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RESEARCH ARTICLE

Is reproduction costly? No increase of oxidative damage in breeding bank voles

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SUMMARY

According to life-history theory, investment in reproduction is associated with costs, which should appear as decreased survival to the next reproduction or lower future reproductive success. It has been suggested that oxidative stress may be the proximate mechanism of these trade-offs. Despite numerous studies of the defense against reactive oxygen species (ROS) during reproduction, very little is known about the damage caused by ROS to the tissues of wild breeding animals. We measured oxidative damage to lipids and proteins in breeding bank vole (*Myodes glareolus*) females after rearing one and two litters, and in non-breeding females. We used bank voles from lines selected for high maximum aerobic metabolic rates (which also had high resting metabolic rates and food intake) and non-selected control lines. The oxidative damage was determined in heart, kidneys and skeletal muscles by measuring the concentration of thiobarbituric acid-reactive substances, as markers of lipid peroxidation, and carbonyl groups in proteins, as markers of protein oxidation. Surprisingly, we found that the oxidative damage to lipids in kidneys and muscles was actually lower in breeding than in non-breeding voles, and it did not differ between animals from the selected and control lines. Thus, contrary to our predictions, females that bred suffered lower levels of oxidative stress than those that did not reproduce. Elevated production of antioxidant enzymes and the protective role of sex hormones may explain the results. The results of the present study do not support the hypothesis that oxidative damage to tissues is the proximate mechanism of reproduction costs.

Key words: bank vole, cost of reproduction, oxidative damage, oxidative stress.

INTRODUCTION

Producing viable offspring and, in this way, increasing fitness is inevitably coupled with costs; increased current investment in reproduction decreases future reproduction either through direct effects on future reproductive output or through effects on parental survival. This negative trade-off is one of the core assumptions of life-history theory (Williams, 1966; Reznik, 1985; Roff, 1992; Stearns, 1992), but its physiological basis is not well understood. The traditional explanation of the cost of reproduction focuses on the allocation of the limited amounts of materials and energy to competing processes (Sibly and Calow, 1986). The raised investment of resources in the current reproduction may limit the allocation of resources to self-maintenance in parents and, consequently, lower the prospects of reproduction in the future. Trade-offs may also result from the physiological costs that are an inevitable side effect of reproduction. For mammals, these obligatory costs include hyperthermia, bone loss, disruption of sleep patterns and oxidative stress (Speakman, 2008). Oxidative stress has received a great deal of interest recently from evolutionary ecologists as it may be a universal constraint in life-history evolution (Dowling and Simmons, 2009; Monaghan et al., 2009; Metcalfe and Alonso-Alvarez, 2010; Isaksson et al., 2011).

Oxidative stress occurs when there is an imbalance between the rate of production of reactive oxygen species (ROS) and the capacity of enzymatic and non-enzymatic antioxidants to neutralize their damaging effects (Monaghan et al., 2009; Metcalfe and Alonso-Alvarez, 2010). ROS are byproducts of normal metabolism that can cause extensive damage to lipids, proteins and DNA when not quenched by the antioxidant machinery (Finkel and Holbrook, 2000; Van Remmen and Richardson, 2001). Time of reproduction is potentially the period when animals may be most prone to oxidative stress, because then their long-term (sustained) metabolic rate reaches the highest levels in the annual cycle, which may increase ROS production. Birds feeding their young operate at metabolic rates 33–50% higher than non-breeding individuals (Drent and Daan 1980). Resting metabolic rates at thermoneutral temperatures and food intake in lactating females of small mammals exceed the non-reproductive levels on average 1.6 (up to 2.2) and two (up to four) times, respectively (Cretegny and Genoud, 2006; Speakman, 2008). It has to be noted, however, that there is no simple link between the rate of energy expenditure and ROS production (Speakman, 2003).

The studies of oxidative stress in reproduction have concentrated on the relationship between the reproductive effort and the antioxidative capacity, especially in birds. In captive zebra finches (*Taeniopygia guttata*), males co-raising experimentally increased broods showed weaker resistance to ROS-induced hemolysis (Alonso-Alvarez et al., 2004). A negative relationship was also shown between the number of breeding attempts by zebra finches and the resistance of their red blood cells to a controlled ROS attack (Alonso-Alvarez et al., 2006). Finally, the number of eggs laid by the breeding pair was negatively correlated with the change in

resistance to oxidative stress, and the correlation disappeared when birds were provided with dietary antioxidant (Bertrand et al., 2006). However, none of these results demonstrate that the birds incurred more oxidative damage (or that production of ROS was increased), which is necessary to prove the existence of oxidative stress (Monaghan et al., 2009).

Papers investigating tissue damage caused by ROS in wild vertebrates as a consequence of reproduction are very scarce, and we are aware of only three such studies on mammals. These studies provide an inconsistent general picture. In a population of wild Soay sheep (*Ovis aries*), Nussey et al. found no correlation between recent or total reproductive output and a measure of phospholipid oxidative damage in blood plasma samples (Nussey et al., 2009). In addition, no evidence of increased oxidative damage was found in female house mice (*Mus musculus domesticus*), neither after a long period of reproductive investment nor during peak lactation, a period of extremely high energetic demands (Garratt et al., 2011). Instead, markers in the liver revealed lower oxidative damage in reproductive than in non-reproductive females. However, a positive relationship between litter mass and protein oxidation in female tissues indicated oxidative stress. A weak positive relationship between oxidative damage and litter size has been reported in the eastern chipmunk (*Tamis striatus*) (Bergeron et al., 2011).

We studied oxidative damage in breeding bank voles, *Myodes* (*Clethrionomys*) *glareolus* (Schreber 1780). Taking into account that ROS production and the damage to tissues are expected to increase with increasing metabolic rates, the objective of the present study was to test two hypotheses: (1) females with higher reproductive effort suffer higher levels of oxidative damage to tissues than non-breeding females, and (2) females with higher genetically determined energy demands suffer higher oxidative damage.

A multiway artificial long-term selection experiment performed in a laboratory colony of bank voles at the Jagiellonian University (Sadowska et al., 2008) created an excellent opportunity to test these hypotheses. We investigated oxidative damage to tissues in two groups of bank vole females from the experiment: animals from lines selected for high aerobic metabolic rate achieved during locomotor activity (swimming), and animals from unselected control lines. The animals from the selected groups, besides having high maximum metabolic rates, were characterized by approximately 13.0% higher daily food intake (in generation 4) and approximately 14.6% higher basal metabolic rates [in generation 11 (Koteja et al., 2009; Koteja et al., 2011)]. In each of the two selection groups (selected and control), we manipulated the reproductive effort by allowing females to wean one or two litters, and other females were not permitted to reproduce. Assuming that oxidative damage is the proximate cost of reproduction, we expected that: (1) female voles that weaned more litters would have higher oxidative damage than non-breeding animals, and (2) bank voles from lines selected for high maximum metabolic rates would have higher oxidative damage than controls. We determined thiobarbituric acid-reactive substances (TBARS) and protein-bound carbonyl levels as markers of oxidative damage to lipids and proteins, respectively, in chosen tissues of females to test the above hypotheses.

MATERIALS AND METHODS

Study animals

Both the selected group and the control group of bank voles in the artificial selection experiment conducted at the Institute of Environmental Sciences, Jagiellonian University, Kraków, were represented by four replicate lines. In the lines selected for high metabolic rates, the selection criterion was the highest 1-min rate of oxygen consumption achieved by voles during swimming in water at 38°C. This temperature ensured that the increase of metabolism was solely due to locomotor activity and not thermoregulatory demand. The animals were kept in standard plastic mouse cages (26×20×16 cm) with sawdust bedding, at a constant temperature (20±1°C) and photoperiod (16 h:8 h light:dark) and had *ad libitum* access to water and food (standard rodent chow, Labofeed H, Kcynia, Poland; no antioxidants as preservatives, vitamin E=90 mg kg^{-1}). The detailed breeding and selection protocols have been described elsewhere (Sadowska et al., 2008).

Experimental design

At the beginning of the experiment, all animals were approximately 90 days old. We used 72 females from the eighth generation, including 36 sampled randomly from four lines selected for high metabolic rates, and 36 sampled randomly from four control lines (i.e. nine females from each of eight lines). Selection group with nested replicate lines was the first factor of our two-factor experimental design, and number of weaned litters, representing the breeding effort, was the second. Females were randomly assigned to three subgroups (three animals in each replicate line). Females in group 0, which were not permitted to breed, had no contact with males throughout the experiment and were kept in standard mouse cages with sawdust bedding. Group 1 consisted of females that weaned one litter (one complete lactation) and group 2 consisted of females that weaned two litters (two complete lactations). Females from groups 1 and 2 were kept with males (which were removed after weaning of the first litter) in bigger cages (43×27×16 cm), with sawdust bedding and a clay pot and paper towel for nesting. All females of groups 1 and 2 were therefore pregnant during the first lactation, as is the natural state for bank vole females in the wild. The number of pups in each litter was recorded at weaning (on the 17th day after parturition) and pups were then weighed to the nearest 0.01 g.

All females were killed by cervical dislocation. Because females from group 1 were in their second pregnancy while rearing the first litter, they were killed, for ethical reasons, within 1 to 3 days after the second parturition. Females from group 2 were killed when the second litter was weaned. Females from group 0 were killed randomly with the females from group 1 and 2. All females were weighed to the nearest 0.1 g, and liver, heart (drained of blood), both kidneys and a sample of skeletal muscles associated with the femur were dissected, weighed (±0.001 g) and snap-frozen in liquid nitrogen within 2–3 min of death. Frozen samples were transported to the Institute of Biology, University of Białystok, and kept at –80°C awaiting analyses of the oxidative damage.

Oxidative damage analysis

Tissue samples were homogenized in a cold mortar in 1.5 ml cold 50 mmol l^{-1} phosphate buffer containing 1 mmol l^{-1} EDTA, pH 7.0. Supernatant was obtained by centrifugation at 10,000 ***g*** for 10 min at 4°C and kept on ice for determination of oxidative damage.

Malondialdehyde (MDA) formation was used to quantify the lipid peroxidation in tissues and was measured as TBARS by means of an MDA colorimetric assay kit (Northwest Life Science Specialities LCC, Vancouver, WA, USA). Protein content in each sample was determined by the Lowry method (Lowry et al., 1951) with Peterson modification (Peterson et al., 1977), and TBARS concentrations were expressed in nmol mg^{-1} protein.

Tissue protein oxidation was assayed by the reaction of 2,4-dinitrophenylhydrazine (DNPH) with protein carbonyls (Stadtman and Olivier, 1991; Levine et al., 1994). All determinations were

made using the Protein Carbonyl Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA) following the manufacturer's instructions. The protein carbonyl content was expressed in nmol mg^{-1} of protein measured in each sample according to the manufacturer's instructions.

Repeatability [calculated following Lessells and Boag (Lessells and Boag, 1987)] of TBARS concentration in samples, assayed in duplicate in kidneys, heart and muscle, was 0.89, 0.94 and 0.86, respectively. Repeatability of protein carbonyl content in kidneys and heart, also assayed twice, was 0.72 and 0.68, respectively. All these coefficients were highly significant (P<0.0001).

Statistical analyses

We analyzed concentrations of the markers of oxidative damage (TBARS and protein carbonyls) with a nested ANOVA model with selection group (animals from lines selected for maximum metabolic rate, and from control lines), number of litters (0, 1 and 2), and their interaction as fixed factors. Replicate line nested in selection group and the interaction between number of litters and the replicate line were random factors. We used the MIXED procedure [with the restricted maximum likehood method (REML)] in SAS version 9.1 (SAS Institute, Cary, NC, USA) to estimate the model.

Body masses of females, masses of internal organs and numbers of offspring produced were analyzed with the same model. To adjust the masses of internal organs for the variation of body mass of females, we added a covariate to the model, which was the difference between body mass and the mass of a given organ. This was to avoid the autocorrelation between these two masses. Data were transformed logarithmically (body and organ masses) or square root transformed (TBARS and protein carbonyl concentrations in heart) where necessary to meet parametric assumptions.

RESULTS

Females allowed to produce two litters weaned on average 2.6 and 2.4 times more pups than those that produced one litter in selected and control groups, respectively (Fig. 1). In the two-factor ANOVA of the number of offspring, the number of litters was highly significant ($F_{1,6}$=85.35, P<0.001), with no effect of selection group ($F_{1,6}$=1.79, P=0.229) or the interaction between the two factors ($F_{1,6}$=0.91, P=0.376).

Body masses, recorded upon collection for oxidative damage analyses, were significantly lower in non-reproducing females than in females that weaned one or two litters, with no difference between the two latter groups (overall effect of reproduction group in the two-factor ANOVA: $F_{2,12}$=22.81, P<0.001; Table 1). At the same time, females from lines selected for high metabolic rates were significantly heavier than control females ($F_{1,6}$=7.23, P=0.0361), and the difference was consistent across litter number groups ($F_{2,12}$=0.05, P=0.948 for the interaction).

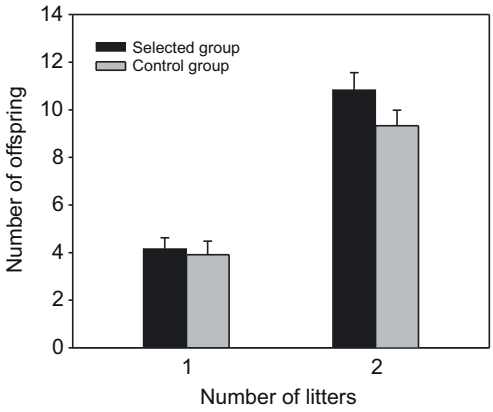

Fig. 1. Total numbers of offspring at weaning born by bank vole females assigned to two experimental groups: allowed to produce one litter, and two litters. One group of females was taken from lines selected for high aerobic metabolic rates, and the other from control (unselected) lines. Means + 1 s.e.m.

Mass of liver, kidneys and heart showed the same pattern of variation depending on the selection group and litter size, as observed in body mass (Table 2A). When corrected for body mass differences, non-reproducing females had significantly lower masses of liver and kidneys than females allowed to reproduce (Table 2). Selection group had no impact on body-size-corrected masses of organs, except of kidneys, which were larger in animals from lines selected for high metabolic rates (Table 2).

We found that the oxidative damage to lipids in kidneys and skeletal muscles (as measured by TBARS concentration) was significantly lower in breeding than in non-breeding females (Table 3, Fig. 2). Oxidative damage to proteins in the heart (protein carbonyl concentrations) also tended to be lower in breeding than in non-breeding females, but the effect did not reach statistical significance (Table 3, Fig. 3). No impact of the number of litters on other combinations of TBARS/carbonyl concentrations and organs was found. The oxidative damage to lipids and proteins did not differ between animals from the selected and control lines (Table 3, Figs 2, 3).

Individual females in both reproduction groups varied greatly in the number of weaned pups at the end of the experiment (ranging from 1 to 14) and total litter mass at weaning (from 9.9 to 137 g). Because female food consumption rises with litter size and mass, which can in turn increase oxidative stress, we also tested for the relationship between TBARS or protein carbonyls in the investigated tissues and the reproductive investment. However, there were no significant relationships between these markers of oxidative damage and the total number of produced offspring or litter mass (one-way nested ANCOVA; selection group as a factor and number of

Table 1. Mean (±s.e.m.) body masses in bank vole females selected for high maximum metabolic rates and controls, assigned to three experimental groups: non-reproducing, allowed to wean one litter and allowed to wean two litters

	Number of litters		
Selection group	0	1	2
Selected	23.5±1.6	29.8±1.4	29.3±1.0
Control	20.6±1.2	25.6±0.6	25.5±0.5
	a	b	b

Body masses were recorded upon collection for oxidative damage analysis (see Materials and methods). N=12 for each mean. Different letters (a, b) show significant differences between body masses of females with 0, 1 and 2 litters, as revealed by multiple contrasts after ANOVA.

Table 2. Mass of internal organs (g) of bank vole females (A), and the results of the two-way nested ANCOVA of organ masses with selection group and number of litters as the main factors and body mass as a covariate (B)

A		Number of litters		
Organ	Selection group	0	1	2
Liver	Selected	1.31±0.11	1.97±0.12	1.95±0.13
	Control	0.98±0.06	1.76±0.06	1.63±0.08
		a	b	b
Kidneys	Selected	0.316±0.021	0.430±0.017	0.385±0.013
	Control	0.251±0.012	0.351±0.015	0.344±0.015
		a	b	b
Heart	Selected	0.150±0.009	0.169±0.007	0.174±0.006
	Control	0.116±0.004	0.143±0.006	0.153±0.005
		a	a	a

B				
Organ	Number of litters	Selection group	Interaction	Body mass[a]
Liver	$F_{2,12}$=19.78	$F_{1,6}$=0.18	$F_{2,12}$=4.22	$F_{1,46}$=114.9
	P=0.0002	P=0.682	P=0.041	P<0.0001
Kidneys	$F_{2,12}$=7.56	$F_{1,6}$=6.31	$F_{2,12}$=1.78	$F_{1,47}$=60.92
	P=0.0075	P=0.046	P=0.210	P<0.0001
Heart	$F_{2,12}$=3.15	$F_{1,6}$=4.65	$F_{2,12}$=2.34	$F_{1,47}$=59.67
	P=0.080	P=0.075	P=0.139	P<0.0001

Different letters (a, b) in A show significant differences between females with 0, 1 and 2 litters, as revealed by multiple contrasts after ANCOVA. N=12 for each mean.

[a]Body mass minus the mass of a given organ.

offspring as a covariate; P-values for the covariate ranged between 0.101 and 0.912).

DISCUSSION

Our measurements of oxidative damage in bank vole females did not support the hypothesis that reproduction increases oxidative stress. Instead, counterintuitively, the markers of oxidative damage of lipids in kidneys and muscles, and perhaps also of proteins in heart, were lower in breeding than in non-breeding females, with other differences between the two groups being non-significant. To our knowledge, this is the second time that the lower oxidative damage in breeding females has been reported. Garratt et al. (Garratt et al., 2011) recently found significantly lower damage to lipids in liver and skeletal muscle, and to protein in liver, in breeding female wild house mice. The lack of signs of increased oxidative damage caused by reproduction in the liver in bank voles in our experiment, in contrast with Garratt's study, points at the importance of testing various organs and tissues for the presence of oxidative stress. It is also worth mentioning that female lizards with larger reproductive investment (larger clutch size corrected for female body size) had lower cellular levels of ROS than females with relatively smaller investment (Olsson et al., 2009). The novelty of our approach lies in using voles with genetically differing metabolic rates. However, this did not result in higher oxidative stress in voles from lines selected for high aerobic metabolic rates.

The assumption of our hypothesis was that lactation significantly raises metabolic rates above the non-reproductive levels in bank vole females. Significantly elevated resting metabolic rate at the thermoneutral zone (RMR_t) has been reported in a variety of small mammalian species during lactation (Cretegny and Genoud, 2006; and references therein), with the highest rise in the absolute RMR_t values by 123 and 151%, and mass-specific values by 101 and 61% in two murids, *Akodon azarae* and the laboratory mouse, respectively (Speakman and McQueenie, 1996; Antinuchi and Busch, 2001). Bank voles in our breeding colony had peak lactation RMR_t 49% higher than in non-breeding controls, and 30% higher when corrected for body mass differences in the ANCOVA model (P<0.001; A. Ryś and P.K., unpublished). In contrast with our voles, Trebaticka et al. (Trebaticka et al., 2007) found that RMR_t in bank vole females remained unchanged throughout the reproductive cycle. The authors acknowledge, however, that the method of measurement of RMR_t might underestimate energy expenditure in females while they are nursing pups. The fact that voles in our experiment increased metabolic rates during lactation may be confirmed by the significantly higher mass of liver and kidneys, corrected for body mass, in breeding *versus* non-breeding females (Table 2). In other words, these organs constituted a higher proportion of body mass in breeding females. Liver and kidneys, together with the small intestine and the heart, are metabolically very active organs that enable females to transfer ingested food to milk, and to process

Table 3. Results of the two-level nested ANOVA (see Materials and methods) for the markers of the oxidative damage of lipids and proteins in various organs of bank vole females, with number of litters and the selection group as the main factors

Oxidative damage	Organ	Number of litters	Selection group
Lipids (TBARS)	Liver	$F_{2,12}$=0.38, P=0.689	$F_{1,6}$=0.87, P=0.387
	Kidneys	$F_{2,12}$=12.21, P=**0.0013**	$F_{1,6}$=2.99, P=0.134
	Heart	$F_{2,12}$=1.40, P=0.284	$F_{1,6}$=2.17, P=0.191
	Muscle	$F_{2,12}$=5.03, P=**0.0259**	$F_{1,6}$=1.29, P=0.299
Proteins (carbonyls)	Liver	$F_{2,12}$=1.53, P=0.256	$F_{1,6}$=0.08, P=0.791
	Kidneys	$F_{2,12}$=1.07, P=0.373	$F_{1,6}$=0.00, P=0.991
	Heart	$F_{2,12}$=3.05, P=0.085	$F_{1,6}$=2.38, P=0.174

P-values of statistically significant effects are shown in bold. The interaction between the two factors was non-significant in all cases.

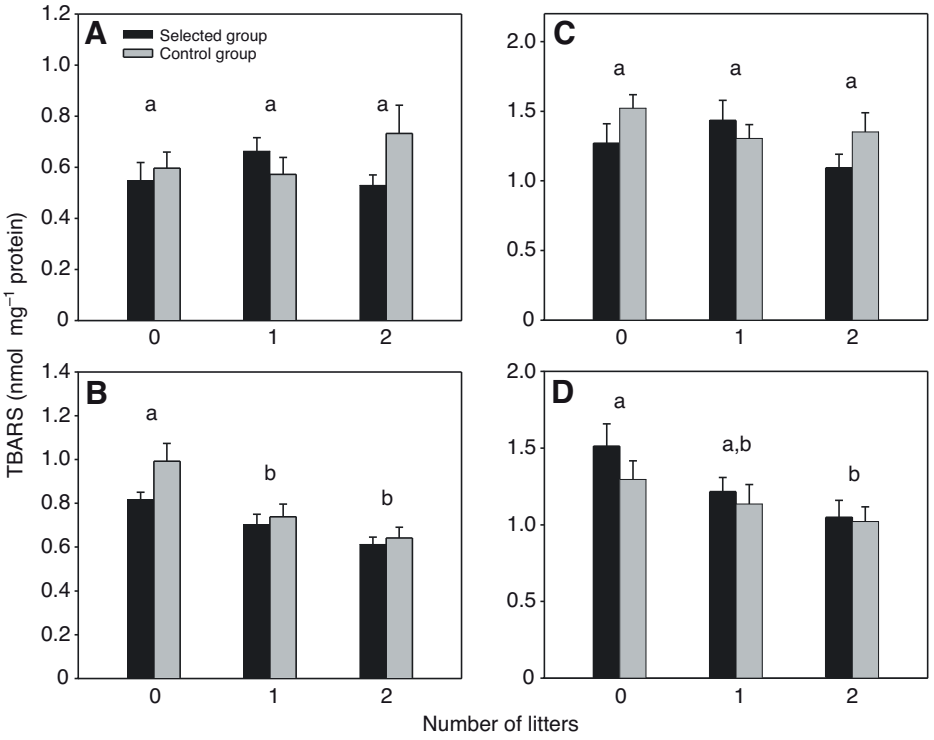

Fig. 2. Mean (+1 s.e.m.) concentrations of thiobarbituric-acid-reactive substances (TBARS; markers of oxidative damage of lipids) in internal organs and in the skeletal muscle of non-reproducing female bank voles and female bank voles allowed to wean one and two litters. (A) Liver; (B) kidneys; (C) heart; (D) muscles. Different letters above the bars show significant differences between females with 0, 1 and 2 litters, as revealed by multiple contrasts after ANOVA (P<0.05).

metabolic waste (Speakman and McQueenie, 1996). Consequently, larger mass of these organs, combined with the high energy cost of their maintenance, is reflected in higher RMR_t (Konarzewski and Diamond, 1995; Speakman and McQueenie, 1996; Książek et al., 2004). Similarly, significantly higher mass of kidneys and marginally larger hearts in voles from selected lines than in control animals might indicate their higher metabolic rates. The concurrent lactation and pregnancy in our voles when nursing the first litter might additionally increase their metabolic expenditure (Oswald and McClure, 1987; Oswald and McClure, 1990) (but see Johnson et al., 2001). Therefore, it is unlikely that the lack of increased oxidative damage in breeding voles was caused by an insufficient increase in metabolic rates in comparison with non-breeding animals.

What might be the mechanism of avoiding oxidative damage to tissues in reproducing females? The simplest explanation is that increased energy expenditure and ROS production induced the animal to increase its antioxidant levels, making it better able to cope with oxidative levels (Monaghan et al., 2009). In the study of wild house mice, the lower oxidative damage to liver tissue in females after a long period of reproductive investment, as compared with non-reproducing females, was correlated with an increased level of glutathione (Garratt et al., 2011), probably the most active antioxidant in biological systems (Halliwell and Gutteridge, 1999). However, the analogically lower damage to gastrocnemius muscle in reproducing mice had to be produced by factors other than glutathione, as no increase of this antioxidant was observed in this tissue (Garratt et al., 2011).

The protective role of sex hormones, especially estradiol, might also explain lack of the oxidative stress in our reproducing voles. As in other mammals, the concentration of estradiol in the peripheral plasma in rodents reaches high levels during oestrus and late pregnancy, whereas in females that have not been exposed to males, as in our non-breeding group, the concentration remains at levels difficult to detect (McCormack and Greenwald, 1974; Cushing et al., 1995). It has been shown that ovariectomized rats produce significantly more peroxide than control females, and this effect disappears after estrogen supplementation (Borras et al., 2003). Estradiol supplementation to ovariectomized females has been shown to significantly increase the activity of glutathione peroxidase and the concentration of glutathione (Borras et al., 2003; Kireev et al., 2007). Most importantly, suppression of the ROS generating system by estrogens has been shown to result in lower lipid peroxidation in various tissues in rats (Huh et al., 1994; Persky et al., 2000). In our experiment, all females were pregnant during the first lactation but not during the second (see Materials and methods). Females with a single litter or with two litters were killed at different stages of their breeding cycle, i.e. soon after parturition of the second litter and during the weaning of the second litter, respectively. Because estradiol concentration reaches the highest values in late pregnancy and at parturition, it is possible (assuming the important role of estradiol in protecting female tissues) that the damage in females with two litters would have been even lower if they had been killed when pregnant or soon after parturition of the third litter.

Besides sex hormones, uncoupling proteins (UCPs), mitochondrial transporters of the inner membrane, play an important role in determining ROS production. UCP2 and UCP3 catalyze proton leak, which decreases ROS emission from mitochondria (Mailloux and Harper, 2011). However, it has been shown that UCP3 is downregulated and UCP3 gene expression in muscle is decreased during lactation, whereas UCP2 remains unchanged [Speakman (Speakman, 2008) and citations therein]. Uncoupling cannot, therefore, explain the lack of oxidative stress in lactating voles. It would be very interesting, however, to test a hypothesis that the damaged lipids and proteins are broken down and excreted at a faster rate during a demanding time such as during lactation (the oxidative damage would increase without assays detecting it).

It is still possible that oxidative stress in reproducing bank voles may appear in a more natural, ecologically relevant situation. Our female voles did not have to work for food or defend pups, and they nursed not more than two consecutive litters. In natural

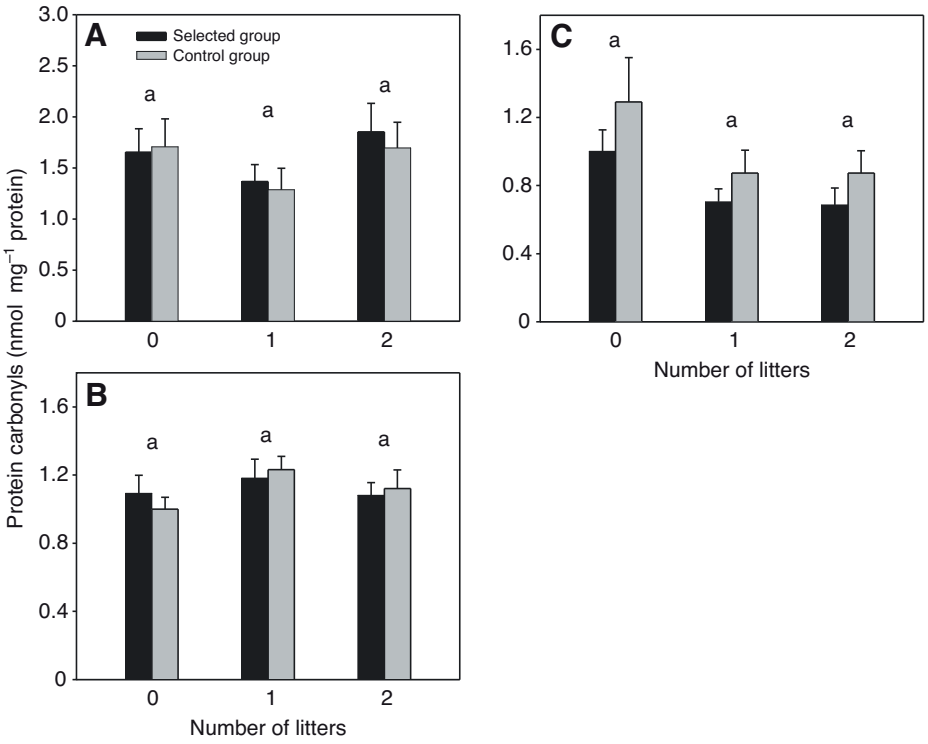

Fig. 3. Mean (+1 s.e.m.) concentrations of protein carbonyls (markers of oxidative damage of proteins) in internal organs of non-reproducing female bank voles and female bank voles allowed to wean one and two litters. (A) Liver; (B) kidneys; (C) heart. Other explanations as in Fig. 2.

situations, bank vole females give birth to up to four litters during the breeding season (Koivula et al., 2003). In the natural environment, in free-ranging bank vole females, the litter enlargement experiment affected both fecundity of mothers in subsequent breeding attempts and their survival [although the proximate mechanism of these costs was not known (Koivula et al., 2003)]. No such reproductive costs were detected in similar experiments on bank vole females conducted in the laboratory or in seminatural enclosures (Mappes et al., 1995; Koskela, 1998; Koskela et al., 2009). Oxidative damage has been reported very recently in a free-ranging rodent, the eastern chipmunk, in which peroxidation of lipids increased with litter size, along with increasing daily energy expenditure; however, the increase of the oxidative damage was very weak (Bergeron et al., 2011).

Our study adds to a very mixed picture of the relationship between reproductive effort and female oxidative status in wild mammalian species, ranging from better oxidative status in some tissues of breeding females (Garratt et al., 2011) (present study) to no detected impact of reproduction [as in wild Soay sheep (Nussey et al., 2009)] to weak oxidative stress [in eastern chipmunk (Bergeron et al., 2011)]. It has to be mentioned here that there is accumulating evidence against a model assuming a simple link between increased energy expenditure and reduced survival mediated by free-radical damage (Speakman et al., 2004; Speakman and Selman, 2011). The lack of such a simple link might also explain why we did not find support for our second hypothesis, that bank vole females with higher genetically determined energy demands would suffer higher oxidative damage. Oxidative damage to tissues did not differ between females from the group selected for high metabolic rates and females from the non-selected control group. However, the upregulation of the antioxidative defence in females from the selected group would be the simplest explanation of this finding.

In summary, our results do not support the hypothesis that oxidative damage to tissues is the proximate mechanism of reproduction costs acting through elevated energy expenditure during reproduction.

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