDATION AND PROTEIN OXIDATION

Oxidative damage was estimated as lipid peroxidation and protein oxidation. Lipid peroxidation was assessed by quantifying malonaldehyde (MDA)(Del Rio, Stewart & Pellegrini 2005) using a TBARS assay kit (Nanjing jiancheng, Nanjing, China) following the manufacturer's instructions. The absorbance of the eluent was monitored spectrophotometrically at 532 nm (BioTek Synergy™ 4 Hybrid Microplate Reader; BioTek, Vermont, USA). Both within- and among-sample variations for this assay were <1·5%. Lipid peroxidation was expressed as nmol of MDA per mg protein or mL serum. It should be noted that this assay has been reported to have low specificity and a substantial degree of the MDA measured can also be generated during the assay process (Moore & Roberts 1998), making the data potentially less reliable than studies of protein oxidation.

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Protein oxidation was assessed by the determination of levels of protein carbonyls (Mateos & Bravo 2007) using a kit (Nanjing jiancheng) according to the manufacturer's instructions. Briefly, proteins in liver and serum were reacted with 2,4-dinitrophenylhydrazine (DNPH) in hydrochloric acid for 30 min at 37 °C, precipitated with trichloroacetic acid and washed four times by resuspension in ethanol/ethyl acetate (1:1 v/v). Proteins were solubilized in guanidine hydrochloride and centrifuged to remove insoluble material. Carbonyl groups were monitored spectrophotometrically at 370 nm (Beckman Coulter DU 800 UV/Vis Spectrophotometer; Beckman, California, USA). Carbonyls were expressed as nmol mg^{-1} of protein.

ANTIOXIDANT ASSAY

Superoxide dismutase (SOD) activity was measured using kits (Nanjing jiancheng) according to the manufacturer's instructions. One unit of SOD was defined as the amount of enzyme that causes 50% inhibition of superoxide radical produced by the reaction between xanthine and xanthine oxidase at 37 °C. SOD activity was expressed as U mg^{-1} protein in liver and U mL^{-1} in serum.

STATISTICAL ANALYSIS

Data were analysed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Prior to all statistical analyses, data were examined for normality using the Kolmogorov–Smirnov test. Differences in liver and serum markers of oxidative stress (MDA, protein carbonyls, SOD activity) and litter mass were analysed by independent sample *t*-test in the first and second experiments, and in the third experiment, they were analysed by one-way ANOVA followed by Tukey's *post hoc* comparisons. Differences between group means were considered statistically significant at $P < 0{\cdot}05$. Given the sample sizes and assay variability between individuals, we had 90% power to detect an effect size of 35% in the assays of protein carbonyls in serum and 90% power to detect an effect size of 65% in the assays of protein carbonyls in the liver in experiments I and II, both at alpha = 0·05. In experiment III, we had 90% power to detect an effect size of 38% in assays of protein carbonyls in serum and 90% power to detect an effect size of 76% in assays of protein carbonyls in the liver, both at alpha = 0·05. Power for the other assays exceeded these values.

Results

EXPERIMENT I

Liver MDA ($t = -1{\cdot}001$, $P = 0{\cdot}091$; Fig. 1a) and serum MDA ($t = -0{\cdot}434$, $P = 0{\cdot}521$; Fig. 1b) in Brandt's voles were not significantly different between lactating and non-reproductive voles. In contrast, liver protein carbonyls were significantly lower in lactating compared with non-reproductive voles ($t = 2{\cdot}226$, $P = 0{\cdot}046$; Fig. 1c), but serum protein carbonyls were significantly higher in lactating compared with non-reproductive voles ($t = -2{\cdot}603$, $P = 0{\cdot}025$; Fig. 1d).

Liver SOD activity was significantly higher in lactating compared with non-reproductive voles ($t = -2{\cdot}739$, $P = 0{\cdot}018$; Fig. 2a), but serum SOD activity was significantly lower in lactating compared with non-reproductive voles ($t = 3{\cdot}230$, $P = 0{\cdot}007$; Fig. 2b). These differences in oxidative protection (SOD) corresponded in direction to the respective changes in damage levels as evaluated by protein carbonyls.

EXPERIMENT II

The litter sizes and litter masses in natural large and small litter size groups are shown in Table 1. There was a significant difference between natural large litter size group and natural small litter size group (day 0 of lactation: $t = 7{\cdot}997$, $P < 0{\cdot}001$; day 18 of lactation: $t = 5{\cdot}137$,

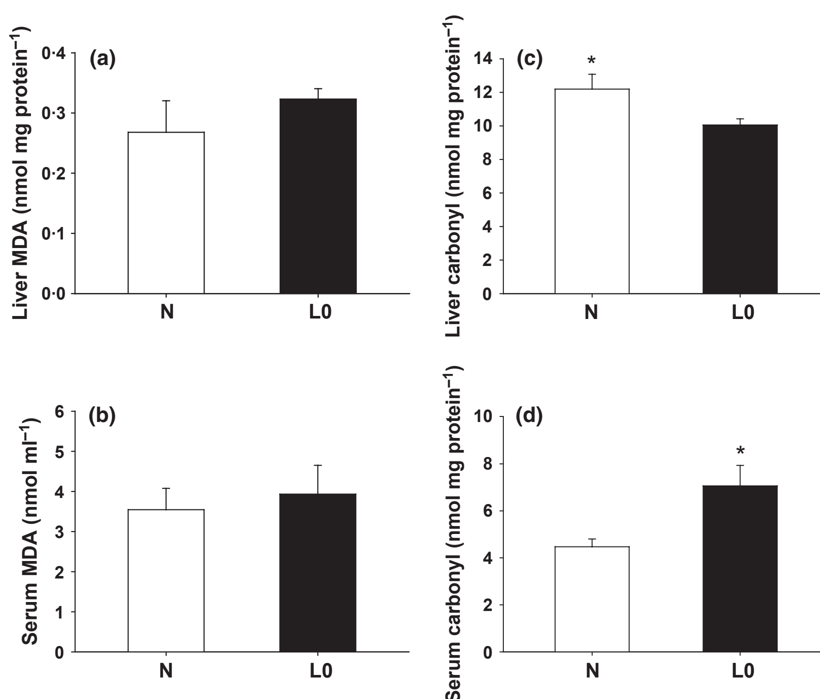

Fig. 1. Liver MDA (a), serum MDA (b), liver protein carbonyl (c) and serum protein carbonyl (d) in lactating (L_0) and non-reproductive (N) Brandt's voles. Values are means ± SEM. Significant difference among groups is indicated by an asterisk if $P < 0{\cdot}05$. MDA, malonaldehyde.

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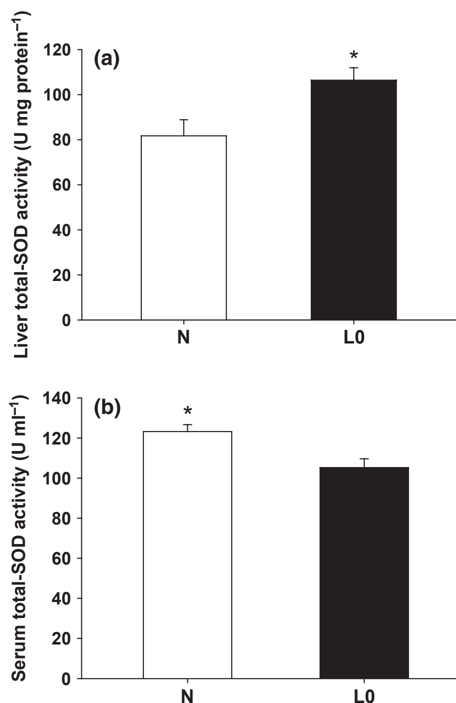

Fig. 2. Liver SOD activity (a) and serum SOD activity (b) in lactating (L_0) and non-reproductive (N) Brandt's voles. Values are means ± SEM. Significant difference between groups is indicated by an asterisk if $P < 0{\cdot}05$. SOD, superoxide dismutase.

$P < 0{\cdot}001$; Table 1). Liver MDA was significantly higher in voles with large litter size than those with small litter size ($t = 2{\cdot}205$, $P = 0{\cdot}042$; Fig. 3a). Serum MDA tended to be higher in natural small litter group compared with natural large litter group although the difference was not statistically significant ($t = -1{\cdot}429$, $P = 0{\cdot}08$; Fig. 3b). There were also no differences in liver protein carbonyls

Table 1. Litter sizes and litter masses in natural large (L) and small (S) litter size groups

Parameters	L	S
Litter size	9·7 ± 1·4	3·3 ± 0·3
Litter mass		
Day 0 of lactation	28·4 ± 2·8	9·9 ± 1·2
Day 18 of lactation	79·9 ± 7·9	39·9 ± 4·7

Values are means ± SEM.

($t = -0{\cdot}096$, $P = 0{\cdot}925$; Fig. 3c) and serum protein carbonyls ($t = -1{\cdot}499$, $P = 0{\cdot}156$; Fig. 3d) between natural large and small litter size groups.

Liver SOD activity was significantly higher in the large litter size group compared with small litter size group ($t = 2{\cdot}252$, $P = 0{\cdot}038$; Fig. 4a). However, there was no difference between the large litter size group and small litter size group in serum SOD activity levels ($t = -1{\cdot}568$, $P = 0{\cdot}135$; Fig. 4b).

EXPERIMENT III

The mean numbers of offspring and litter masses in enlarged, control and reduced groups were shown in Table 2. There was a significant difference among enlarged, control and reduced groups (day 0 of lactation: $F_{2,18} = 145{\cdot}859$, $P < 0{\cdot}001$; day 18 of lactation: $F_{2,19} = 38{\cdot}053$, $P < 0{\cdot}001$; Table 2). Liver MDA ($F_{2,21} = 0{\cdot}688$, $P = 0{\cdot}515$; Fig. 5a) and serum MDA ($F_{2,20} = 1{\cdot}025$, $P = 0{\cdot}379$; Fig. 5b) did not differ among enlarged, control and reduced groups. Liver protein carbonyls ($F_{2,21} = 3{\cdot}117$, $P = 0{\cdot}068$; Fig. 5c) and serum protein carbonyls ($F_{2,20} = 1{\cdot}212$, $P = 0{\cdot}321$; Fig. 5d) were not significantly different among the three groups (Table 2).

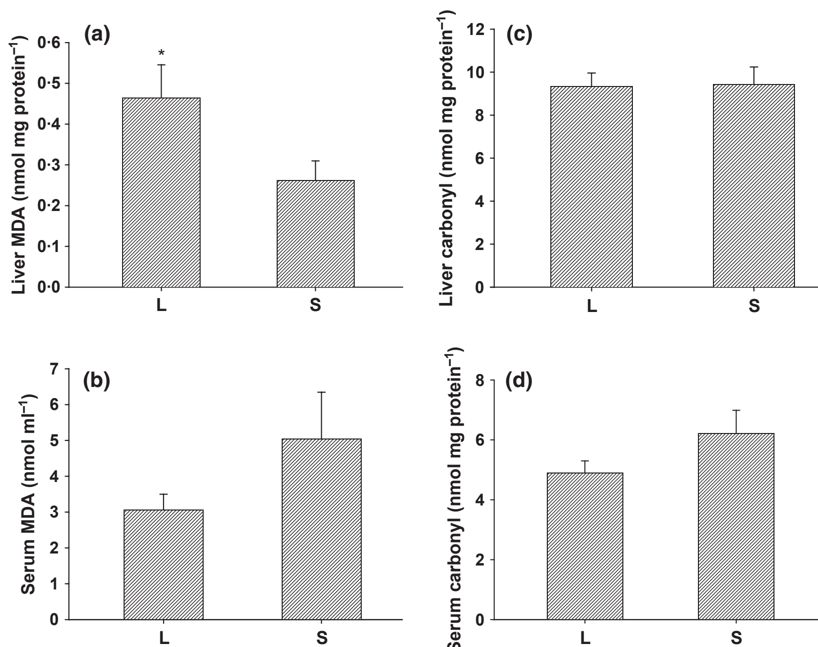

Fig. 3. Liver MDA (a), serum MDA (b), liver protein carbonyl (c) and serum protein carbonyl (d) in Brandt's voles with natural large (L) or small (S) litter size. L, large litter size group; S, small litter size group; MDA, malonaldehyde. Values are means ± SEM. Significant difference between groups is indicated by an asterisk if $P < 0{\cdot}05$.

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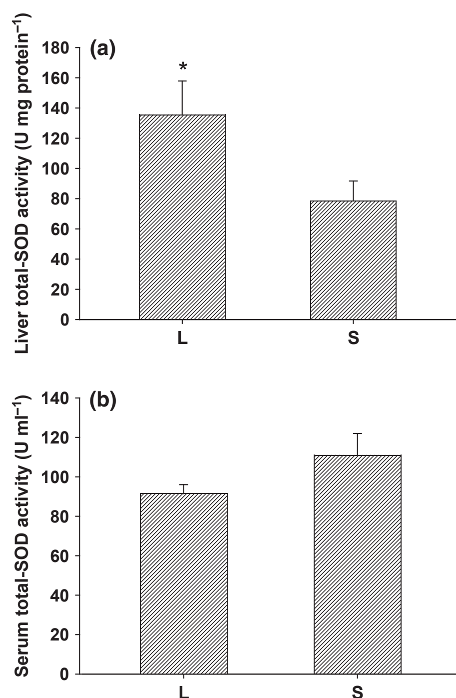

Fig. 4. Liver SOD activity (a) and serum SOD activity (b) in Brandt's voles with natural large (L) or small (S) litter size. L, large litter size group; S, small litter size group; SOD, superoxide dismutase. Values are means ± SEM. Significant difference between groups is indicated by an asterisk if $P < 0{\cdot}05$.

Liver SOD activity ($F_{2,21} = 0{\cdot}419$, $P = 0{\cdot}627$; Fig. 6a) and serum SOD activity ($F_{2,20} = 1{\cdot}665$, $P = 0{\cdot}216$; Fig. 6b) also did not differ among enlarged, control and reduced groups. Combined data for all voles with natural litters (i.e. natural large and small litter size groups and the control group) revealed that litter size (at day 18 of lactation) was positively correlated with liver MDA ($r = 0{\cdot}54$, $P = 0{\cdot}004$; see Fig. S1, Supporting information) and liver SOD activity ($r = 0{\cdot}553$, $P = 0{\cdot}003$). There were no other significant correlations between litter size and other markers of oxidative stress. In addition, liver SOD activity was positively correlated with liver MDA ($r = 0{\cdot}923$, $P < 0{\cdot}001$; Fig. S2, Supporting information). No other significant correlations were detected between markers of oxidative damage and antioxidants for natural large, control and small litters groups combined.

Table 2. Litter sizes and litter masses in enlarged (E), control (C) and reduced (R) groups

Parameters	E	C	R
Litter size	10·3 ± 0·4	6·3 ± 0·3	1·6 ± 0·3
Litter mass			
Day 0 of lactation	33·9 ± 1·6	18·4 ± 1·9	5·2 ± 1·2
Day 18 of lactation	76·4 ± 5·3	63·5 ± 4·7	20·7 ± 4·3

Values are means ± SEM.

Discussion

Oxidative stress has been suggested as a proximate cost of reproduction (Monaghan, Metcalfe & Torres 2009; Selman *et al.* 2012). In the first experiment, we found that protein oxidative damage in serum proteins (protein carbonyls) was increased in lactating compared with virgin non-reproductive Brandt's voles, consistent with the predictions of life-history theory. The observed reduction in levels of serum SOD activity was consistent with the elevated protein oxidative damage observed in the serum. These changes in the serum were consistent with previous field studies of lactating small mammals where elevated

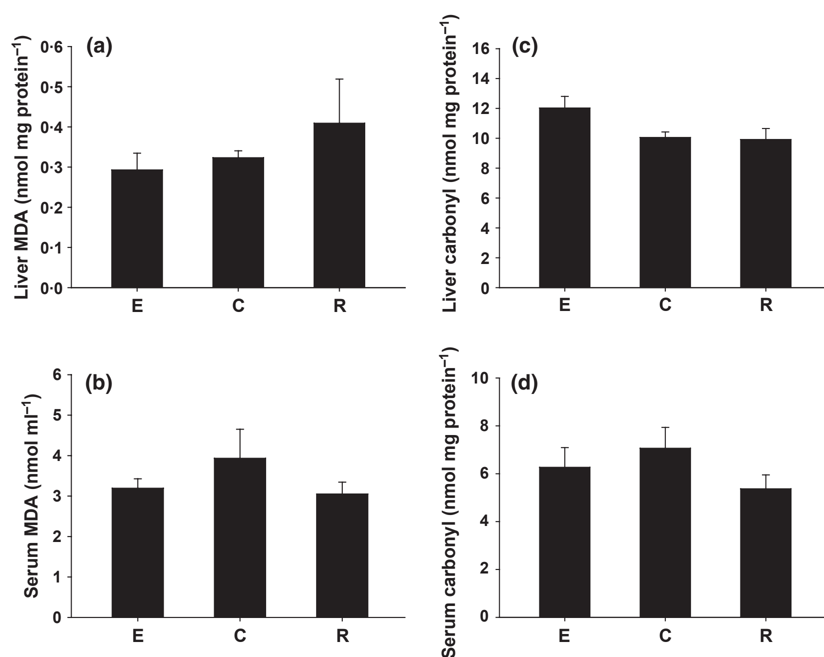

Fig. 5. Liver MDA (a), serum MDA (b), liver protein carbonyl (c) and serum protein carbonyl (d) in E, C and R group. E, enlarged litter size group, C, control group, R, reduced litter size group; MDA, malonaldehyde. Values are means ± SEM.

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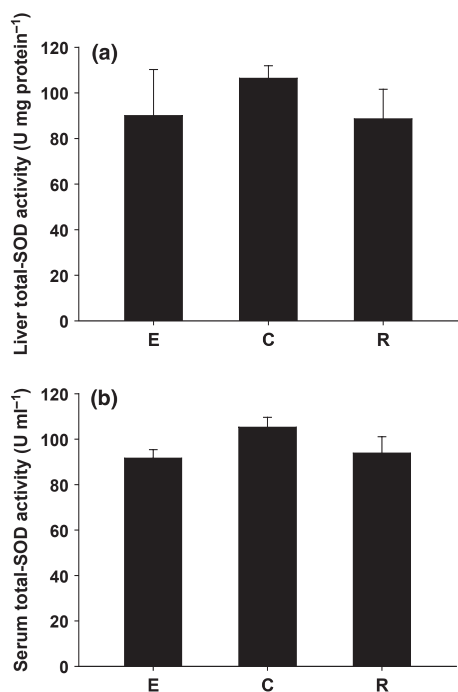

Fig. 6. Liver SOD activity (a) and serum SOD activity (b) in E, C and R group. E, enlarged litter size group; C, control group; R, reduced litter size group; SOD, superoxide dismutase. Values are means ± SEM.

oxidative damage was associated with reproduction using serum as the sample source. We presume that this damage occurs during lactation. However, it is possible that the damage occurred during pregnancy and was carried over into lactation. We suspect that this is less likely because during lactation, the energy demands on the female increase enormously relative to the levels in gestation – in Brandt's voles the difference is about twofold to 2·5-fold higher (Wu *et al.* 2009). If the resource allocation model is correct, then we would not anticipate major effects in pregnancy because individuals could simply elevate their intake to cover the costs of protecting themselves. This option may be unavailable in lactation because the females work under a physiological constraint that caps their intake (Speakman & Krol 2010). Hence, it is anticipated that if there is an oxidative cost of reproduction, it will arise during lactation rather than gestation.

However, lactating voles had significantly lower protein carbonyls in their livers compared with non-reproductive voles, indicating oxidative damage in the liver was decreased during lactation. This reduction in protein oxidative damage was consistent with the observation that oxidative damage (measured by protein thiols and MDA in the liver and muscle) was reduced during lactation in house mice (Garratt *et al.* 2011) and in kidneys and muscles (measured by MDA) of bank voles (Oldakowski *et al.* 2012). These latter data suggest that reproductively active females might develop compensatory antioxidant mechanisms to limit oxidative damage to their tissues (Monaghan, Metcalfe & Torres 2009). The observed elevation in levels of liver SOD activity might explain why oxidative damage was reduced in the liver, and the similar situation was also reported in reproducing house mice (Garratt *et al.* 2011). Peroxidation of lipids (MDA) in both the liver and serum was unaffected by reproductive status, consistent with the results found in wild Soay sheep (*Ovis aries*) in which no relation between reproductive effort and oxidative damage after reproduction was completed was found (Nussey *et al.* 2009). These changes are directly opposite to the expectations based on life-history theory which predicts that investment in antioxidant defences should decline during reproduction leading to elevated oxidative damage.

Although it has been previously observed that oxidative stress may differ between tissues (Sohal, Agarwal & Sohal 1995; Costantini 2008), the reasons for the diametrically opposite responses in the liver and serum remain unclear. Several previous studies have attempted to establish whether serum oxidative stress biomarkers are indicative of oxidative stress in tissues (Argüelles *et al.* 2004; Veskoukis *et al.* 2009), and they found positive correlations between oxidative stress measurements in serum and tissues for some biomarkers but not for others (Argüelles *et al.* 2004; Veskoukis *et al.* 2009). In the present study, differences in damage markers were consistent with the different activities of serum and liver antioxidants. Whatever the cause of these differences, it is evident that if we had only measured one tissue, we would have reached opposite conclusions regarding the predictions of life-history theory depending on the tissue we had chosen for analysis. Notably previous field studies have focused primarily on blood samples because of the difficulties in assaying other tissues in the field without compromising other aspects of the field-based studies (Nussey *et al.* 2009; Bergeron *et al.* 2011; Fletcher *et al.* 2013). Since our study has indicated opposite effects in serum and the liver, we should be cautious in interpreting studies based only on serum samples as supporting the suggestion that oxidative damage is a proximate mediator of life-history trade-offs. We have assumed that reproductive status causes these effects, but it is also possible that the effects arise from the hormonal and body composition changes that accompany reproductive attempts. If this was correct, we would not expect to observe links between oxidative stress and the level of reproductive effort.

We also examined therefore how markers of oxidative damage vary in response to variations in female reproductive effort. In the second experiment, we found that voles with natural large litter sizes had significantly higher liver lipid damage (MDA) than those with natural small litter sizes. Surprisingly, SOD activity in the liver was also higher in voles with natural large litter sizes compared those with small litter sizes. MDA in the serum and protein carbonyls in both the liver and serum were unaffected by natural litter size. Clearly, when observing natural litter sizes, the female may have chosen her own

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level of reproductive investment (Metcalfe & Monaghan 2013). In this case, 3 of 4 assays of damage were non-significant, but levels of lipid peroxidation in the liver were higher in the voles raising large litters, consistent with the oxidative stress hypothesis. Moreover, the effect of natural litter sizes on liver SOD activity was positive, opposite to the prediction from the resource allocation model. One interpretation of these data is that females adjusted their reproductive effort to match their own capability to cope with the resultant oxidative stress, and hence, 3 out of 4 assays provided non-significant associations to reproductive effort and they tailored their protection system to defend themselves from the anticipated damage, leading to a positive rather than negative association between protection and effort (Metcalfe & Monaghan 2013).

This was why it was important for us to include an experimental manipulation of the litter size in Experiment III. However, subjecting breeding female Brandt's voles to experimentally enlarged litter size did not lead to a reduction in SOD activity or to an increase in the lipid or protein oxidative damage. This is unlikely to have been a power issue in the analysis. This result contrasts the data for birds and *Drosophila melanogaster* which showed that an increased reproductive effort was associated with a decrease in activity of antioxidants and/or resistance to oxidative stress (Salmon, Marx & Harshman 2001; Wang, Salmon & Harshman 2001; Alonso-Álvarez *et al.* 2004; Wiersma *et al.* 2004), although in the latter studies, oxidative damage was not measured directly. A potential explanation for the absence of an effect of manipulations of litter size on oxidative damage is that female reproductive effort may be independent of litter size (e.g. in laboratory mice, elevating litter size did not result in elevated food intake or milk production (Johnson, Thomson & Speakman 2001; Duah *et al.* 2013 – see also Speakman & Garratt, in press). However, in this species, experimentally enlarging litter size is known from previous studies to increase energy requirements (i.e. food intake) and energy expenditure (i.e. resting metabolic rate; Xu, Yang & Wang 2012), so it is unlikely that the lack of increased oxidative damage in breeding voles was caused by an insufficient increase in investment. Similarly, reduced litter size did not affect oxidative stress markers measured. These results suggest that manipulated reproductive effort (litter size) during lactation had no effect on oxidative damage in this species.

In conclusion, we found that the oxidative damage to proteins in livers was lower and in serum was higher, in lactating compared with virgin non-reproductive Brandt's voles. These data show that the inferred effects of reproduction status on oxidative damage are critically dependent on the exact markers used and the samples in which they are measured. Although some data suggested greater damage in voles raising naturally larger litters, this effect was not replicated when litter size was experimentally manipulated, contrasting with the results from birds.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Correlations between litter size and markers of oxidative stress for natural large, control and small litters groups combined.

Fig. S2. Correlations between markers of oxidative damage and antioxidants for natural large, control and small litters groups combined.

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