High basal metabolic rate does not elevate oxidative stress during reproduction in laboratory mice

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Summary

Increased oxidative stress (OS) has been suggested as a physiological cost of reproduction. However, previous studies reported ambiguous results, with some even showing a reduction of oxidative damage during reproduction. We tested whether the link between reproduction and OS is mediated by basal metabolic rate (BMR), which has been hypothesised to affect both the rate of radical oxygen species production and anti-oxidative capacity. We studied the effect of reproduction on OS in females of laboratory mice divergently selected for high (H-BMR) and low (L-BMR) BMR, previously shown to differ with respect to parental investment. Non-reproducing L-BMR females showed higher oxidative damage to lipids (quantified as the level of malonaldehyde in internal organ tissues) and DNA (quantified as the level of 8-oxodG in blood serum) than H-BMR females. Reproduction did not affect oxidative damage to lipids in either line; however, it reduced damage to DNA in L-BMR females. Reproduction increased catalase activity in liver (significantly stronger in L-BMR females) and decreased in kidneys. We conclude that the effect of reproduction on OS depends on the initial variation in BMR and varies between studied internal organs and markers of OS.

Introduction

The evolution of high basal metabolic rate (BMR) and endothermy are hypothesized to result from selection for intensive parental care (Farmer, 2000; Koteja, 2000). The ability to maintain a high, sustained level of energy expenditure and locomotor activity could allow for more efficient feeding, guarding or brooding of offspring, which in turn can decrease juvenile mortality and thus increase parental fitness (Kozłowski, 1992). However, one of the key assumptions of life history theory is that intense parental effort should lead to higher costs of reproduction, revealed by a lower survival of parents, or their reduced future reproductive success (Roff, 1992; Stearns, 1992). Thus, even if higher BMR of parents enables better survival of their offspring, it may confer no evolutionary advantage if it simultaneously incurs an increase of the physiological costs of reproduction and thereby parental mortality.
It is unclear however, to what extent elevated BMR is associated with such costs, most notably, with increased oxidative stress (OS), hypothesized to represent a significant reproductive cost at the molecular level (Speakman, 2008; Monaghan et al., 2009).

OS occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the capacity of antioxidant mechanisms to control their damaging effects (Monaghan et al., 2009). ROS are primarily byproducts of normal metabolic processes that can cause damage to lipids, proteins, and DNA when not quenched by antioxidant mechanisms (e.g. enzymes like catalase and superoxide dismutase, or non-enzymatic antioxidants like glutathione; Monaghan et al., 2009; Pamplona and Constantini, 2011). Reproduction represents the period when animals may be particularly prone to OS, because elevated energy expenditure in reproduction can potentially increase the rate of ROS production and /or reduce investment in the antioxidative systems (Speakman, 2008; Monaghan et al., 2009). However, the relationship between BMR and the magnitude of oxidative damage is not obvious. First, higher metabolic rates do not universally elevate the rate of ROS production (Barja, 2007). For example, higher mitochondrial uncoupling may increase the rate of oxygen consumption but simultaneously decrease the ROS production (Speakman et al., 2004). Second, higher BMR may allow for more effective anti-oxidative mechanisms (Speakman et al., 2002). Finally, previous studies (reviewed in Stier et al., 2012; Metcalfe and Monaghan, 2013; Speakman and Garratt, 2013) on the relationship between OS and reproduction have produced ambiguous results. For example, laboratory experiments on small rodents reported both an increase (Stier et al., 2012) and decrease (Garratt et al., 2011; Ołdakowski et al., 2012; Garratt et al., 2013) in oxidative damage during reproduction. In

conclusion, although variation in BMR is likely to affect both the magnitude of OS and its changes during reproduction, the direction of the relationship between these parameters is difficult to predict. This issue is particularly important because variation in individual parental quality may mediate the importance of OS as the cost of reproduction (Metcalfe and Monaghan, 2013). Moreover, if BMR is an important predictor of parental quality, then variation in BMR (both within and between experiments) may be one of the factors responsible for the inconclusive results of previous studies of the changes of OS during reproduction.

In the present paper, we explore the presumed links between BMR and OS elicited by reproduction. We used laboratory mice from two line types with BMR manipulated by means of artificial selection for high (H-BMR) and low (L-BMR) body-mass-corrected BMR (Książek et al., 2004). The relative between-line-type difference in BMR reaches 40-50% [see the results of the present study]. Such a considerable difference makes testing correlations between BMR and life history parameters on an intra-specific level possible. Although selection is aimed at BMR measured before reproduction, it resulted in a significantly higher parental investment during lactation in H-BMR females, enabling faster growth of pups (Sadowska et al., 2013). Thus, selection for BMR affected parental effort and both line types are therefore particularly suited as a model for studies on the association between OS, BMR and reproduction (see Metcalfe and Monaghan, 2013). Recently, it was shown that selection for high maximum aerobic metabolic rate in bank voles did not affect the level of oxidative damage though it elevated BMR (Ołdakowski et al., 2012). However, the difference in BMR between mice from H-BMR and L-BMR line types is ca. 3 times higher than between control and selected line types of bank voles (compare Ołdakowski et al., 2012), and thus is more likely to reveal the effect of BMR on OS. Moreover, OS in females of bank vole was measured after weaning of their litters (Ołdakowski et al., 2012), whereas in the present experiment we assayed females at the peak lactation, when energetic costs of reproduction are highest.

In the present experiment, we predicted that: (i) if higher BMR increases the rate of ROS production and oxidative damage, the oxidative damage should be higher in non-reproducing H-BMR than L-BMR females. Moreover, elevated energy expenditures during reproduction are likely to further increase OS, particularly in H-BMR line type; (ii) alternatively, if higher BMR decreases the rate of ROS production (e.g. via more uncoupled mitochondria), enables more effective antioxidant mechanisms or is related to lower susceptibility to ROS-related damage, then non-reproducing H-BMR females should have

lower oxidative damage than non-reproducing L-BMR females. Under such a scenario (positive correlation between BMR and anti-oxidative defence or ROS resistance and/or negative correlation between BMR and ROS production), elevated energy expenditures during reproduction may not affect or may even reduce oxidative damage. To test these hypotheses, we measured two parameters quantifying oxidative damage to different types of molecules (Monaghan et al., 2009) in reproducing and non-reproducing females of both line types: the level of malonaldehyde (MDA; the product of lipid peroxidation) in liver, kidneys, and heart; and the concentration of 8-oxo-2'-deoxyguanosine (8-oxodG; the product of repair of ROS-mediated damage of guanosine) in blood serum as markers of oxidative damage to lipids, and DNA, respectively. We also measured the activity of catalase in liver and kidneys, an enzyme that represents an important component of anti-oxidative defence (Pamplona and Constantini, 2011).

Results

Body-mass corrected BMR of reproducing females differed consistently between line types both before (LS means ± s.e.m.; H-BMR: 65.11 ± 0.88 ml O_2 h^{-1}, L-BMR: 43.28 ± 0.83 ml O_2 h^{-1}) and after first reproduction (H-BMR: 71.24 ± 1.27 ml O_2 h^{-1}, L-BMR: 54.53 ± 1.16 ml O_2 h^{-1}). A significant interaction between the line type and the order of measurement reveals that the effect of first reproduction on BMR was line-type-specific (Table 1). Indeed, BMR increased between the measurements in the L-BMR (P=0.0002) but not in the H-BMR line type (P=0.96). However, the standardized between-line type differences were higher than d_{drift} for BMR measured both before (d = 5.11 versus d_{drift} = 0.71) and after (d = 2.70 versus d_{drift} = 0.71) first reproduction, suggesting that the difference in BMR arose as a result of selection rather than genetic drift and was still highly significant in females after first reproduction.

Among non-reproducing females, all parameters quantifying oxidative damage were higher in mice from the L-BMR line type, and the magnitude of most differences between line types exceeded the values expected under the effect of genetic drift (Table 2, Fig. 1). Oxidative damage to lipids was unaffected by reproduction (Table 2, Fig. 1). There was a significant interaction between line type affiliation and reproductive status for the level of 8-oxodG in blood serum (Table 2, Fig. 1D): non-reproducing L-BMR females had higher level of 8-oxodG than H-BMR ones (P=0.0004), but this difference disappeared in reproducing females (P=0.86).

There was also a significant interaction between reproductive status and line type for the activity of catalase in liver (Table 2; Fig. 2A). A Tukey's test showed that the activity of catalase did not differ between non-reproducing females from L-BMR and H-BMR line types (P>0.99). Although it was elevated during reproduction in both line types, this increase was stronger in L-BMR than in H-BMR mice (Tukey test, respectively: P<0.0001 and P=0.014), resulting in a significant difference between reproducing females from both line types (P=0.016). Catalase activity in the kidneys was not affected by the line type, and significantly decreased during reproduction in both line types (Table 2; Fig. 2B).

Discussion

Differences in BMR between line types of studied mice significantly affected all examined markers of oxidative damage; however, reproduction did not change oxidative damage to lipids in internal organs, whereas the concentration of 8-oxodG in blood serum was reduced only in females with low BMR (Table 2, Fig. 1). For most traits quantifying oxidative damage, the magnitudes of phenotypic differences (*d*) between line types for non-reproducing females were large enough to attribute it to the selection on BMR, rather than genetic drift (Table 2). This finding agrees with the results of other studies suggesting that the resistance to OS may have a significant genetic component (e.g. (Kim et al., 2010) and studies cited there). Interestingly, in non-reproducing females the level of oxidative damage was higher in mice selected for low BMR (L-BMR), whereas earlier studies reported positive inter- and intra-specific correlation between concentration of 8-oxodG and basal/standard metabolic rate (Foksinski et al., 2004; Topp et al., 2008).

This study does not allow us to pinpoint the exact mechanisms underlying the effect of the between-line-type variation in BMR on oxidative damage. However, the magnitude of oxidative damage reflects the balance between the rate of ROS generation and neutralisation, susceptibility to ROS, and the efficiency of repair mechanisms (Monaghan et al., 2009). Between-line type difference in BMR did not affect the activity of catalase in liver and kidneys (Fig. 2), and we have demonstrated earlier that males of both line types did not differ with respect to anti-oxidative capacity of blood serum (Brzęk et al., 2012). Thus, lower oxidative damage in the H-BMR mice probably cannot be attributed to their enhanced antioxidative defences, at least those assayed here and in Brzęk et al. (2012). Alternatively, mice with high BMR may have lower rate of ROS production because of higher mitochondrial uncoupling (Speakman et al., 2004). Finally, L-BMR mice may be more susceptible to OS due to higher proportion of ROS-susceptible polyunsaturated fatty acids in their cell membrane lipids (Brzęk et al., 2007). The products of lipid peroxidation may induce DNA damage (Evans and Cooke, 2006; Hulbert et al., 2007), and these mechanisms may explain the higher concentration of both MDA and 8-oxodG in L-BMR mice.

Our study is not the first one that reported no change or a reduction of oxidative damage in internal organs of reproducing rodents (Garratt et al., 2011; Ołdakowski et al., 2012; Garratt et al., 2013). The simplest explanation for the lack of increase in OS during reproduction is an improvement of anti-oxidative mechanisms. Indeed, we found a significant increase in the activity of catalase in the liver during reproduction (Fig. 2A), which agrees

with observations of elevated level of glutathione (Garratt et al., 2011) and the activity of superoxide dismutase (Garratt et al., 2013) in the same organ. Moreover, the increase of catalase activity was significantly higher in L-BMR females. Such a pattern may indicate that elevated energy metabolism during reproduction triggered higher up-regulation of ROS-neutralizing mechanisms in this line type, presumably to counterbalance a higher propensity to oxidative damage incurred by their more ROS-susceptible cell membrane lipids (Brzęk et al. 2007). Unexpectedly, the activities of catalase in the kidneys were significantly reduced during reproduction, indicating that other anti-oxidative mechanisms must be responsible for the lack of increase in oxidative damage in this organ (compare Fig. 1B and 2B). This result also suggests that the effect of reproduction on anti-oxidative defences can vary even between vital organs, such as liver and kidneys (similarly, the effect of reproduction on the activity of superoxide dismutase in Brandt's vole and Mongolian gerbil is tissue dependent; Xu et al., in press; Yang et al., 2013).

Although reproduction did not change the magnitude of oxidative damage to lipids in internal organs, it significantly reduced the concentration of 8-oxodG in blood serum of the L-BMR line type (Fig. 1D). However, the concentration of 8-oxodG in blood serum is a general marker of OS at the whole-body level, and imbalance between ROS production and neutralisation may differ between organs (Garratt et al., 2011; Garratt et al., 2012; Speakman and Garratt, 2013). One possibility is that elevated energy metabolism during reproduction triggered an enhanced up-regulation of ROS-neutralizing mechanisms in the L-BMR line type, similar to the pattern observed for the catalase activity in the liver. Alternatively, whereas excretion rates of 8-oxodG in a steady state are largely unaffected by repair capacities (Loft et al., 2008) and thus mainly reflects the rate of DNA damage, one might hypothesize that reproduction reduced activity of mechanisms excreting damaged DNA in L-BMR line type. We cannot exclude such a scenario; however, we emphasize that it would mean that reproducing females from L-BMR line type accumulated more mutagenic DNA lesions than females from H-BMR line type, a pattern that still indicates that higher BMR does not result in relatively higher oxidative damage during reproduction.

To the best of our knowledge, this is the first analysis of the link between variation in BMR and changes in OS during reproduction. Our results have three important evolutionary implications. First, the observed patterns suggest that the evolution of high BMR and endothermy via selection for more effective parental care (as proposed in Koteja, 2000) does not necessarily elevate the costs of reproduction in terms of an increased oxidative damage (since its value in reproducing H-BMR females never exceeded that observed in L-BMR line

type). In fact, our results suggest a negative association between energy expenditures and OS. An important caveat is that reproducing mice in our experiment had unlimited access to food, whereas the fitness effects of BMR are often context-dependent (Burton et al., 2011), and reproduction is more likely to increase OS when breeding individuals face limited food resources (Fletcher et al., 2013), or must cope with additional stresses (van de Crommenacker et al., 2012). However, reproducing females from the H-BMR line type cope better with lower ambient temperature than those from the L-BMR line type (Sadowska et al., 2013). Thus, it is unlikely that even under worse conditions, elevated OS in H-BMR line type would counter-balance profits resulting from better parental care. Second, results for DNA damage and the activity of catalase in the liver show that the initial differences in BMR can affect how parameters related to OS change during reproduction, confirming that inter-individual variation may be important in studies of the link between OS and the cost of reproduction (Metcalfe and Monaghan, 2013). Thus, we recommend that - whenever possible - future studies on the association between the costs of reproduction and OS should take into account systematic variation in the level of basal energy expenditures (such as BMR) specific to studied organisms. Finally, contrasting directions of changes of the catalase activity in liver and kidneys during reproduction (without simultaneous changes in markers of OS in these organs) suggest the presence of significant variation between organs in the activation of anti-oxidative mechanisms during reproduction, a pattern found also in other recent studies (Xu et al., in press; Yang et al., 2013; Speakman and Garratt, 2013).

Materials and methods

Animals and their maintenance

Subjects in our experiment were females of Swiss-Webster mice (*Mus musculus* Linnaeus 1758) from generation 36 of an artificial selection experiment for high and low body-mass-corrected BMR. The selection experiment, and the BMR assays are described in detail elsewhere (Książek et al., 2004; Gębczyński and Konarzewski, 2009). Briefly, males and females characterized by the highest and lowest mass-corrected BMR measured at age 12-16 week were chosen as progenitors of the H-BMR and L-BMR line types, respectively. A similar procedure was repeated in subsequent offspring generations, yielding significant differentiation of the line types with respect to BMR, without simultaneous changes in body mass. Although the described selection experiment has no replications, between-line-type differences in BMR and several other traits have been shown several times to be large enough to claim that they represent a genuine change in frequencies of alleles directly related to BMR rather than genetic drift (Książek et al., 2004; Brzęk et al., 2007; Gębczyński and Konarzewski, 2009).

Throughout the course of the selection experiment, mice were maintained in a climatic chamber at an ambient temperature of 23^0C and 12:12 light-dark cycle, and offered *ad libitum* water and food (murine laboratory chow, Labofeed H, Wytwórnia Pasz A. Morawski, Kcynia, Poland). The same conditions were applied during the present experiment.

Experimental procedures

Following BMR measurements, being a part of the selection procedure, females used in the present study were assigned randomly to reproducing and non-reproducing (control) treatments. Virgin, non-reproducing females (32 in H-BMR and 30 in L-BMR line type) were maintained in separate cages. Reproducing females (23 in H-BMR and 26 in L-BMR line type) were concurrently bred at 22 week of age (this reproduction was a part of our selection procedure). After weaning they were paired again with males from their respective line types and gave birth at 30 week of age (males were removed before parturition). Cages were bedded with sawdust and provided with paper towels for nest construction. Litter size did not differ between both line types ($P>0.05$ for both reproductive attempts).

All females were killed on day 17 of the second lactation of reproducing dams by cervical dislocation, and theirs liver, kidneys, and heart were dissected and immediately frozen in liquid nitrogen. Blood samples were taken and centrifuged to collect blood serum.

All samples were stored at -80^{0}C. Samples from control females were collected at the same age like in reproducing females.

Measurements of resting metabolic rate in reproducing females do not represent BMR (i.e. the primary target of artificial selection) because they include the cost of pregnancy or milk synthesis. Therefore, we did not quantify metabolic rate of females during reproduction. However, to estimate whether the between-line-type difference in BMR was still significant after the first reproduction, we measured BMR in reproducing females right after the weaning of their first litter and compared with values found in the same individuals before experiment (see (Książek et al., 2004) for description of BMR assays).

Analysis of oxidative damage and activity of catalase

MDA was measured by means of the NWK-MDA01 assay kit (Northwest Life Science Specialties LCC, Vancouver, WA, USA) according to the manufacturer's instructions (before spectrophotometric analyses, samples were extracted with butane-pyridine mixture v/v 15:1). Activity of catalase was assayed according to the method of Aebi (Aebi, 1983). Protein content in the supernatant was determined by means of the Lowry method using Sigma Aldrich TP0300 kit (Sigma-Aldrich, St. Louis, MO, USA). Repeatability of all assays was $r \geq 0.9$ (with the exception for protein content in heart, where $r = 0.83$). Concentration of MDA was expressed in nmol per mg^{-1} protein, and activity of catalase was expressed (following recommendation by Aebi (Aebi, 1983)) as the rate constant of a first order reaction (k) per mg^{-1} protein.

The concentration of 8-oxodG in blood serum was quantified with Trevigen HT 8-oxodG ELISA kit (4370-096-K; Trevigen Inc., Gaithersburg, MD, USA; analyzed in duplicate, repeatability $r > 0.93$), and expressed as ng of 8-oxodG per mL of blood serum. Immunoassays may overestimate 8-oxodG content (Cooke et al., 2008; Cadet et al. 2011). Still, they can be a reliable test (Cooke et al., 2006), particularly when the aim of the study is to compare the relative level of 8-oxodG in different groups (Cooke et al., 2008).

Sample sizes for different assay differed because of limitations in the quantity of sample available for analysis but the number of non-analyzed animals never exceeded two per group.

Data analysis

Variables were analyzed with an ANOVA with the line type, reproductive status (reproducing vs. non-reproducing females), the order or measurements (first and second, for changes in

BMR between two measurements) as main factors, their respective interaction terms, and the family affiliation (nested within line type) as random factor. Body mass was added as a covariate in analyses of BMR. Besides the line type and reproductive status, all other terms were included in the final model only when significant (P<0.05). We subsequently tested between-group differences by means of Tukey post-hoc test. The magnitude of oxidative damage in lipids in liver, the concentration of 8-oxodG, and BMR were log-transformed, and catalase activity in liver was exponentially transformed before analyses to improve homogeneity of variation. All analyses were carried using procedure MIXED in SAS software.

Mice for this study came from a selection experiment without replicated lines. Therefore, the observed differences between line types might have arisen as a result of genetic drift rather than representing a genuine effect of artificial selection. To control for the possible effect of genetic drift, we analysed the between-line-type differences in markers of oxidative damage in non-reproducing females according to Henderson's guidelines (Henderson, 1997; Konarzewski et al. 2005). We also analysed in the same way BMR measured in reproducing females before and after first reproduction to check whether the between-line-type difference in BMR caused by selection was still significant before second reproduction when OS was quantified. First, we expressed the magnitude of difference between H-BMR and L-BMR line types for a given trait X as the difference between the within-line-type mean values divided by the weighted phenotypes (d_X) (see Konarzewski et al., 2005). Then, we estimated the 95% confidence intervals (95% CI, hereafter d_{drift}) for d_X, using equation 16 from Henderson (Henderson, 1997):

$$\sigma_{(dX)} = 2\sqrt{(h_X^2 F + 1/n)} \quad (1),$$

where h_X^2 is the narrow-sense heritability of analyzed trait X, F is the inbreeding coefficient (F = 0.25 in generation F36 of the studied selection experiment, calculated from equation 3.5 from Falconer and Mackay (Falconer and Mackay, 1996)), and n is the number of families used for studying the particular trait. We assumed h^2 = 0.4 for BMR (Konarzewski et al., 2005); however, we are aware of only one estimate of heritability for parameters we used to quantify oxidative damage (h^2 = 0.17 calculated for 8-oxodG urinary content in humans; Broedbaek et al., 2011). Therefore, we calculated d_{drift} assuming either low (h^2 = 0.1) or high (h^2 = 0.4) heritability of studied parameters. All differences where $d > d_{drift}$ can be ascribed to selection effect, rather than genetic drift. We emphasize that all calculated d_{drift} for $h^2 < 0.7$ were lower than values of d estimated for BMR, MDA content in kidneys, and 8-oxodG (see

Results), and thus $d > d_{drift}$ for these traits even if assumed values of the narrow-sense heritability were inaccurate.

List of abbreviations

8-oxodG	8-oxo-2'-deoxyguanosine
BMR	basal metabolic rate
L-BMR, H-BMR	mice selected for high and low BMR, respectively
MDA	malonaldehyde
OS	oxidative stress
ROS	reactive oxygen species

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Competing interests statement

The authors declare that they have no competing interests.

Author contributions

P. Brzęk and M. Konarzewski developed the concept of the study. P. Brzęk performed the experiment. P. Brzęk, A. Książek, and Ł. Ołdakowski carried out biochemical analyses. P. Brzęk carried out statistical analysis of results. P. Brzęk, A. Książek, and M. Konarzewski prepared the paper. All authors approved the final manuscript.

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Figure legends

Figure 1. Oxidative damage in experimental animals. Oxidative damage to lipids in liver (A), kidneys (B), and heart (C), and blood serum concentration of 8-oxodG (D). Means ± s.e.m. are presented.

Figure 2. Activity of catalase in experimental animals. Activity of catalase in liver (A), and kidneys (B). Means ± s.e.m. are presented.

Table 1. Summary of ANOVA of the effect of the line type (H-BMR vs. L-BMR), the order of measurement (before and after the first reproduction) and body mass on BMR.

	F	Df	*P*
Line type	261.41	1,36	<0.0001
Order of measurement	5.74	1,56	0.02
Line type × order of measurement	20.16	1,56	<0.0001
Body mass	45.88	1,56	<0.0001

Table 2. Summary of ANOVA of the effect of reproductive status (reproducing vs. non-reproducing females) and the line type (H-BMR vs. L-BMR) on markers of oxidative damage and the activity of catalase. Standardized between-line type differences *d* are shown for parameters with significant effect of the line type in non-reproducing females. d_{drift} = 0.43 for h^2 =0.1 and d_{drift} = 0.69 for h^2 = 0.4.

	Reproductive status			Line type			Reproductive status × line type			*d*
	F	df	*P*	*F*	df	*P*	*F*	df	*P*	
Content of MDA in liver	2.07	1,104	0.15	9.81	1,104	0.0023				0.62
Content of MDA in kidneys	0.73	1,102	0.39	13.28	1,102	0.0004				0.89
Content of MDA in heart	0.01	1,104	0.92	4.19	1,104	0.043				0.05
Concentration of 8-oxodG in blood serum	0.31	1,106	0.58	11.24	1,106	0.0011	4.69	1,106	0.033	1.33
Activity of catalase in liver	45.93	1,98	<0.0001	4.89	1,98	0.029	5.50	1,98	0.021	
Activity of catalase in kidneys	58.43	1,107	<0.0001	0.20	1,107	0.66