

## RESEARCH ARTICLE

# Reproduction is not costly in terms of oxidative stress

Łukasz Oldakowski<sup>1</sup>, Aleksandra Wasiluk<sup>1</sup>, Edyta T. Sadowska<sup>2</sup>, Paweł Koteja<sup>2</sup> and Jan R. E. Taylor<sup>1,\*</sup>

## ABSTRACT

One of the core assumptions of life-history theory is the negative trade-off between current and future reproduction. Investment in current reproduction is expected to decrease future reproductive success or survival, but the physiological mechanisms underlying these costs are still obscure. To test for a role of oxidative stress, we measured oxidative damage to lipids and proteins in liver, heart, kidneys and muscles, as well as the level of antioxidants (total glutathione and catalase), in breeding and non-breeding bank voles. We used females from lines selected for high aerobic metabolism and non-selected control lines and manipulated their reproductive investment by decreasing or increasing litter size. Unlike in most previous studies, the females reared four consecutive litters (the maximum possible during a breeding season). Contrary to predictions, oxidative damage in reproducing females was decreased or not changed, and did not differ between the selected and control lines. Oxidative damage to lipids and proteins in the liver was lower in females that weaned enlarged litters than in non-breeding ones, and was intermediate in those with reduced litters. Oxidative damage to proteins in the heart also tended to be lower in breeding females than in non-breeding ones. A negative relationship between the level of oxidative damage and activity of catalase in kidneys indicated a protective action of antioxidants. In conclusion, our study falsified the hypothesis that oxidative stress is a part of the proximate physiological mechanism underlying the fundamental life-history trade-off between current and future reproduction.

**KEY WORDS:** Cost of reproduction, Manipulation of litter size, Oxidative stress, Oxidative damage, Antioxidants, *Myodes glareolus*

## INTRODUCTION

The trade-offs in resource and time allocation that animals face throughout their lives are the fundamental concepts of evolutionary ecology and life-history theory; investing more energy or time in one process decreases the amounts available for other processes (Roff, 1992; Stearns, 1992). Such trade-offs often focus on reproduction, one of the most energetically costly processes in life. The increased energy allocation to reproduction may limit the energy invested in self-maintenance and, consequently, lead to decreased future reproduction success and survival. Although such costs of reproductive investment have been shown in numerous studies (e.g. Clutton-Brock et al., 1982; Dijkstra et al., 1990; Gustafsson and Pärt, 1990; Koivula et al., 2003; Boonekamp et al., 2014), their physiological basis remains obscure. It has been reported, however, that increased reproductive effort is associated with reduced immunocompetence (Nordling et al., 1998; Norris and

Evans, 2000), hyperthermia and bone (calcium) loss, as well as disruption of sleep patterns (Speakman, 2008).

During the past decade, evolutionary ecologists have debated whether oxidative stress is a potential proximate cost of reproduction (Monaghan et al., 2009; Stier et al., 2012; Metcalfe and Monaghan, 2013; Speakman and Garratt, 2014). Oxidative stress is an imbalance between the production of reactive oxygen species (ROS) and their neutralization by enzymatic and non-enzymatic antioxidants (Monaghan et al., 2009; Metcalfe and Alonso-Alvarez, 2010). ROS are by-products of normal metabolism. The overproduction of ROS often has detrimental effects on animals, leading to oxidative damage to proteins, lipids and DNA (Finkel and Holbrook, 2000; Van Remmen and Richardson, 2001; Monaghan et al., 2009). Such a result may be expected, especially during reproduction, assuming that highly elevated metabolic rates at that time (Drent and Daan, 1980; Cretegy and Genoud, 2006) bring about proportionally high ROS production.

Despite the growing number of studies of oxidative stress during reproduction, empirical support for the hypothesis that oxidative damage to tissues underpins life-history trade-offs is weak (Stier et al., 2012; Metcalfe and Monaghan, 2013; Speakman and Garratt, 2014). Some studies have shown a positive relationship between reproduction and oxidative damage, whereas numerous others have concluded that reproduction causes no increase, or even causes a decrease, in parental levels of oxidative damage. For example, in our previous study (Oldakowski et al., 2012), we investigated the oxidative stress in breeding female bank voles, *Myodes glareolus* (Schreber 1780). This species is a good model for examining proximate physiological costs of reproduction, since litter enlargements have been shown to reduce the survival and fecundity of females in the field (Koivula et al., 2003). We used both bank voles from lines selected for high aerobic metabolic rates (characterized by high energy requirements) and unselected control voles (Sadowska et al., 2008, 2015; Konczal et al., 2015). Contrary to our predictions, females that weaned one or two litters exhibited lower oxidative damage to lipids in kidneys and in skeletal muscles than did non-breeding females, and the damage did not differ between the selected and control lines. We suggested that elevated production of antioxidant enzymes and the protective role of oestrogens (Huh et al., 1994; Persky et al., 2000) might explain these results (Oldakowski et al., 2012).

Metcalfe and Monaghan (2013) argued that the inconsistency in results among studies investigating the link between oxidative stress (OS) and reproduction could be due to flaws in experimental design. In these studies, food has often been supplied *ad libitum* to the reproducing animals, and they might simply have increased their food intake so as to allocate sufficient resources simultaneously to both repair mechanisms, such as antioxidant defences and reproduction. A prerequisite to examine the link between OS and reproduction is to manipulate reproductive effort by increasing or decreasing numbers of offspring in the litter or clutch. When animals can freely reproduce, they may vary the number of offspring

<sup>1</sup>Department of Animal Ecology, Institute of Biology, University of Białystok, Ciołkowskiego 1 J, Białystok 15-245, Poland. <sup>2</sup>Institute of Environmental Sciences, Jagiellonian University, ul. Gronostajowa 7, Kraków 30-387, Poland.

\*Author for correspondence (taylor@uwb.edu.pl)

produced depending on their body condition, thereby potentially avoiding OS. Reproductive costs may therefore only be evident when animals are induced to increase their reproductive effort above that intended, e.g. by enlarging their litter/clutch size or making them work hard to get food (Metcalf and Monaghan, 2013). We are aware of four studies that aimed to measure the oxidative costs of reproduction in small mammals by manipulation of litter size (Garratt et al., 2013; Aloise King et al., 2013; Plumel et al., 2014; Xu et al., 2014). Interestingly, in only one of these experiments (and in only one reported tissue, the blood; Plumel et al., 2014) was there any increase in oxidative damage in females with greater reproductive load, and in some tissues the damage in breeding females was even lower than in control non-breeding females.

However, in all the above experiments, the manipulation of reproductive effort was limited to a single reproductive attempt (Garratt et al., 2013; Plumel et al., 2014 and Xu et al., 2014) or two litters (Aloise King et al., 2013). Garratt et al. (2013) did not find signs of oxidative stress in house mice with enlarged first litters, but suggested that they might occur during or after later reproductive attempts. Speakman and Garratt (2014) drew attention to the fact that young females have a high future reproductive potential, and it may be more important for them than for older females to invest in protection against oxidative stress to increase their chances of survival for later successful breeding. This could potentially explain the observed lower oxidative damage in primiparous females than in virgin females in these experiments (and also in Oldakowski et al., 2012).

Here, we present the results of a further experiment on the same animal model, namely bank voles from the selection experiment (Sadowska et al., 2008, 2015; Konczal et al., 2015), designed to solve both the above-mentioned methodological problems. First, as recommended by Metcalfe and Monaghan (2013), by reducing or enlarging the original litter, we manipulated female reproductive investment. Non-breeding females served as a reference. Second, to examine the effects of several breeding attempts on OS, the females weaned four consecutive litters. The females were housed with males until the birth of the fourth litter, and, consequently, during the three first lactations, they became pregnant during the short postpartum oestrus. This concentrated the energy-demanding periods of consecutive lactations in time, as would occur in the wild. Four litters represent the maximum number during a breeding season in wild bank voles, and presumably also nearly the maximum lifetime output for an individual vole (Bujalska, 1983).

We investigated oxidative damage to lipids and proteins in different tissues and determined the concentration of two antioxidant compounds that are important for protection against oxidative stress (total glutathione and activity of catalase) in female bank voles from two generations of the selection experiment. If the 'oxidative stress' hypothesis holds true, then increased oxidative damage and/or reduced antioxidant defences should be observed in females with enlarged litters. Conversely, females with reduced litters, which maintain reproductive status but are not challenged by high costs of reproduction, should have high antioxidant defences

and low and stable levels of oxidative damage. Specifically, we tested three hypotheses: (1) females with low breeding effort (with reduced litters) should suffer lower oxidative damage to tissues than non-breeding ones (as in our previous study; Oldakowski et al., 2012); (2) females with high breeding effort (with enlarged litters) should have higher oxidative damage to tissues than non-breeding females; and (3) females from selected lines (characterized by higher basal and routine metabolic rates; see Materials and methods) should have higher oxidative damage than those from control lines (both in breeding and non-breeding groups).

## MATERIALS AND METHODS

### Study animals

In the experiment, we used bank voles (*Myodes glareolus*) that came from generations 12 and 14 of an artificial selection experiment conducted at the Institute of Environmental Sciences, Jagiellonian University, Kraków (Sadowska et al., 2008, 2015; Konczal et al., 2015). We used females from four lines selected for 1 min maximum rate of oxygen consumption during swimming in water at 38°C (i.e. measured without the cost of thermoregulation) and four non-selected (control) lines. Females from the selected lines had 45% higher mass-corrected maximum metabolic rates than control females (generation 13; Konczal et al., 2015), 10% higher mass-corrected basal metabolic rates (generation 11; Sadowska et al., 2015) and about 20% higher mass-corrected food consumption measured at 20°C (generations 11 and 18; G. Dheyongera and K. Grzebyk, unpublished results). In the main colony, the average litter size at weaning in generations 12 and 14 (used in this study) was 5.3 and 4.8 in the selected and control lines, respectively (values averaged from subsequent litters, typically litters 1–3, and the two generations; P.K., unpublished results).

For this experimental purpose, adult voles (from generation 11 and 13) from the main colony were transferred to animal facilities of the Institute of Biology, University of Białystok. As in the main colony, the voles were maintained at a constant temperature (20±1°C) and photoperiod (16 h light:8 h dark), with unlimited access to food (Labofeed H, Morawski, Kcynia, Poland) and water. The pairs were allowed to reproduce and their offspring were used as a basis of this experiment. Non-breeding experimental females were kept in groups of 2–3 individuals in standard plastic mouse cages (27×21×14 cm) with sawdust bedding. Breeding pairs were kept in larger cages (43×27×16 cm) with bedding, plastic houses and paper towel for nesting, in the same room as non-breeding animals. All the experimental procedures were approved by the local ethical committee in Białystok (permission number 49/2010).

### Experimental design and tissue sampling

The voles were virgin at the beginning of the experiment and were about 3.5 months old. We used 89 females from the selected group and 83 from the control group (92 from generation 12 and 80 from generation 14; Table 1). In each generation, females from each of the four replicate lines in the two selection groups were randomly assigned to three reproductive treatments: (1) non-breeding, (2) breeding with reduced litters (two pups in each consecutive litter) or (3) breeding with enlarged litters (two extra pups added to original litter size in each consecutive litter) (Table 1). In both reproductive groups, manipulations of the number of pups were made during the first 2 days after parturition. Female bank voles readily accept foreign pups if they are of similar age and size to their own (Mappes et al., 1995). On the 17th day after parturition, the number of weaned pups was

**Table 1. Numbers of bank vole females used in various groups of the experiment**

Generation no. (year)	Selected			Control			Total
	Non-breeding	Reduced litters	Enlarged litters	Non-breeding	Reduced litters	Enlarged litters	
12 (2012)	15	18	18	15	15	11	92
14 (2013)	13	13	12	15	14	13	80
Total	28	31	30	30	29	24	172

Numbers in selection groups (selected and control), in reproductive treatment groups (non-breeding females, females with reduced and enlarged litters) and in two generations of animals.

recorded; the litters were weighed and separated from parents. The pair of parental voles was then placed in a clean cage where it awaited the next parturition. After the birth of the fourth litter, the male was removed from each breeding cage.

After weaning this last litter, females were killed by cervical dislocation. Non-breeding females were killed at the same time as females from groups with enlarged and reduced litters. All females were weighed to the nearest 0.1 g and liver, heart (drained of blood), both kidneys and skeletal muscles associated with the femur were dissected, weighed ( $\pm 0.001$  g) and immediately snap-frozen in liquid nitrogen. The whole intestine (small intestine, caecum and large intestine) was dissected, cleaned, dried and weighed (to 0.001 g). The mass of these metabolically active organs was used as a representation of the animals' energy expenditure (Konarzewski and Diamond, 1995; Książek et al., 2004). Frozen samples were kept at  $-80^{\circ}\text{C}$  awaiting analyses of oxidative damage and antioxidant levels.

### Analyses of oxidative damage and antioxidants

Tissues were homogenized with a CAT X120 homogenizer in cold  $50\text{ mmol l}^{-1}$  phosphate buffer with  $1\text{ mmol l}^{-1}$  EDTA and pH 7.0. Supernatant was obtained by centrifugation at  $10,000\text{ g}$  for 15 min at  $4^{\circ}\text{C}$  and kept on ice to determine oxidative damage. We measured the level of malondialdehyde (MDA) formation to quantify the lipid peroxidation in tissues (Ohkawa et al., 1979). Briefly, tissue supernatant, 20% acetic acid and 0.5% thiobarbituric acid (TBA) were mixed and incubated for 1 h in  $95^{\circ}\text{C}$  water bath. Next, samples were extracted with butane-pyridine mixture v/v 15:1 and centrifuged. Absorption was recorded at 532 nm and 600 nm with a Beckman Coulter Du 730 spectrophotometer and the difference between the two absorption values was used for further calculations (Jentzsch et al., 1996). To prepare the calibration curve, tetraethoxypropane was used. Protein content in each sample was determined by the Lowry method with Peterson modification (Lowry et al., 1951; Peterson, 1977) and was expressed as TBARS (thiobarbituric-acid-reactive substances) in  $\text{nmol mg}^{-1}$  protein.

The level of protein damage was determined by the reaction of 2,4-dinitrophenylhydrazine (DNPH) with protein carbonyls (Stadtman and Oliver, 1991; Levine et al., 1994). Briefly, the supernatant and 10 mmol DNPH in  $2.5\text{ mol l}^{-1}$  HCl were added to the sample tube and  $2.5\text{ mol l}^{-1}$  HCl to the control tube. After incubation for 1 h at room temperature, 50% trichloroacetic acid (TCA) was added to each tube, which was then vortexed and cooled at  $-20^{\circ}\text{C}$  for the next 20 min. After the incubation, tubes were centrifuged, the supernatant was discarded and the pellet washed three times with a 1:1 ethanol:ethyl acetate mixture. Next, the pellet was dissolved in  $6\text{ mol l}^{-1}$  guanidine hydrochloride and the absorbance of sample and control tubes were measured at 370 nm. The level of protein carbonyls was calculated from the difference between sample and control tube absorbance. The protein content was determined in control tubes by measuring absorbance at 280 nm, and final protein carbonyl concentration was expressed in  $\text{nmol mg}^{-1}$  protein.

Catalase activity in liver and kidneys was determined according to Aebi's (1986) method and was expressed as the rate constant of a first-order reaction ( $k$ ) per mg protein. The total glutathione concentration in liver was measured according to Anderson (1996) along with modification for use with a 96-well plate reader (Vasilaki et al., 2006) and expressed in nmol per mg protein. In both analyses, protein content was measured with the Lowry method with Peterson modification (Lowry et al., 1951; Peterson, 1977).

Assays were carried out in duplicate. Repeatability (intra-class correlation coefficient; Lessells and Boag, 1987) of the assays of oxidative damage and antioxidants was  $r_1 > 0.96$  and of protein concentration assays was  $r_1 > 0.84$ .

### Statistical analyses

The main ANOVA model – used for testing the differences in number and mass of offspring, time periods between parturitions, mass of females and their internal organs, and concentrations of markers of oxidative damage and antioxidants – included reproductive treatment group (non-breeding, reduced litters and enlarged litters), selection group (selected versus control) and generation (12, 14) as fixed factors and replicate lines nested in selection group as a random factor. All appropriate interactions between factors were

also included in initial models. Non-significant interactions were then sequentially removed, except for the reproductive treatment  $\times$  selection interaction, which was considered a fundamental part of the model and was therefore retained irrespective of its significance.

In order to meet parametric assumptions, the comparisons of the number and mass of offspring across treatment groups, selection groups and generations were performed after square-root transformation. The time intervals between consecutive parturitions of females were log-transformed. In comparisons of the mass of liver, kidneys, heart and intestine, the total body mass was added to the main model as a covariate and all masses of these organs, as well as the covariate, were log-transformed. Log-transformation was applied to the concentration of TBARS prior to the analysis of the concentration of markers of oxidative damage in liver, kidneys, heart and muscles, as well as to the concentration of carbonyls in liver. The same log-transformation of TBARS and carbonyls was applied when testing their correlation with the number or mass of offspring (the latter variables were used as covariates and were not transformed; these models did not include the reproductive treatment group). The levels of antioxidants, glutathione and catalase were log-transformed in all analyses. The relationship between the concentration of log-transformed TBARS and carbonyls and the levels of antioxidants were tested by including the concentration of glutathione or activity of catalase as a covariate in the main model.

The assumption of homogeneity of slopes was always checked prior to performing ANCOVA with a common slope. Tukey–Kramer adjustment was applied for pair-wise *a posteriori* comparisons between groups of factors with more than two levels. All statistical analyses were carried in SAS v9.3 (SAS Institute, Cary, NC, USA) using the MIXED procedure with the restricted maximum-likelihood method (REML).

## RESULTS

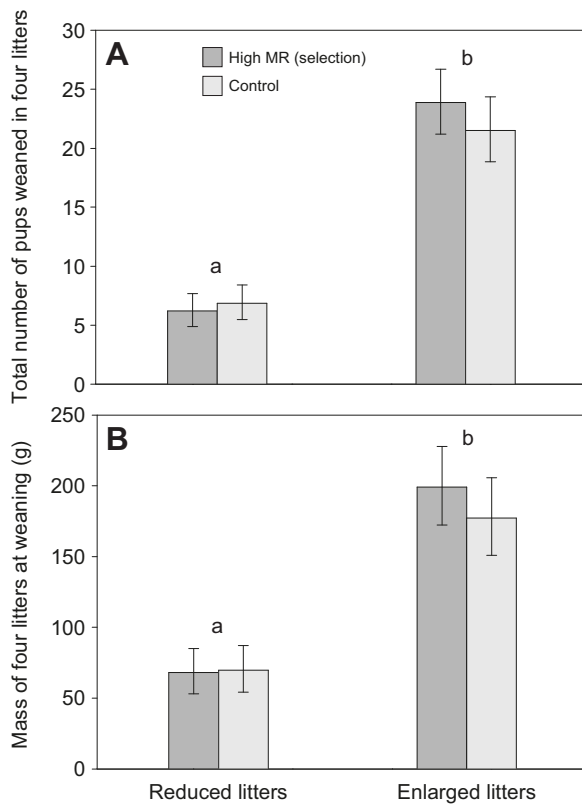
### Litter size and mass

All females gave birth to four consecutive litters. Females from the enlarged litter group weaned 3.5 times more pups than females from reduced litter group, with no significant difference between the two selection groups (voles from the selected and control lines) or between the two generations (ANOVA; reproductive treatment:  $F_{1,6}=765.2$ ,  $P<0.0001$ ; selection group:  $F_{1,6}=0.17$ ,  $P=0.698$ ; generation:  $F_{1,7}=1.02$ ,  $P=0.347$ ; reproductive treatment  $\times$  selection interaction:  $F_{1,6}=5.57$ ,  $P=0.056$ ) (Fig. 1A). Selected and control females with enlarged litters weaned on average 0.89 (95% CI, 0.53–1.25) and 0.93 (0.56–1.31) more pups, respectively, than recorded in their litters before manipulation. The average litter size at weaning was 6.26 (5.88–6.64) and 5.86 (5.36–6.36) in these females, respectively. The average litter size at weaning in females with reduced litters was 1.63 (1.46–1.80) and 1.70 (1.55–1.85) in selected and control females, respectively.

The total mass of four weaned litters was 2.7 times higher in the enlarged litter group than in the reduced litter group ( $F_{1,6}=402.6$ ,  $P<0.0001$ ) and larger in generation 14 than in generation 12 ( $F_{1,7}=8.91$ ,  $P=0.020$ ), but it did not differ significantly between the selected and control lines ( $F_{1,6}=0.48$ ,  $P=0.516$ ). The interaction between the reproductive treatment and selection was also non-significant ( $F_{1,6}=2.79$ ,  $P=0.146$ ) (Fig. 1B).

The average time between birth of two consecutive litters was longer in females with enlarged litters than with reduced litters; the median was 24.4 and 20.7 days, respectively (reproductive treatment:  $F_{1,6}=6.49$ ,  $P=0.044$ ; selection:  $F_{1,6}=0.40$ ,  $P=0.551$ ; generation:  $F_{1,7}=0.01$ ,  $P=0.943$ ; reproductive treatment  $\times$  selection:  $F_{1,6}=0.14$ ,  $P=0.719$ ). These intervals indicate that 84% of the 2nd to 4th litters were conceived in postpartum oestrus so that females were pregnant during the first to the third lactation (83.3% and 84.4% in females with enlarged and reduced litters, respectively; assuming an interval between birth of two consecutive litters of less than 28 days; Steven, 1957; Gustafsson et al., 1980).





**Fig. 1. Total number of pups and total mass of four consecutive litters weaned by bank vole females with experimentally reduced and enlarged litters.** (A) Total number of pups. (B) Total mass of all four litters at weaning. Adjusted means from ANOVA models ( $\pm 95\%$  confidence limits) (see text for a description of the model). Animals were from lines selected for high metabolic rate (MR) or from control lines. Different letters above bars show significant differences between two reproductive treatment groups.

### Body mass and mass of metabolically active organs in females

The body mass of females differed between reproductive treatment groups (ANOVA;  $F_{2,12}=50.68$ ,  $P<0.0001$ ); it was lower in the non-breeding group than in either the enlarged or reduced litter groups (Tukey–Kramer test;  $P<0.0001$ ), but did not differ between the two breeding groups (Tukey–Kramer test;  $P=0.593$ ; Table 2). Body mass in the selected lines was higher than in the control lines ( $F_{1,6}=9.07$ ,  $P=0.024$ ) and higher in generation 14 than in generation 12 ( $F_{1,7}=24.59$ ,  $P=0.0016$ ).

The mass of energetically demanding organs (liver, kidneys, heart and intestine) increased with total body mass, but in the case of the liver and heart, there was also a significant interaction between body mass and reproductive treatment (ANCOVA with

body mass as a covariate; Table 3). The interaction resulted from the relationship having a higher regression slope in the non-reproducing group [liver:  $b=1.292\pm 0.092$ ; heart:  $b=0.867\pm 0.083$  (means $\pm$ s.e.m.)] than in the two reproducing groups (which were not different; liver:  $b=0.759\pm 0.128$ ; heart:  $b=0.351\pm 0.070$ ). Despite this interaction, in order to determine a general pattern we calculated ANCOVA models with a common slope for all the organs and all three reproductive treatments (i.e. without the interaction term). Mass-adjusted values of all the four organ masses were larger in the selected than control groups, but the difference was marginally non-significant for liver and intestine (Table 3). The adjusted mass of the liver, kidneys and intestine, but not of the heart, was larger in females from the enlarged litter group than in the two remaining reproductive treatment groups. However, the comparison of reproductive groups was unreliable for the liver and heart because the assumption of homogeneity of slopes was not met. Therefore, we performed similar ANCOVA analysis for these two organs in just the two groups of breeding females (for which the homogeneity of slopes assumption was met). The analysis confirmed that the adjusted mass of the liver but not of the heart was larger in the enlarged litter group than in the reduced litter group (Table 3).

### Oxidative damage to tissues

The concentration of TBARS, which are markers of oxidative damage to lipids, only differed significantly between reproductive treatment groups for the liver (Table 4, Fig. 2). Contrary to predictions, the highest concentration of TBARS in the liver was found in non-breeding females, while females with enlarged litters had the lowest concentration (Fig. 2A). The same pattern was found for the concentration of protein carbonyls (markers of the damage to proteins); the concentration in the liver was significantly higher in non-breeding females than in those with enlarged litters. The effect of reproductive treatment was also significant for carbonyl concentration in the heart, but there was a significant interaction between the reproductive treatment and generation (Table 4). In ANOVA models that examined patterns in each of the two generations, the difference between reproductive treatment groups was significant in generation 12 (Fig. 3C) ( $F_{2,12}=15.52$ ,  $P=0.0005$ ; selection:  $F_{1,6}=1.36$ ,  $P=0.287$ ) but not in generation 14 ( $F_{2,12}=0.30$ ,  $P=0.746$ ; selection:  $F_{1,6}=0.37$ ,  $P=0.565$ ).

Females selected for high metabolic rates did not differ from control females either in TBARS or in carbonyl levels in any of the investigated tissues (Table 4, Figs 2 and 3). However, the two generations of females differed in the level of oxidative damage to lipids and proteins in almost all investigated tissues. In all the cases of significant difference, the damage was larger in the 12th generation than in the 14th generation, except for the damage to lipids in the heart, where the inverse situation was found.

Breeding females varied greatly in the total number of pups weaned in four litters (ranging from 3 to 34 per female) and the total mass of all weaned pups (from 28 to 282 g). Therefore, we also tested whether these measures of reproductive effort were predictors of the markers of oxidative damage, TBARS or protein carbonyls, in the investigated tissues. However, no significant relationships were found (Table 5; ANCOVAs with either number of pups or offspring mass as an additional covariate). The relationship that was the closest to attaining significance, between TBARS in the heart and the total mass of weaned pups ( $P=0.075$ ; Table 5), had a negative slope ( $-0.19\times 10^{-3}$  [ $-0.39\times 10^{-3}$ ,  $0.002\times 10^{-3}$ ]).

**Table 2. Body mass (grams) of bank vole females from lines selected for high maximum metabolic rates and control lines in three reproductive treatment groups**

Selection group	Reproductive treatment group		
	Non-breeding	Reduced litters	Enlarged litters
Selection	24.3 (22.0–26.5) <sup>a</sup>	33.2 (30.9–35.4) <sup>b</sup>	33.9 (31.7–36.2) <sup>b</sup>
Control	22.0 (19.7–24.2) <sup>a</sup>	30.2 (27.9–32.4) <sup>b</sup>	31.5 (29.1–33.9) <sup>b</sup>

Adjusted means derived from ANOVA models (see text). The 95% confidence intervals are shown in the parentheses. Different letters (a, b) show significant differences between body masses of females in three groups (Tukey–Kramer pair-wise comparisons;  $P<0.0001$ ).

**Table 3. Mass of internal organs of bank vole females**

A		Reproductive treatment group		
Organ*	Selection group	Non-breeding	Reduced litters	Enlarged litters
Liver	Selected	1.52 (1.42–1.64) <sup>a</sup>	1.57 (1.46–1.69) <sup>a</sup>	1.75 (1.63–1.88) <sup>b</sup>
	Control	1.41 (1.30–1.52) <sup>a</sup>	1.46 (1.36–1.56) <sup>a</sup>	1.66 (1.55–1.79) <sup>b</sup>
Kidneys	Selected	0.360 (0.339–0.382) <sup>a</sup>	0.373 (0.352–0.395) <sup>a</sup>	0.381 (0.359–0.404) <sup>b</sup>
	Control	0.323 (0.303–0.345) <sup>a</sup>	0.329 (0.311–0.348) <sup>a</sup>	0.376 (0.354–0.399) <sup>b</sup>
Heart	Selected	0.166 (0.156–0.178) <sup>a</sup>	0.171 (0.160–0.182) <sup>a</sup>	0.167 (0.157–0.179) <sup>a</sup>
	Control	0.141 (0.131–0.151) <sup>a</sup>	0.144 (0.135–0.153) <sup>a</sup>	0.152 (0.142–0.162) <sup>a</sup>
Intestine	Selected	0.322 (0.302–0.344) <sup>a</sup>	0.354 (0.333–0.378) <sup>a</sup>	0.382 (0.358–0.408) <sup>b</sup>
	Control	0.318 (0.296–0.342) <sup>a</sup>	0.311 (0.292–0.330) <sup>a</sup>	0.374 (0.350–0.401) <sup>b</sup>

B		Reproductive treatment			Body mass
Organ	Reproductive treatment	Selection	Generation	Reproductive treatment×selection	Body mass
All three reproductive treatment groups					
Liver	$F_{2,12}=13.09$ $P=0.001$	$F_{1,6}=4.25$ $P=0.085$	$F_{1,7}=5.03$ $P=0.060$	$F_{2,12}=0.19$ $P=0.831$	$F_{1,138}=230.26$ $P<0.0001$
Kidneys	$F_{2,12}=8.18$ $P=0.006$	$F_{1,6}=9.72$ $P=0.021$	$F_{1,7}=0.00$ $P=0.962$	$F_{2,12}=3.52$ $P=0.063$	$F_{1,138}=125.0$ $P<0.0001$
Heart	$F_{2,12}=0.86$ $P=0.447$	$F_{1,6}=20.28$ $P=0.004$	$F_{1,7}=9.95$ $P=0.016$	$F_{2,12}=1.59$ $P=0.243$	$F_{1,138}=91.73$ $P<0.0001$
Intestine	$F_{2,12}=14.70$ $P=0.001$	$F_{1,6}=4.99$ $P=0.067$	$F_{1,7}=4.51$ $P=0.071$	$F_{2,12}=3.04$ $P=0.085$	$F_{1,138}=85.26$ $P<0.0001$
Females with reduced and enlarged litters only					
Liver	$F_{1,6}=21.81$ $P=0.003$	$F_{1,6}=3.70$ $P=0.103$	$F_{1,7}=2.41$ $P=0.165$	$F_{1,6}=0.38$ $P=0.559$	$F_{1,88}=82.26$ $P<0.0001$
Heart	$F_{1,6}=1.62$ $P=0.251$	$F_{1,6}=23.51$ $P=0.003$	$F_{1,7}=5.97$ $P=0.045$	$F_{1,6}=3.77$ $P=0.100$	$F_{1,88}=25.29$ $P<0.0001$

Mean adjusted mass (grams; 95% CI in parentheses) from ANCOVA with reproductive treatment, selection, selection line nested in selection group and generation as factors, and female body mass as a covariate (A) and the significance of factors and the covariate in ANCOVA model (B). Since there were significant interactions between the reproductive treatment and females' body mass in the analyses of liver and heart samples, the lower part of B shows results for these organs from females with reduced and enlarged litters only; the assumption of the homogeneity of slopes was fulfilled in these models. Different letters (a, b) in A show significant differences between reproductive treatment groups (Tukey–Kramer test;  $P<0.05$ ).  $P$  values of statistically significant effects in B are shown in bold.

\*Wet mass of liver, kidneys and heart, and dry mass of intestine.

### Antioxidant defences

We found no significant differences in catalase activity between the reproductive treatment groups, selection groups or generations, either in the liver (ANOVA;  $F_{2,12}=2.69$ ,  $P=0.108$ ;  $F_{1,6}=0.01$ ,  $P=0.938$ ;  $F_{1,7}=0.37$ ,  $P=0.562$ , respectively) or kidneys ( $F_{2,12}=2.13$ ,  $P=0.162$ ;  $F_{1,6}=0.09$ ,  $P=0.773$ ;  $F_{1,7}=1.48$ ,  $P=0.264$ ). Moreover, the level of total glutathione (GSH) in the liver did not differ between reproductive treatment and selection groups ( $F_{2,12}=1.66$ ,  $P=0.231$ ;  $F_{1,6}=0.11$ ,  $P=0.757$ , respectively). However, the difference between the two generations was highly significant ( $F_{1,7}=53.58$ ,  $P=0.0002$ ), with the GSH level higher in the generation 14 voles (in which oxidative damage to proteins and lipids in the liver was lower). All interactions were non-significant.

Next, we asked whether the oxidative damage to lipids or proteins in the liver and kidneys was related to the level of antioxidant defence. To this end, we tested ANCOVA models with the markers of oxidative damage as the dependent variables, and with either GSH concentration or catalase activity as a covariate (Table 6). Damage levels were not related to the level of the studied antioxidants in the liver, but in the kidneys, oxidative damage decreased with increasing activity of catalase (GSH was not studied in kidneys; Table 6). All the above analyses showed that the oxidative damage adjusted for the level of antioxidants was higher in voles for generation 12. The damage in the liver, when adjusted

for variation in either GSH concentration or catalase activity, was still higher in the non-breeding than in the two groups of breeding females (Table 6; Tukey–Kramer test;  $P<0.05$ ). However, damage levels in the kidneys did not differ between the reproductive treatment groups when adjusted for catalase activity (GSH was not studied) (Table 6).

### DISCUSSION

The fundamental prediction of the hypothesis relating life history to oxidative stress is that reproductive effort leads to an inevitable increase in free-radical production, which causes lasting oxidative damage that persists after the reproductive event. Previous laboratory tests of this hypothesis conducted on small mammals produced inconsistent results that might result from favourable laboratory conditions and consequently sub-maximal parental effort (Metcalf and Monaghan, 2013). Therefore, by adding an extra two pups (or leaving only two pups in a litter), we manipulated litter size in bank voles. Importantly, however, average litter size at weaning in the enlarged litters was only 0.9 pup larger than the original litter size recorded within 2 days of parturition, and only about one pup larger than in non-manipulated litters in the main colony (see Materials and methods for litter size in the main colony). Apparently, females could not or refused to nurse two additional pups, which indicates that they achieved an upper limit of their

**Table 4. Results from the ANOVA for TBARS and carbonyls, markers of oxidative damage to lipids and proteins, respectively, in internal organs**

	Reproductive treatment	Selection	Generation	Reproductive treatment×selection
<b>TBARS</b>				
Liver	$F_{2,12}=5.25$ <b><math>P=0.023</math></b>	$F_{1,6}=0.27$ $P=0.622$	$F_{1,7}=106.6$ <b><math>P&lt;0.0001</math></b>	$F_{2,12}=0.59$ $P=0.567$
Kidneys	$F_{2,12}=3.29$ $P=0.072$	$F_{1,6}=0.45$ $P=0.526$	$F_{1,7}=30.52$ <b><math>P=0.001</math></b>	$F_{2,12}=1.38$ $P=0.289$
Heart	$F_{2,12}=0.60$ $P=0.564$	$F_{1,6}=0.36$ $P=0.573$	$F_{1,7}=17.14$ <b><math>P=0.004</math></b>	$F_{2,12}=1.73$ $P=0.219$
Muscles	$F_{2,12}=0.66$ $P=0.535$	$F_{1,6}=0.08$ $P=0.785$	$F_{1,7}=2.34$ $P=0.170$	$F_{2,12}=0.53$ $P=0.600$
<b>Carbonyls</b>				
Liver	$F_{2,12}=5.09$ <b><math>P=0.025</math></b>	$F_{1,6}=1.77$ $P=0.231$	$F_{1,7}=15.90$ <b><math>P=0.005</math></b>	$F_{2,12}=1.16$ $P=0.345$
Kidneys	$F_{2,12}=0.09$ $P=0.912$	$F_{1,6}=0.24$ $P=0.643$	$F_{1,7}=69.68$ <b><math>P&lt;0.0001</math></b>	$F_{2,12}=1.12$ $P=0.357$
Heart <sup>a</sup>	$F_{2,12}=7.62$ <b><math>P=0.007</math></b>	$F_{1,6}=1.87$ $P=0.221$	$F_{1,7}=2.41$ $P=0.165$	$F_{2,12}=1.49$ $P=0.263$
Muscles	$F_{2,12}=2.48$ $P=0.126$	$F_{1,6}=0.13$ $P=0.729$	$F_{1,7}=5.36$ <b><math>P=0.005</math></b>	$F_{2,12}=0.32$ $P=0.729$

*P* values of statistically significant effects are shown in bold. See Materials and methods for the models.

<sup>a</sup>The interaction between reproductive treatment and generation was significant ( $F_{2,132}=5.59$ ,  $P=0.0047$ ), and therefore it was left in the ANOVA model.

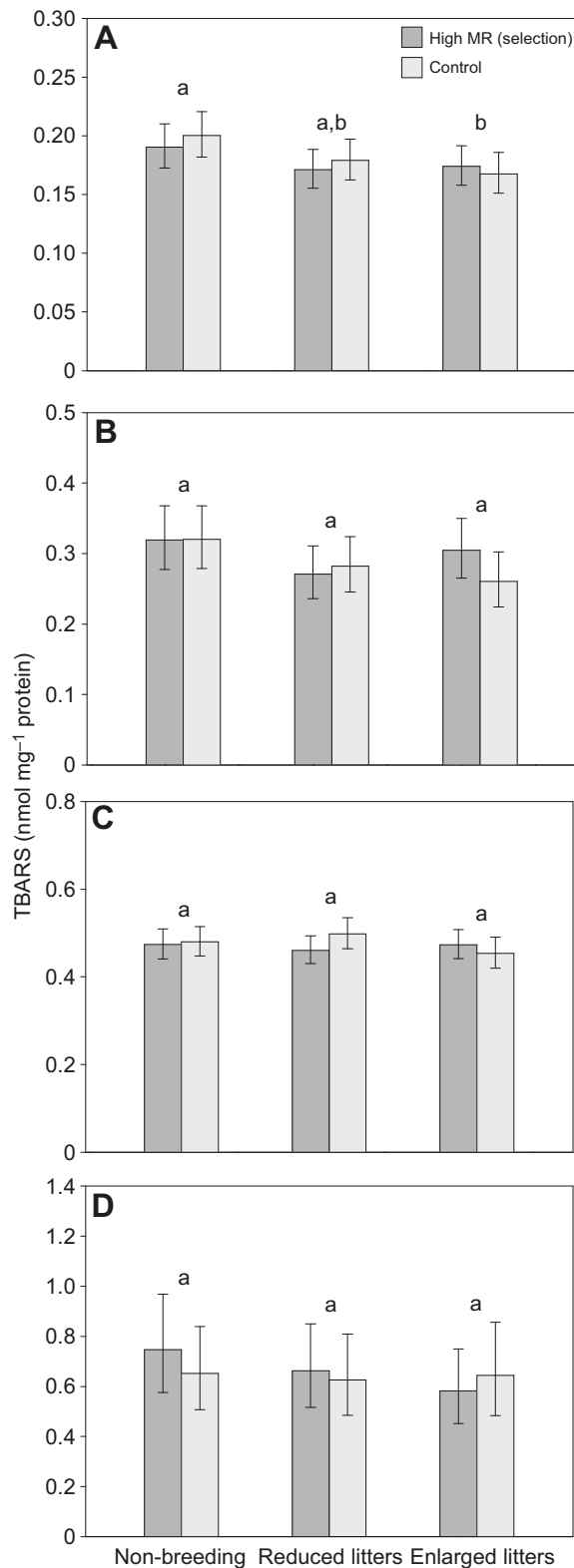
reproductive effort (set by physiological limitations or life-history optimization mechanisms).

An increased energy turnover of females with enlarged litters was indicated by an increase in the size of their liver, kidneys and intestine, which are metabolically active organs processing food and metabolic waste (cf. Konarzewski and Diamond, 1995; Książek et al., 2004). The kidney and intestine mass, adjusted for total body mass, was significantly larger in breeding females than in non-breeding females (Table 3). The comparison of liver mass was difficult because of heterogeneous regression slopes, but the mass-adjusted liver mass was clearly larger in the enlarged litter group than in the reduced litter group (Table 3). It is well documented that an increased mass of these organs is associated with an increased resting metabolic rate measured at thermoneutral temperatures (Konarzewski and Diamond, 1995; Speakman and McQueenie, 1996; Książek et al., 2004) and it indicated increased metabolic rates in our voles rearing enlarged litters.

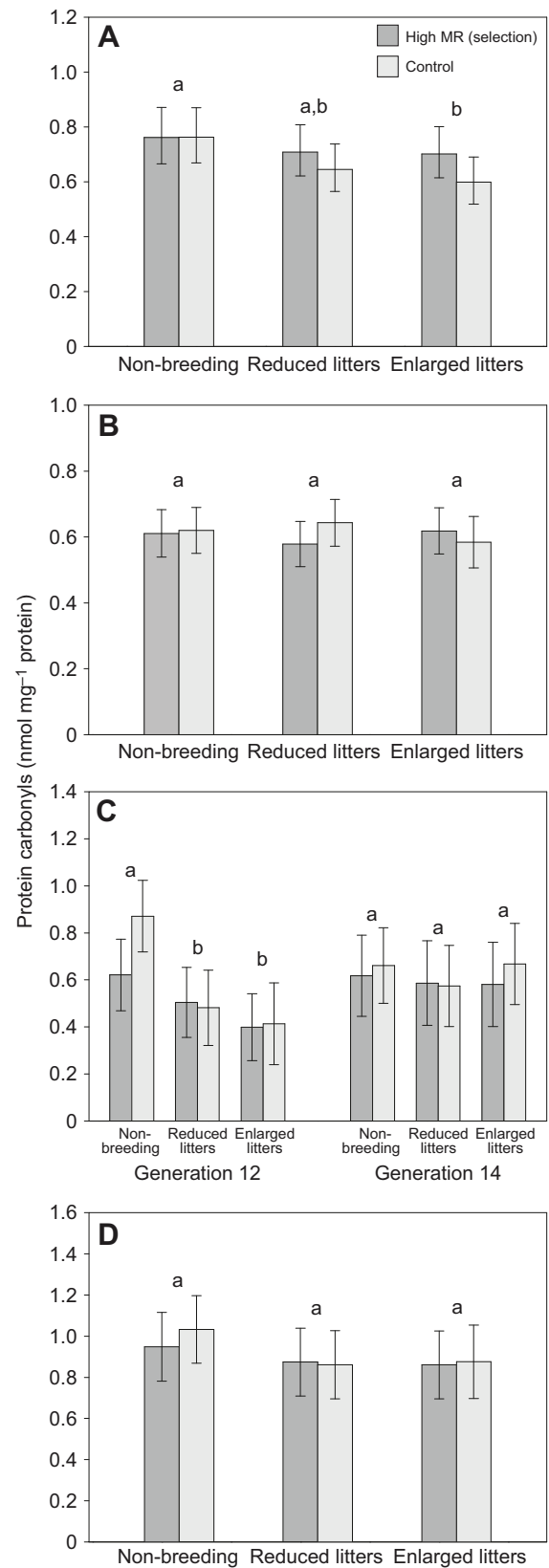
The high overall rate of energy processing in lactating females is associated with increased resting metabolic rate (RMR; Cretegy and Genoud, 2006). In our breeding colony, mass-adjusted RMR in females at peak lactation was 30% higher than in non-breeding ones (A. Rys and P.K., unpublished results). In this experiment, during the first three lactations, the value could be further increased by concurrent pregnancy (observed in the majority of females). For example, during concurrent pregnancy and lactation, RMR in Norway rats, wood rats and cotton rats was 11–26% higher than the RMR during lactation alone (Oswald and McClure, 1987, 1990), although such an increase was not observed in laboratory mice (Johnson et al., 2001). In our experiment, the proportion of concurrent pregnancies did not differ between the groups with enlarged and reduced litters, but females from the former group had longer intervals between litters. This could compensate to some degree for the increased metabolic rates associated with concurrent pregnancy. However, the observation of extended intervals between litters demonstrates that females rearing the enlarged litters had to trade-off the rate of development of the next litter for the ability to successfully nurse the current one, which is evidence that the females were working at or close to their maximum capacity to provide energy.

Females reared four consecutive litters in our experiment. Thus, females with enlarged litters were challenged up to or near their upper limit of energy turnover for a period equivalent to the entire adult life span of a wild vole. Therefore, the experiment offers an adequate model to test the hypothesis that oxidative damage to lipids and proteins appears as a cost of maintaining high levels of reproductive effort, and hence a possible biochemical mechanism underlying the fundamental life-history trade-off between current reproductive effort and future reproductive value. Contrary to the prediction of the ‘oxidative stress hypothesis’, however, the oxidative damage to lipids and proteins in the liver (in both generations studied) and the damage to proteins in the heart (in one of the generations) were significantly lower in females rearing enlarged litters than in non-breeding ones (Table 4, Figs 2 and 3). The level of damage to lipids in the heart and to lipids and proteins in the kidneys and skeletal muscles did not differ between the reproductive groups (Table 4, Figs 2 and 3), which is also inconsistent with the hypothesis. The indices of oxidative damage did not differ between voles from the lines selected for high rates of aerobic metabolism and those from unselected control lines, which provides another argument against the oxidative stress hypothesis.

The results from our experiment, which imposed both an enlarged and prolonged energy burden on the breeding females, were qualitatively similar to those of other experiments that imposed litter manipulation. In wild-derived house mice, the damage to proteins in heart and skeletal muscles did not differ between non-reproducing females and those feeding enlarged or reduced litters, whereas in the liver, the damage either did not differ between these groups or was even decreased in the group with enlarged litters (Aloise King et al., 2013; Garratt et al., 2013). The results of the few studies on wild-derived small rodents showed that the damage to liver and other organs was unaffected or diminished by rearing non-manipulated litters (Garratt et al., 2011; Ołdakowski et al., 2012; Schmidt et al., 2014). Interestingly, however, increased damage to proteins was found in the blood serum of reproducing females, even though the same studies showed no increased damage in the organs (Yang et al., 2013; Plumel et al., 2014; Xu et al., 2014). Similarly, two studies that reported increased damage due to reproductive load in small mammals under natural conditions only considered damage



**Fig. 2. Concentration of thiobarbituric-acid-reactive substances as a marker of oxidative damage to lipids in internal organs of bank voles.** Concentration in (A) liver, (B) kidneys, (C) heart, and (D) skeletal muscles of voles in three reproductive treatment groups: non-breeding females and in females that weaned four consecutive litters, with reduced litters and enlarged litters. Adjusted means from ANOVA models  $\pm$ 95% CL. Different letters above the bars show significant differences between the reproductive treatment groups ( $P < 0.05$ ; Tukey–Kramer pair-wise comparisons).



**Fig. 3. Concentration of protein carbonyls as a marker of oxidative damage to proteins in internal organs of bank voles.** Concentration in (A) liver, (B) kidneys, (C) heart and (D) skeletal muscles of voles in three reproductive treatment groups. Concentration of carbonyls in heart (C) was shown for two generations separately due to the significant interaction between reproductive treatment and generation. See Fig. 2 for other explanations.

**Table 5. Significance of relationships between the concentration of the markers of oxidative damage to tissues (TBARS and carbonyls) and the reproductive effort measured as the total number of weaned pups and the total mass of weaned offspring**

	Total number of pups	Total mass of offspring
TBARS		
Liver	$F_{1,97}=0.09$ $P=0.770$	$F_{1,97}=0.00$ $P=0.994$
Kidneys	$F_{1,97}=0.20$ $P=0.659$	$F_{1,97}=0.60$ $P=0.441$
Heart	$F_{1,97}=1.82$ $P=0.180$	$F_{1,97}=3.25$ $P=0.075$
Muscles	$F_{1,96}=0.45$ $P=0.505$	$F_{1,96}=0.78$ $P=0.381$
Carbonyls		
Liver	$F_{1,97}=0.06$ $P=0.808$	$F_{1,97}=0.10$ $P=0.753$
Kidneys	$F_{1,97}=0.03$ $P=0.874$	$F_{1,97}=0.19$ $P=0.667$
Heart	$F_{1,91}=0.46$ $P=0.499$	$F_{1,91}=0.41$ $P=0.522$
Muscles	$F_{1,96}=0.00$ $P=0.992$	$F_{1,96}=0.09$ $P=0.770$

ANCOVA with either number of pups or offspring mass as a covariate.

in the serum, not the organs (Bergeron et al., 2011; Fletcher et al., 2013). It is not known, however, whether damage detected in the blood is more important for the future fitness of the individual than that detected in its organs.

The simplest explanation of the lack of increase or even a decrease in oxidative damage to tissues due to reproduction is an improvement in antioxidative defences. Indeed, the catalase was more active in kidneys of bank vole females with lower oxidative damage to lipids and proteins in that organ. However, no correlation was found between the concentration of markers of oxidative damage in the liver and the activity of catalase or the concentration of glutathione (Table 6), which is one of the most active antioxidants in biological systems (Halliwell and Gutteridge, 1999). There is growing evidence that reproduction in mammals prompts an upregulation of antioxidants, and consequent reduction in markers of oxidative stress, although examples of no correlation of antioxidants with reproductive status are also common (Garratt et al., 2011, 2013; Yang et al., 2013; Xu et al., 2014). It is possible that the kinetics of oxidative damage and antioxidant defences may not be the same, suggesting that single point measurements of antioxidant concentrations may not capture the relationship between

oxidative damage and antioxidant defences. The lack of oxidative stress in our breeding voles may also be explained by the pleiotropic effects of sex hormones. Oestrogens have antioxidant and protective properties as they upregulate the gene expression of antioxidant enzymes that are directed to mitochondria (Borrás et al., 2010). It has been shown, for example, that oestrogens decrease lipid peroxidation in liver, cardiac and skeletal muscles of female rats (Huh et al., 1994; Persky et al., 2000), the same tissues that we studied in our bank voles. Recently it has been hypothesized that transition to the reproductive state triggers a pre-emptive reduction in levels of oxidative damage to tissues in order to shield mothers, their gametes and developing offspring from harm caused by inevitable increase in oxidative damage resulting from reproductive effort (Blount et al., 2015). This 'oxidative shielding hypothesis' may explain the low levels of oxidative damage in our breeding females, but the cost of shielding is not known. It has also been hypothesized that females may be selected to diminish the level of the damage to protect their offspring (Blount et al., 2015).

Also surprisingly, contrary to our predictions, we did not find any significant differences in the damage to lipids and proteins in any of the three organs, or in muscles between lines selected for high metabolic rates and control lines, either in breeding or non-breeding groups. In a recent study by Brzęk et al. (2014), significant differences between the oxidative damage to lipids were found in liver, kidneys and heart between laboratory mice selected for high and for low basal metabolic rates (BMRs), both in non-reproducing and reproducing females. Surprisingly, however, the damage was higher in the low-BMR animals, which was also not consistent with the oxidative stress hypothesis (Brzęk et al., 2014).

To summarize, our results (1) undermine the hypothesis that oxidative stress is the key proximate mechanism underlying the fundamental life-history trade-off between the current investment in reproduction and future survival or reproductive success, and (2) provide moderate support for the hypothesis that low oxidative damage to tissues in breeding females is maintained owing to upregulation of antioxidant enzymes. It could be argued that the high rate of metabolism associated with reproductive effort may become an important factor leading to increased oxidative stress, with potential consequences for further life, if energy budgets of reproducing animals are additionally challenged by other stressors, such as parasite or pathogen load (Speakman and Garratt, 2014). We agree that such a scenario is plausible. However, such an argument itself undermines the classical paradigm of a trade-off between current reproductive effort and future success, with oxidative stress

**Table 6. Relationship between the concentration of the markers of oxidative damage to tissues (TBARS and carbonyls) and the amount of antioxidants (total glutathione [GSH] level and activity of catalase)**

Organ	Marker	Anti-oxidant	Antioxidant	Reproductive treatment	Selection	Generation	Reproductive treatment×selection
Liver	TBARS	GSH	$F_{1,139}=0.04$ $P=0.838$	$F_{2,12}=5.29$ $P=0.023$	$F_{1,6}=0.27$ $P=0.625$	$F_{1,7}=75.24$ $P<0.0001$	$F_{2,12}=0.58$ $P=0.577$
		Catalase	$F_{1,139}=0.00$ $P=0.977$	$F_{2,12}=5.17$ $P=0.024$	$F_{1,6}=0.27$ $P=0.622$	$F_{1,7}=106.3$ $P<0.0001$	$F_{2,12}=0.59$ $P=0.569$
	Carbonyls	GSH	$F_{1,139}=2.89$ $P=0.091$	$F_{2,12}=5.81$ $P=0.017$	$F_{1,6}=1.99$ $P=0.208$	$F_{1,7}=6.96$ $P=0.034$	$F_{2,12}=0.95$ $P=0.415$
		Catalase	$F_{1,139}=0.28$ $P=0.599$	$F_{2,12}=5.20$ $P=0.024$	$F_{1,6}=1.69$ $P=0.241$	$F_{1,7}=15.64$ $P=0.0055$	$F_{2,12}=1.23$ $P=0.326$
Kidneys	TBARS	Catalase	$F_{1,136}=5.01$ $P=0.027$	$F_{2,12}=3.77$ $P=0.054$	$F_{1,6}=0.36$ $P=0.571$	$F_{1,7}=24.90$ $P=0.002$	$F_{2,12}=1.79$ $P=0.209$
	Carbonyls	Catalase	$F_{1,136}=6.59$ $P=0.011$	$F_{2,12}=0.33$ $P=0.726$	$F_{1,6}=0.14$ $P=0.719$	$F_{1,7}=65.97$ $P<0.0001$	$F_{2,12}=1.49$ $P=0.264$

Results from ANCOVA with the antioxidant level as a covariate.



as a mediator, because it assumes that the trade-off must include some additional components, such as the cost of operating the immune system.

#### Acknowledgements

We thank Iwona Ciereszko for making her laboratory available for our project. We also thank Anna Koszewnik, Małgorzata Lewoc, Stanisław Plonowski and Bogusław Lewończuk for their assistance in animal maintenance. This article benefited from the comments of Jonathan D. Blount, Neil B. Metcalfe and an anonymous reviewer on the earlier version of the manuscript. Neil B. Metcalfe improved the English.

#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

The experiments were performed by Ł.O. and A.W. E.T.S. was in charge of the selection experiment in the main breeding colony and provided voles for the study. The experiments were conceptualized and designed by J.R.E.T. and P.K. Ł.O. and J.R.E.T. analysed the data and wrote the paper with substantial contributions from P.K.

#### Funding

The main source of funding was the National Science Centre [NN304280840 to J.R.E.T.]. The cost of the source animals was covered by the National Science Centre [NN303 816740] and Jagiellonian University [DS/WBINOZ/INOS/757] to P.K.

#### References

- Aebi, H. E. (1986). Catalase. In *Methods of Enzymatic Analysis*, 3, 3rd edn (ed. H. U. Bergmeyer), pp. 273-286. Weinheim: VCH Verlagsgesellschaft mbH.
- Aloise King, E. D., Garratt, M. and Brooks, R. (2013). Manipulating reproductive effort leads to changes in female reproductive scheduling but not oxidative stress. *Ecol. Evol.* **3**, 4161-4171.
- Anderson, M. E. (1996). Measurement of antioxidants: glutathione. In *Free Radicals, A Practical Approach* (ed. N. A. Punchard and F. J. Kelly), pp. 213-226. Oxford: Oxford University Press.
- Bergeron, P., Careau, V., Humphries, M. M., Réale, 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.
- Blount, J. D., Vitikainen, E. I. K., Stott, I. and Cant, M. A. (2015). Oxidative shielding and the cost of reproduction. *Biol. Rev.* doi: 10.1111/brv.12179.
- Boonekamp, J. J., Salomons, M., Bouwhuis, S., Dijkstra, C. and Verhulst, S. (2014). Reproductive effort accelerates actuarial senescence in wild birds: an experimental study. *Ecol. Lett.* **17**, 599-605.
- Borrás, C., Gambini, J., López-Gruoso, R., Pallardó, F. V. and Viña, J. (2010). Direct antioxidant and protective effect of estradiol on isolated mitochondria. *Biochim. Biophys. Acta* **1802**, 205-211.
- Brzek, P., Książek, A., Oldakowski, Ł. and Konarzewski, M. (2014). High basal metabolic rate does not elevate oxidative stress during reproduction in laboratory mice. *J. Exp. Biol.* **217**, 1504-1509.
- Bujalska, G. (1983). Reproduction. In *Ecology of the bank vole* (ed. K. Petruszewicz). *Acta Theriol.* **28**, 148-161.
- Clutton-Brock, T. H., Guinness, F. E. and Albon, S. D. (1982). *Red Deer: Behavior and Ecology of Two Sexes*. Chicago: University of Chicago Press.
- Cretegy, 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.
- Dijkstra, C., Bult, A., Bijlsma, S., Daan, S., Meijer, T. and Zijlstra, M. (1990). Brood size manipulations in the kestrel (*Falco tinnunculus*): effects on offspring and parent survival. *J. Anim. Ecol.* **59**, 269-285.
- Drent, R. H. and Daan, S. (1980). The prudent parent: energetic adjustments in avian breeding. *Ardea* **68**, 225-252.
- Finkel, T. and Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature* **408**, 239-247.
- Fletcher, Q. E., Selman, C., Boutin, S., McAdam, A. G., Woods, S. B., Seo, A. Y., Leeuwenburgh, C., Speakman, J. R. and Humphries, M. M. (2013). Oxidative damage increases with reproductive energy expenditure and is reduced by food-supplementation. *Evolution* **67**, 1527-1536.
- 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. Lond. B Biol. Sci.* **278**, 1098-1106.
- Garratt, M., Pichaud, N., King, E. D. A. and Brooks, R. C. (2013). Physiological adaptations to reproduction. I. Experimentally increasing litter size enhances aspects of antioxidant defence but does not cause oxidative damage in mice. *J. Exp. Biol.* **216**, 2879-2888.
- Gustafsson, L. and Pärt, T. (1990). Acceleration of senescence in the collared flycatcher *Ficedula albicollis* by reproductive costs. *Nature* **347**, 279-281.
- Gustafsson, T., Andersson, B. and Westlin, L. (1980). Reproduction in a laboratory colony of bank vole, *Clethrionomys glareolus*. *Can. J. Zool.* **58**, 1016-1021.
- Halliwell, B. and Gutteridge, J. M. C. (1999). *Free Radicals in Biology and Medicine*. Oxford: Oxford University Press.
- Huh, K., Shin, U.-S., Choi, J.-W. and Lee, S.-I. (1994). Effect of sex hormones on lipid peroxidation in rat liver. *Arch. Pharm. Res.* **17**, 109-114.
- Jentzsch, A. M., Bachmann, H., Fürst, P. and Biesalski, H. K. (1996). Improved analysis of malondialdehyde in human body fluids. *Free Radic. Biol. Med.* **20**, 251-256.
- Johnson, M. S., Thomson, S. C. and Speakman, J. R. (2001). Limits to sustained energy intake. III. Effects of concurrent pregnancy and lactation in *Mus musculus*. *J. Exp. Biol.* **204**, 1947-1956.
- 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.
- Konarzewski, M. and Diamond, J. (1995). Evolution of basal metabolic rate and organ masses in laboratory mice. *Evolution* **49**, 1239-1248.
- Konczal, M., Babik, W., Radwan, J., Sadowska, E. T. and Koteja, P. (2015). Initial molecular-level response to artificial selection for increased aerobic metabolism occurs primarily through changes in gene expression. *Mol. Biol. Evol.* **32**, 1461-1473.
- Książek, A., Konarzewski, M. and Łapo, I. B. (2004). Anatomic and energetic correlates of divergent selection for basal metabolic rate in laboratory mice. *Physiol. Biochem. Zool.* **77**, 890-899.
- Lessells, C. M. and Boag, P. T. (1987). Unrepeatable repeatabilities: a common mistake. *Auk* **104**, 116-121.
- Levine, R. L., Williams, J. A., Stadtman, E. R. and Shacter, E. (1994). Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol.* **233**, 346-357.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.
- Mappes, T., Koskela, E. and Ylönen, H. (1995). Reproductive costs and litter size in the bank vole. *Proc. R. Soc. Lond. B Biol. Sci.* **261**, 19-24.
- 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.
- Metcalfe, N. B. and Monaghan, P. (2013). Does reproduction cause oxidative stress? An open question. *Trends Ecol. Evol.* **28**, 347-350.
- 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.
- Nordling, D., Andersson, M., Zohari, S. and Lars, G. (1998). Reproductive effort reduces specific immune response and parasite resistance. *Proc. R. Soc. Lond. B Biol. Sci.* **265**, 1291-1298.
- Norris, K. and Evans, M. R. (2000). Ecological immunology: life history trade-offs and immune defense in birds. *Behav. Ecol.* **11**, 19-26.
- Ohkawa, H., Ohishi, N. and Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* **95**, 351-358.
- Oldakowski, Ł., Piotrowska, Ż., Chrzęścik, 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. *J. Exp. Biol.* **215**, 1799-1805.
- Oswald, C. and McClure, P. A. (1987). Energy allocation during concurrent pregnancy and lactation in Norway rats with delayed and undelayed implantation. *J. Exp. Zool.* **241**, 343-357.
- Oswald, C. and McClure, P. A. (1990). Energetics of concurrent pregnancy and lactation in cotton rats and woodrats. *J. Mammal.* **71**, 500-509.
- Persky, A. M., Green, P. S., Stubble, L., Howell, C. O., Zaulyanov, L., Brazeau, G. A. and Simpkins, J. W. (2000). Protective effect of estrogens against oxidative damage to heart and skeletal muscle in vivo and in vitro. *Proc. Soc. Exp. Biol. Med.* **223**, 59-66.
- Peterson, G. L. (1977). A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* **83**, 346-356.
- Plumel, M. I., Stier, A., Thiersé, D., van Dorsselaer, A., Criscuolo, F. and Bertile, F. (2014). Litter size manipulation in laboratory mice: an example of how proteomic analysis can uncover new mechanisms underlying the cost of reproduction. *Front. Zool.* **11**, 41.
- Roff, D. A. (1992). *The Evolution of Life Histories*. New York: Chapman and Hall.
- Sadowska, E. T., Baliga-Klimczyk, K., Chrzęścik, K. M. and Koteja, P. (2008). Laboratory model of adaptive radiation: a selection experiment in the bank vole. *Physiol. Biochem. Zool.* **81**, 627-640.
- Sadowska, E. T., Stawski, C., Rudolf, A., Dheyongera, G., Chrzęścik, K. M., Baliga-Klimczyk, K. and Koteja, P. (2015). Evolution of basal metabolic rate in bank voles from a multidirectional selection experiment. *Proc. R. Soc. Lond. B Biol. Sci.* **282**, 20150025.
- Schmidt, C. M., Blount, J. D. and Bennett, N. C. (2014). Reproduction is associated with a tissue-dependent reduction of oxidative stress in eusocial female damaraland mole-rats (*Fukomys damarensis*). *PLoS ONE* **9**, e103286.
- Speakman, J. R. (2008). The physiological costs of reproduction in small mammals. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **363**, 375-398.

- Speakman, J. R. and Garratt, M.** (2014). Oxidative stress as a cost of reproduction: beyond the simplistic trade-off model. *BioEssays* **36**, 93-106.
- 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.
- Stadtman, E. R. and Oliver, C. N.** (1991). Metal catalyzed oxidation of proteins. Physiological consequences. *J. Biol. Chem.* **266**, 2005-2008.
- Stearns, S. C.** (1992). *The Evolution of Life Histories*. Oxford: Oxford University Press.
- Steven, D. M.** (1957). The bank vole in captivity. *Nature* **179**, 33-34.
- Stier, A., Reichert, S., Massemmin, S., Bize, P. and Criscuolo, F.** (2012). Constraint and cost of oxidative stress on reproduction: correlative evidence in laboratory mice and review of the literature. *Front. Zool.* **9**, 37.
- Van Remmen, H. and Richardson, A.** (2001). Oxidative damage to mitochondria and aging. *Exp. Gerontol.* **36**, 957-968.
- 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.
- Xu, Y.-C., Yang, D.-B., Speakman, J. R. and Wang, D.-H.** (2014). Oxidative stress in response to natural and experimentally elevated reproductive effort is tissue dependent. *Funct. Ecol.* **28**, 402-410.
- Yang, D.-B., Xu, Y.-C., Wang, D.-H. and Speakman, J. R.** (2013). Effects of reproduction on immuno-suppression and oxidative damage, and hence support or otherwise for their roles as mechanisms underpinning life history trade-offs, are tissue and assay dependent. *J. Exp. Biol.* **216**, 4242-4250.