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RESEARCH ARTICLE

Effect of dietary restriction on metabolic, anatomic and molecular traits in mice depends on the initial level of basal metabolic rate

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SUMMARY

Dietary restriction (DR)-related delay of ageing is hypothesized to be mediated by the reduction of the metabolic rate (MR). However, studies on the effect of DR on MR have produced equivocal results. We demonstrated that this lack of congruency can be due to a variation in the initial level of MR within a given pool of experimental subjects. We subjected laboratory mice from two line types divergently selected for basal MR (BMR) to 30% DR lasting 6 months to test whether the effect of DR depends on the initial variation in BMR and peak MR. BMR and peak MR were independently affected by DR. The effect of DR was stronger in line types with higher initial levels of MR. Line-type-specific changes in the proportions of body components explained contrasting effects of DR on the mass-corrected BMR, which decreased in the high-BMR line type and did not change in the low-BMR line type. We conclude that the initial variation in MR can significantly affect response to DR. However, we found no association between the level of MR and mechanisms underlying the susceptibility to or protection against oxidative stress.

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INTRODUCTION

Dietary restriction (DR) is the most effective treatment known for extending lifespan in a wide range of organisms (reviewed in Weindruch and Walford, 1988; Masoro, 2002; Masoro, 2005). However, the physiological and molecular mechanisms underlying the anti-ageing effect of DR are still not fully understood (Masoro, 2005; Sinclair, 2005). One of the most-cited hypotheses relates this effect to a reduction in metabolic rate (MR) (Sacher, 1977). According to this, a lower rate of metabolism decreases the rate of reactive oxygen species synthesis and, therefore, the magnitude of oxidative stress, which plays a key role in ageing (Finkel and Holbrook, 2000; Masoro, 2005). Although a higher rate of metabolism does not always result in a higher rate of reactive oxygen species synthesis (Barja, 2007), nor shorten lifespan (Speakman et al., 2004; Vaanholt et al., 2009), the existence of a significant link between the rate of metabolic processes and ageing is well documented (Anderson and Weindruch, 2010). However, the ubiquity of DR-induced reduction of MR is far from certain, and numerous studies have reported an increase, decrease or the lack of change in MR of animals subjected to DR (e.g. McCarter et al., 1985; McCarter and Palmer, 1992; Selman et al., 2005; Faulks et al., 2006; Ramsey and Hagopian, 2006; Ferguson et al., 2007; Sohal et al., 2009; Hempenstall et al., 2010).

How DR affects the actual MR is not a trivial question because the long-term effects of DR on metabolism are expected to accumulate over an organism's lifespan and thus slow ageing. A number of recent studies have called attention to the importance of intraspecific genetic variation in response to DR [including cases when DR actually shortens lifespan (Rikke et al., 2003; Rikke and Johnson, 2007; Liao et al., 2010; Rikke et al., 2010)]. In the present study, we demonstrate that genetically based differences in MR can account for equivocal results of the studies on directional effects of DR on metabolism. We propose that physiological responses to DR depend on the level of metabolism characteristic of individuals subject to DR. More specifically, we predict that the magnitude of DR-elicited reduction of MR should be larger in individuals having higher initial MR. Conversely, the effect of DR on individuals characterized by low MR should be smaller.

It is unclear, however, which measure of MR is most relevant to DR. Many studies have adopted basal MR (BMR) or resting MR as a measure of energy expenditure (e.g. Faulks et al., 2006; Ferguson et al., 2007; Sohal et al., 2009). This seems well justified, as BMR may represent a significant part of an animal's total energy expenditure (e.g. Speakman, 1999; Speakman et al., 2003), and is significantly related to lifespan and mortality (Speakman et al., 2004; Ruggiero et al., 2008). In contrast, both DR and ageing may independently reduce the metabolic processes underlying thermogenic and exercise capacity (Keys et al., 1950; Weiss et al., 2007), which may be the most significant drawback of DR treatment from both evolutionary and medical points of view. For this reason, here we studied both BMR and peak MR (PMR) elicited by swimming, which we adopted as a metabolic measure of animal's physical fitness.

From a methodological perspective, a strong test of the effect of DR on MR should be provided by an artificial selection experiment, which allows for manipulation of allele frequencies directly related to the expected outcomes of DR treatment (for a review, see Garland and Rose, 2009). Here, we report the results of an experiment in which we used two line types of laboratory mice divergently selected for BMR (Książek et al., 2004; Brzęk et al., 2007; Gębczyński and

Konarzewski, 2009a; Gębczyński and Konarzewski, 2009b). There are two reasons why these line types of mice are good models for studying DR. First, apart from a 30% difference in BMR, they differ distinctly with respect to daily food consumption [higher in mice with high BMR (H-BMR) (Książek et al., 2004)], PMR [higher in mice with low BMR (L-BMR) (Książek et al., 2004)], and the relative sizes of the small intestine, liver, kidneys and heart [larger in H-BMR mice (Książek et al., 2004)]. Second, our mouse line types also differ with respect to the proportion of unsaturated fatty acids (FAs) in cell membrane phospholipids [higher in L-BMR mice (Brzęk et al., 2007)], which are particularly prone to oxidative stress (Hulbert, 2005).

In our experiment, we analyzed the effects of long-term (6 month), 30% DR in mice from both selected line types differing with respect to initial levels of BMR. We expected that mice from the H-BMR line type should be more likely to show DR-induced decreases in both total and body mass (M_b)-corrected BMR. More specifically, we asked whether long-term DR affects between-line-type differences in BMR through: (1) reduction in the size of metabolically active internal organs (such as the liver and heart) or other body components (such as fat stores), and/or (2) reduction in mass-corrected MR and changes in related traits, such as composition of FA in cell membrane phospholipids. Furthermore, we ascertained the effects of long-term DR on exercise and thermogenic capacity by measuring PMR and hypothermia elicited by 5 min swimming in water at 25°C (ΔT_{swim}), and compared these effects with line-type-specific changes in BMR. Finally, to relate whole-body metabolic responses to the molecular level, we quantified the effect of DR on the anti-oxidative capacity of blood serum (an index of total antioxidant capacity) and the FA composition of cell membrane phospholipids in the liver and kidney (an index of susceptibility to oxidative stress).

MATERIALS AND METHODS

Animals and housing

Subjects in our experiment were Swiss-Webster mice (*Mus musculus* Linnaeus 1758) selectively bred towards high and low M_b-corrected BMR as described in detail elsewhere (Książek et al., 2004; Brzęk et al., 2007; Gębczyński and Konarzewski, 2009a; Gębczyński and Konarzewski, 2009b). Briefly, the BMR of 12–16-week-old mice was measured for 3 h in an open-circuit respirometry system at an ambient temperature of 32°C. Males and females characterized by the highest and lowest mass-corrected BMR were chosen as progenitors of the H-BMR and L-BMR selection lines, respectively. A similar procedure was repeated in subsequent offspring generations, yielding significant differentiation of the lines with respect to BMR, without simultaneous changes in M_b. Although both line types came from an unreplicated selection experiment, between-line-type differences in BMR and several other traits are 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, 2009a). Throughout the course of the selection experiment, mice were maintained in a climatic chamber at an ambient temperature of 23°C under a 12 h:12 h light:dark cycle. The same conditions were also applied during the present experiment. All experimental procedures were approved by the Local Ethical Committee in Białystok (permission 2003/34).

Experimental design and sequence of measurements

Our experiment was carried out on males of generation F24. BMR was measured at the age of 4–5 months (for details, see Książek et al., 2004). After BMR measurement was completed, we measured the food intake of all mice (see below for methods) and then randomly assigned half of the mice within each of the selected lines to one of two feeding regimens: (1) unlimited access to food [*ad libitum* (AL) regimen], or (2) every 2 days mice were fed a precise amount of food equal to 70% of their individually measured, 2 day *ad libitum* food consumption (DR). This design created four experimental groups (34 individuals each): (1) H-BMR mice fed AL, mean (±s.e.m.) 2 day food consumption 12.44±0.18 g; (2) H-BMR mice fed DR, offered 8.74±0.19 g every 2 days; (3) L-BMR mice fed AL, mean 2 day food consumption 10.69±0.18 g; and (4) L-BMR mice fed DR, offered 7.33±0.18 g every 2 days. All mice were housed individually and had unlimited access to water.

The described feeding experiment began when mice were 6 months old and lasted for 6 months. Upon completion of the trial, the 12-month-old mice were subjected to a second BMR measurement. Next, all individuals were subjected to measurements of PMR elicited by forced swimming in 25°C water. PMR was defined as the highest oxygen consumption averaged over 2 min of a 5 min swim [for a detailed description of this procedure, see Gębczyński and Konarzewski (Gębczyński and Konarzewski, 2009b)]. Colonic temperature was measured to the nearest 0.1°C with a thermocouple thermometer (BAT-12, Physitemp Instruments, Clifton, NJ, USA), immediately before (hereafter referred to as pre-swim core body temperature, T) and after measurement of PMR. The difference between pre- and post-trial core temperatures was taken as the magnitude of ΔT_{swim}. All mice were then killed by cervical dislocation, and their metabolically active organs (liver, kidney, heart and small intestine) were excised and weighed. Liver and kidneys were immediately frozen in liquid nitrogen for later analyses of composition of membrane lipids. The remaining carcasses were stored at –20°C for body fat measurements.

Measurement of food intake

For measurements of food intake, mice were housed individually in cages equipped with plastic grids. Each mouse was fed a standard laboratory chow diet (Labofeed B, Wytwórnia Pasz A. Morawski, Kcynia, Poland; dietary proximate analysis was as follows: protein 174 g kg^{-1}, fat 32 g kg^{-1}, starch 290 g kg^{-1}, fiber 70 g kg^{-1}, ash 60 g kg^{-1}, metabolizable energy content 12.5 MJ kg^{-1}). Food remains (orts) dropping to the bottom of the cage were separated from feces, dried in an oven at 65°C, and weighed to the nearest 0.01 g. Average food intake was calculated individually for each mouse during two consecutive 2-day-long trials as the mass of food disappearing from the food dispenser minus orts. Plastic grids were removed from cages after completion of this trial.

Body fat measurement

The thawed carcasses were dried at 65°C to a constant mass, homogenized with an electric mill, and fat was extracted from homogenates with petroleum ether in a Soxhlet extractor. The residues were then re-dried, and the fat content was calculated as the mass lost during extraction (Sawicka-Kapusta, 1975).

FA composition

Liver and kidney samples were pulverized in an aluminum mortar with a stainless steel pestle pre-cooled in liquid nitrogen. The powder was then transferred to clean glass tubes containing methanol at a temperature of –20°C. Butylated hydroxytoluene (Sigma-Aldrich, St Louis, MO, USA) was added, as an antioxidant, to methanol at a dose of 30 mg 100 ml^{-1}. Lipids were extracted by the method of

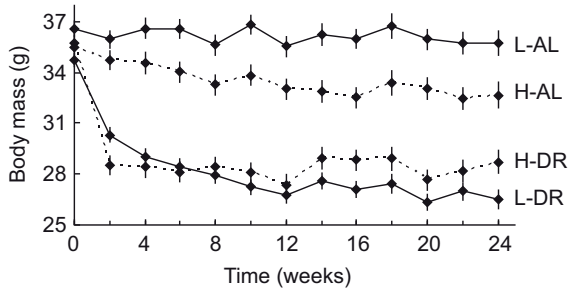

Fig. 1. Changes in body mass of mice in four experimental groups during the course of the present experiment. H-AL, high-basal metabolic rate (BMR) mice fed *ad libitum*; H-DR, high-BMR mice subject to DR; L-AL, low-BMR mice fed *ad libitum*; L-DR, low-BMR mice subject to DR. On this and subsequent graphs, means [or least-square (LS) means when indicated] ± s.e.m. are shown.

Folch et al. (Folch et al., 1957). To isolate phospholipids, the samples were fractionated on silica plates (Kieselgel 60, 0.22 mm, Merck, Darmstadt, Germany) using chloroform-methanol-acetic acid-water (50:37.5:3.5:2, v/v/v/v) as the developing solvent. Lipid bands were visualized under UV light after spraying with a 0.5% solution of 3′,7′-dichlorofluorescein in absolute methanol. The gel bands corresponding to phospholipids were scraped off of the plate and transferred into screw tubes containing methylpentadecanoic acid (Sigma-Aldrich) as an internal standard. FAs were then transmethylated along with the gel in the presence of 1 ml of 14% boron fluoride in methanol at 100°C for 90 min. The resulting methyl esters were extracted with hexane and analyzed by gas-liquid chromatography. A Hewlett-Packard 5890 Series II gas chromatograph with a double flame ionization detector and a fused HP-INNOWax (100 m) capillary column were used (Hewlett-Packard, Palo Alto, CA, USA). The injector and detector temperatures were both set at 250°C. The oven temperature was increased linearly from 160 to 230°C at rate of 5°C min^{-1}. Individual FA methyl esters were quantified using the area corresponding to the internal standards. FA standards were purchased from Sigma-Aldrich.

We expressed relative molar amounts of the most common FAs as their relative percentage of all FA chains. We also calculated the relative abundance of saturated (SFA; no double bonds), monounsaturated (MUFA; one double bond) and polyunsaturated (PUFA; at least two double bonds) FAs, the unsaturation index (UI; the average number of double bonds per 100 FA chains) and the average length of FA chains.

Analysis of antioxidative capacity of blood serum

We quantified the total antioxidative capacity of blood serum with a Cayman Chemical Antioxidant Assay Kit (no. 709001, Ann Arbor, MI, USA). This assay quantifies the overall ability of antioxidants in blood serum to inhibit the oxidation of 2,2′-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) by metmyoglobin. Blood samples of all mice were collected immediately after the animals were killed. Blood samples were centrifuged and blood serum was stored at –20°C and subsequently assayed according to the kit manual.

Statistical analyses

Results of anatomic and metabolic measurements in 12-month-old mice were analyzed by means of ANOVA or analysis of covariance (ANCOVA), with line type and feeding regimen as fixed factors, and family affiliation nested within line type as a random factor controlling for the effect of animals' relatedness (number of families: L-AL, 12; H-AL, 13; L-DR, 18; H-DR, 13). We analyzed data in two ways: (1) without M_b as covariate – here, we tested for changes in total values of measured parameters; and (2) with M_b or mass of body organs as covariates – here, we analyzed for changes in mass-corrected values. Different covariates were applied for particular traits (as listed in the Results). For most traits measured in 12-month-old mice, lean M_b (M_{lean}, i.e. total mass minus the mass of fat and digestive tract content) was used as a covariate. In separate analyses, BMR measured at that age was also corrected for the summed mass of metabolically active internal organs (liver, kidney, heart and small intestines), or for the mass of remaining body carcass (i.e. M_{lean} minus mass of these internal organs).

We applied repeated-measures ANOVA to compare M_b at 6 and 12 months of age (i.e. at the beginning and at the end of experiment). We did not analyze M_b during the whole course of experiment because of the non-linearity of changes in M_b with time (see Fig. 1). FA composition in 12-month-old mice was analyzed by means of ANOVA with line type and feeding regimen as fixed factors, family affiliation nested within line type as a random factor and the respective interaction terms.

Differences were considered significant at $P<0.05$. In all analyses, factors other than line type and feeding regimen were included in the final model only when their P-values were <0.05. Whenever significant interactions between line type and feeding regimen were detected, we tested for inter-group differences by means of a Tukey's test (separate ANOVA/ANCOVA analyses within each of the fixed effects produced the same results). All tests were carried out using the GLM procedure in SAS 9.1.3 (SAS Institute, Cary, NC, USA).

Table 1. Summary of repeated-measures ANOVA on body mass of mice (*Mus musculus*) from both *ad libitum* (AL) and dietary restriction (DR) feeding regimens and from each feeding regimen separately

	AL and DR feeding regimen			AL feeding regimen			DR feeding regimen		
	F	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*
Feeding regimen	47.27	1,49	<0.0001						
Line type	0.06	1,49	0.80	3.40	1,17	0.083	5.89	1,46	0.019
Feeding regimen × line type	7.72	1,49	0.0077						
Family affiliation	2.83	40,49	0.0003	2.77	26,17	0.016	n.s.	n.s.	n.s.
Time	201.36	1,49	<0.0001	11.56	1,17	0.0034	416.22	1,46	<0.0001
Time × feeding regimen	85.41	1,49	<0.0001						
Time × line type	2.04	1,49	0.16	8.51	1,17	0.0096	0.26	1,46	0.61
Time × line type × feeding regimen	7.75	1,49	0.0076						
Time × family affiliation	1.22	40,49	0.25	1.34	26,17	0.27	n.s.	n.s.	n.s.

n.s. not significant.

Table 2. Summary of ANOVA/ANCOVA for total (whole body) and lean body mass (M_{lean})-corrected mass of internal organs of mice

	Feeding regimen			Line type			Family affiliation			M_{lean}[a]		
	F	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*
Total mass												
Fat[b]	30.87	1,83	<0.0001	29.36	1,83	<0.0001	n.s.	n.s.	n.s.			
Liver	38.37	1,84	<0.0001	11.66	1,84	0.001	n.s.	n.s.	n.s.			
Kidneys	44.27	1,46	<0.0001	29.86	1,46	<0.0001	1.95	38,46	0.016			
Heart	91.27	1,46	<0.0001	18.24	1,46	<0.0001	2.99	38,46	0.0002			
Intestine	0.00	1,46	0.98	28.61	1,46	<0.0001	2.12	38,46	0.0077			
M_{lean}-corrected mass												
Fat[c]	1.01	1,82	0.32	60.06	1,82	<0.0001	n.s.	n.s.	n.s.	72.86	1,82	<0.0001
Liver	3.49	1,83	0.065	20.21	1,83	<0.0001	n.s.	n.s.	n.s.	81.52	1,83	<0.0001
Kidneys	1.23	1,45	0.27	49.04	1,45	<0.0001	1.75	38,45	0.037	42.78	1,45	<0.0001
Heart	18.66	1,45	<0.0001	19.75	1,45	<0.0001	2.95	38,45	0.0003	13.51	1,45	0.0006
Intestine	0.80	1,45	0.37	28.92	1,45	<0.0001	2.14	38,45	0.0074	1.33	1,45	0.26

[a]Mass of analyzed organ was subtracted from M_{lean}.
[b]Interaction between feeding regimen and line type: $F_{1,83}$=16.22, P=0.0001.
[c]Interaction between feeding regimen and line type: $F_{1,82}$=6.52, P=0.012.
Interaction between feeding regimen and line type was never significant for internal organs. M_{lean} was not significant as a covariate for intestine mass, but we present this analysis for comparison with other organs.

RESULTS

Effect of feeding regimen on M_b, fat content and mass of internal organs

DR resulted in a reduction of M_b in DR mice (Fig. 1). However, the effect of feeding regimen was line-type-dependent, as indicated by significant three-way interaction between line type, feeding regimen and the time course of the experiment (Table 1). Separate repeated-measures ANOVAs within each of the feeding regimens suggest that this interaction reflected line-type-specific changes of M_b in AL-fed mice (significant interaction between line type and the time course within this feeding regimen; Table 1), whereas DR affected M_b similarly in mice from both line types (no significant interaction between line type and the time course in DR feeding regimen; Table 1).

A significant feeding regimen × line type interaction revealed that the effect of DR on total and M_{lean}-corrected fat mass was also line-type-dependent (Table 2, Fig. 2A,B). A Tukey's test revealed that DR significantly reduced total fat mass in the L-BMR (P<0.0001) but not the H-BMR line type (P=0.72; Fig. 2A). However, when corrected for M_{lean}, DR-elicited changes of fat mass were not significant within either of the line types (P>0.1 in both cases; significant interaction reflected the opposite directions of non-significant trends; Fig. 2B), which indicates that reduction of total fat mass was strictly proportional to changes in M_{lean}.

H-BMR mice retained significantly larger total and M_{lean}-corrected masses of all internal organs (Table 2, Fig. 3). DR significantly reduced total masses of the liver, kidneys and heart but not the small intestine; when corrected for M_{lean}, the effect of DR remained significant only for the heart mass (Table 2, Fig. 3F). The lack of significant interactions between line type and feeding regimen in these analyses revealed that the effect of DR on organ mass was similar in both line types.

Effect of experimental treatment on BMR

The effects of feeding regimen and line type on the final level of BMR (measured at the completion of the experiment, in 12-month-old mice) depended on how we corrected BMR for the effect of body or organ masses. There was a significant interaction between line type and feeding regimen for M_{lean}-corrected and lean-carcass-corrected BMR (Table 3, Fig. 4B,C). A Tukey's test revealed that DR reduced M_{lean}-corrected BMR in H-BMR (P=0.0068) but not in L-BMR mice (P=1; Fig. 4B). We obtained similar results when BMR was corrected for lean carcass mass (H-BMR line type: P=0.0042; L-BMR line type: P=0.98; Fig. 4C). In contrast, there was no significant DR × line type interaction when BMR was corrected for the summed mass of all four metabolically active organs studied here (Table 3, Fig. 4D).

Effect of experimental treatment on PMR and *T*

The effect of feeding regimen on total PMR was line-type-dependent, as indicated by strong line type × feeding regimen interaction (Table 4, Fig. 5A). A Tukey's test showed that DR significantly reduced total PMR in mice of both line types (P<0.0001 in both cases). However, within AL mice, L-BMR individuals had significantly higher PMR than H-BMR individuals (P=0.0006), whereas the between-line-type difference was not significant within DR mice (P=0.96). This indicates that total PMR decreased more in the L-BMR than the H-BMR line type. However, the line type × feeding regimen interaction became non-significant when PMR

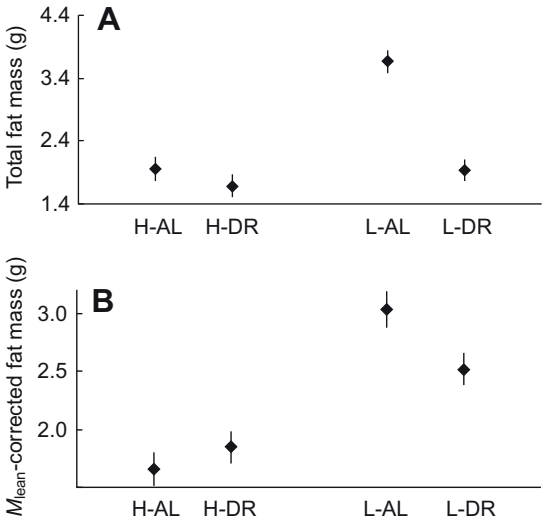

Fig. 2. Total (A) and lean body mass (M_{lean})-corrected (B; LS means) fat mass of mice in four experimental groups after completion of the present experiment. For further details see Fig. 1.

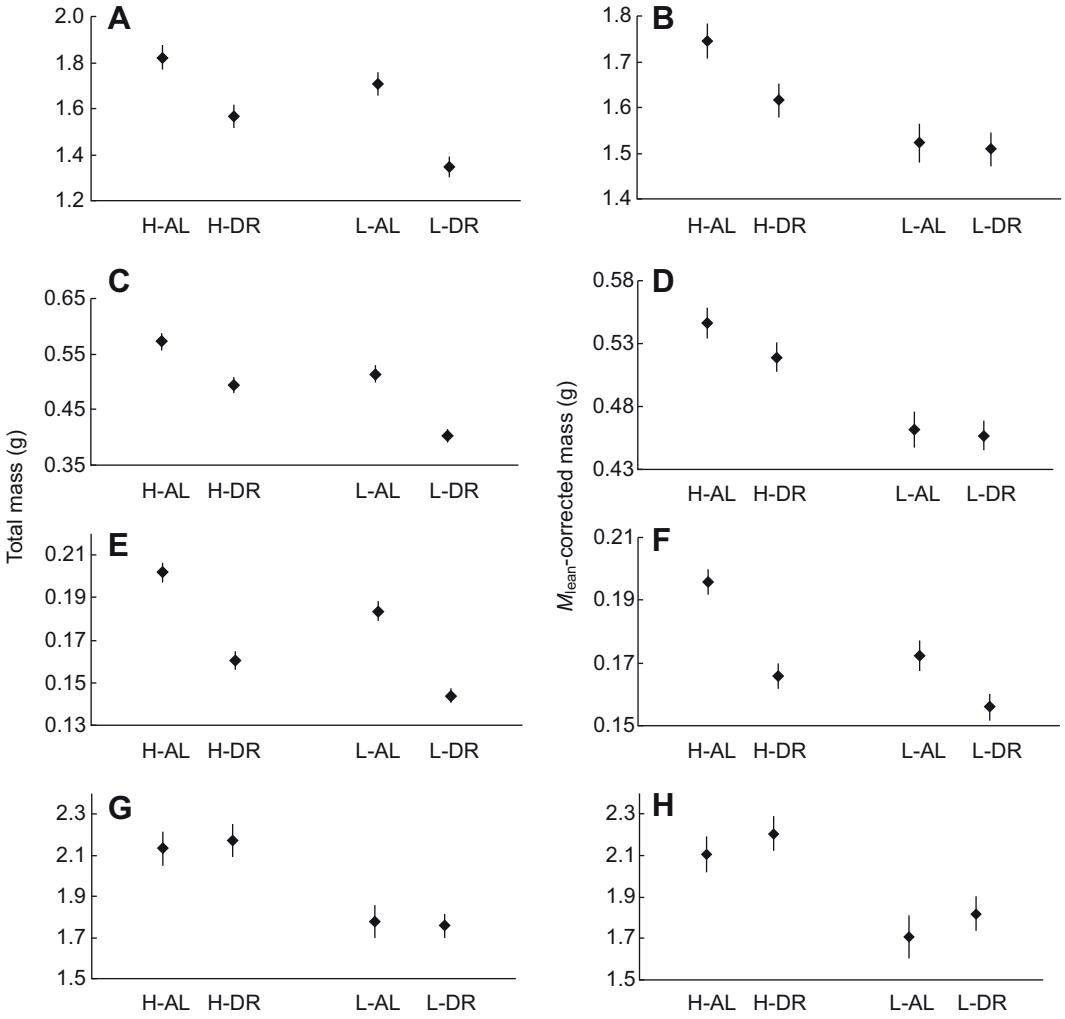

Fig. 3. Total (left column) and M_{lean}-corrected (right column; LS means) masses of the liver (A,B), kidney (C,D), heart (E,F) and small intestine (G,H) of mice in four experimental groups after completion of the present experiment. For further details see Fig. 1.

was controlled for M_{lean} (Table 4, Fig. 5B), which suggests that DR-elicited reduction in PMR largely reflects a reduction in M_b, rather than in mass-corrected MR.

DR significantly reduced T in mice of both line types (Table 4, Fig. 5C). Interestingly, DR reduced body temperature similarly in both line types (Fig. 5C), despite significant differences in BMR. This inconsistency of DR-mediated changes in BMR and T corresponds with the lack of differences in T of H-AL and L-AL mice, despite their conspicuously different M_{lean}-corrected BMRs (Fig. 4, Fig. 5C).

The effect of DR on ΔT_{swim} was much stronger in L-BMR than H-BMR mice (Table 4, Fig. 5D): DR significantly increased ΔT_{swim} in L-BMR but not in H-BMR mice (Tukey's test; $P>0.0001$ and $P=1$, respectively). However, although the interaction between line type and feeding regimen remained significant when ΔT_{swim} was corrected for M_{lean} (Table 4), the effect of DR was no longer significant for either line type ($P>0.1$ in both cases; Fig. 5E). Thus, the interaction most likely reflected opposite, non-significant effects of DR within each of the line types.

Antioxidative capacity of blood serum

Neither feeding regimen ($F_{1,79}=1.05$, $P=0.31$) nor line type ($F_{1,79}=1.93$, $P=0.17$) had significant effects on the antioxidant capacity of blood serum (feeding regimen × line type interaction was non-significant; data not shown).

FA composition of cell membrane phospholipids

DR significantly modified FA composition of membrane phospholipids in liver and kidneys (Table 5, supplementary material Tables S1, S2). In general, DR elevated the content of MUFA at the expense of SFA (only in the liver) and PUFA. As a result, DR significantly reduced UI (Table 5). DR affected the FA profiles of cell membranes similarly in mice from both selected line types, as shown by an almost complete lack of significant interactions between line type and feeding regimen for analyzed parameters (Table 5, supplementary material Tables S1, S2). Line type had little effect on cell membrane phospholipids, except for higher DHA (22:6) content in the livers of L-BMR mice (supplementary material Table S1).

DISCUSSION

DR elicited a significant reduction of both total BMR (Fig. 4A) and total PMR (Fig. 5A). However, because the effect of DR on total M_b, fat mass and M_{lean} depended on the effect of line type (Figs 1, 2), all other results had to be corrected for M_b or M_{lean} to analyze how DR affected mice from both line types. Our findings suggest that the effect of DR frequently depended on the initial between-line-type differences in the studied traits.

Effect of experimental treatment on BMR

Mice used in our study came from line types divergently selected for BMR measured at the age of 3–5 months (Książek et al., 2004).

Table 3. Summary of ANOVA/ANCOVA of total (whole body), M_{lean}-, lean-carcass-mass- and organ-mass-corrected basal metabolic rate measured at the completion of the experiment in 12-month-old mice

	Feeding regimen			Line type			Feeding regimen × line type			Covariate		
	F	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*
Total	18.66	1,84	<0.0001	42.91	1,84	<0.0001	n.s.	n.s.	n.s.			
M_{lean}-corrected	4.72	1,82	0.033	73.45	1,82	<0.0001	5.25	1,82	0.025	50.94	1,82	<0.0001
Lean-carcass-mass-corrected	7.05	1,82	0.0095	77.20	1,82	<0.0001	4.17	1,82	0.044	36.99	1,82	<0.0001
Organ-mass-corrected	5.48	1,83	0.022	10.13	1,83	0.0021	n.s.	n.s.	n.s.	37.21	1,83	<0.0001

The effect of family affiliation was never significant, and is thus not presented here.

Here, we demonstrated that between-line-type differences in M_b-corrected BMR and organ masses observed at that age (Książek et al., 2004; Brzęk et al., 2007) are preserved after 6 months of DR (Figs 3, 4). However, DR reduced M_{lean}-corrected BMR only in mice from the H-BMR line type, and had no significant effect on mice from the L-BMR line type (Fig. 4B).

The DR-induced changes in MR may be due to concurrent reduction in the mass of metabolically active internal organs, fat and/or the proportion of remaining body components (carcasses), or in their mass-corrected MR. BMR mainly reflects the metabolic costs of maintenance of internal organs related to food processing, such as the liver, kidney and heart (Konarzewski and Diamond, 1995). Following DR treatment, these organs became smaller in mice of both line types (Fig. 3), which was reflected in the observed reduction of total BMR (Fig. 4A). In contrast, however, DR did not affect M_{lean}-corrected organ masses, except for the heart (Table 2), which suggests that organ mass reduction was proportional to M_{lean} reduction. Thus, organ downsizing had a weak effect on M_b-corrected BMR. Indeed, the interaction between line type and feeding regimen disappeared when BMR was corrected for summed mass of internal organs (Table 3, Fig. 4D). All of these results suggest that the observed DR-elicited changes in M_{lean}-corrected BMR (Fig. 4B) are due to line-type-specific changes in fat and carcass mass, rather than organ mass. This is supported by the significant interaction between line type and feeding regimen in the analysis of BMR corrected for carcass mass (i.e. M_b without fat and internal organs; Fig. 4C). Likewise, Faulks et al. (Faulks et al., 2006) reported a stronger effect of DR on mice's relative size of skin than that of liver, heart or kidneys.

DR resulted in a significant reduction of the UI in cell membrane phospholipids of the liver and kidneys (Table 5). Low UI of cell membrane phospholipids is considered a hallmark for low rate of metabolic processes (Hulbert, 2007; but see Brzęk et al., 2007), and may suggest that DR not only reduced the size of internal organs but also downregulated their mass-corrected metabolism. This is indirectly supported by significant DR-elicited reduction of BMR corrected for the summed mass of internal organs (Table 3, Fig. 4D). However, because the magnitude of UI reduction was similar in both line types (Table 5), we conclude that the reduction in mass-corrected MR was not affected by a between-line-type difference in BMR.

Our experimental mice came from non-replicated selection (Książek et al., 2004). Nevertheless, we have repeatedly shown that differences between H-BMR and L-BMR line types in BMR, size of internal organs and composition of cell membrane FAs arose as a result of selection rather than genetic drift (Książek et al., 2004; Brzęk et al., 2007). This consistency allows us to assume that the responses to DR reported in the present study also indirectly resulted from the selection on BMR, rather than random fluctuations of phenotypes driven by genetic drift. However, it must be kept in mind that responses to DR reported therein might be specific to the genetic make-up of our selected mice and therefore cannot be considered as universal [particularly because of significant between-strain variation in response to DR in mice (e.g. Rikke et al., 2003; Liao et al., 2010)]. In any case, our study suggests that inconsistent results of earlier studies on associations between metabolism and long-term DR may stem from between-study differences in the initial level of MR. For example, although some authors have reported that DR does not affect or even elevates mass-corrected MR (e.g. McCarter et al., 1985; Selman et al., 2005; Faulks et al., 2006; Hempenstall et al., 2010), others found a significant reduction in the metabolism of DR-treated mice (Ferguson et al., 2007; Sohal et al., 2009). Likewise, some

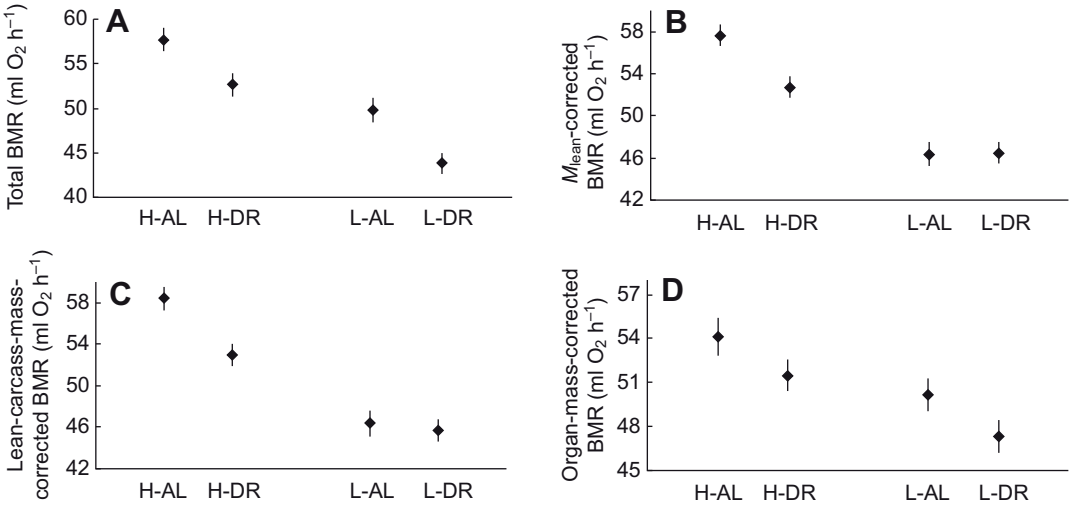

Fig. 4. Total (A), M_{lean}-corrected (B), lean-carcass-mass-corrected (C) and organ-mass-corrected (D) BMR measured in mice during the present experiment (panel A shows means, panels B–D show LS means). For further details, see Fig. 1.

Table 4. Summary of ANOVA/ANCOVA of total (whole body) and M_{lean}-corrected peak metabolic rate (PMR), pre-swim core body temperature (T) and post-swim hypothermia (ΔT_{swim})

	Feeding regimen			Line type			Feeding regimen × line type			M_{lean}		
	F	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*
Total												
PMR[a]	140.34	1,44	<0.0001	9.15	1,44	0.0041	12.43	1,44	0.001			
T	13.05	1,84	0.0005	0.48	1,84	0.49	n.s.	n.s.	n.s.			
ΔT_{swim}	13.18	1,82	0.0005	33.17	1,82	<0.0001	13.00	1,82	0.0005			
M_{lean}-corrected												
PMR[b]	34.53	1,41	<0.0001	8.00	1,41	0.0072	n.s.	n.s.	n.s.	15.54	1,41	0.0003
ΔT_{swim}	1.00	1,81	0.32	39.79	1,81	<0.0001	6.67	1,81	0.012	12.45	1,81	0.0007

M_{lean} was not significant as covariate for T.
[a]Effect of family affiliation: $F_{37,44}$=3.15, P=0.0002.
[b]Effect of family affiliation: $F_{37,41}$=2.05, P=0.013; effect of channel in respirometer: $F_{3,41}$=3.57, P=0.022.

studies reported a significant decrease of BMR with age (e.g. Speakman et al., 2003; Ruggiero et al., 2008; Moe et al., 2009), whereas others found no BMR change (e.g. O'Connor et al., 2002; Chappell et al., 2003; Moe et al., 2007). None of these studies considered the initial level of MR, which, as we have shown here, can significantly affect the responses to DR and age-related changes (i.e. the effect of time course in our experiment). This concurs with a study by Speakman et al. (Speakman et al., 2004), which showed that a 17% difference in initial RMR in mice (i.e. two times lower than in our experiment) resulted in a 36% difference in their lifespan. Similarly, Ruggiero et al. (Ruggiero et al., 2008) demonstrated that variation in BMR is correlated with mortality risk in humans. Thus, we suggest that metabolic responses to DR are more likely to be found in populations with a high initial variation in MR, where at least some individuals can manifest significant DR-related changes in the studied parameters.

Effect of experimental treatment on PMR

At the age of 3–5 months, AL-fed mice from the L-BMR line type have higher PMR than those from the H-BMR line type (Książek et al., 2004) and this difference was preserved over the course of our 6-month-long experiment (Table 4, Fig. 5A,B). However, the PMR of mice subjected to DR was significantly reduced, with a much stronger effect on L-BMR than H-BMR mice (Fig. 5A,B), which was analogous to a larger DR-elicited reduction of BMR in the H-BMR mice. This important observation deserves two comments. First, just like in the case of BMR, changes in PMR elicited by DR seem to be proportional to its initial level, which highlights the importance of variation in the level of metabolic traits of individuals in studies on DR and ageing. Second, changes in body components and composition, discussed above in the context of BMR variation, clearly do not correspond with the magnitude of between-line-type differences in DR-induced

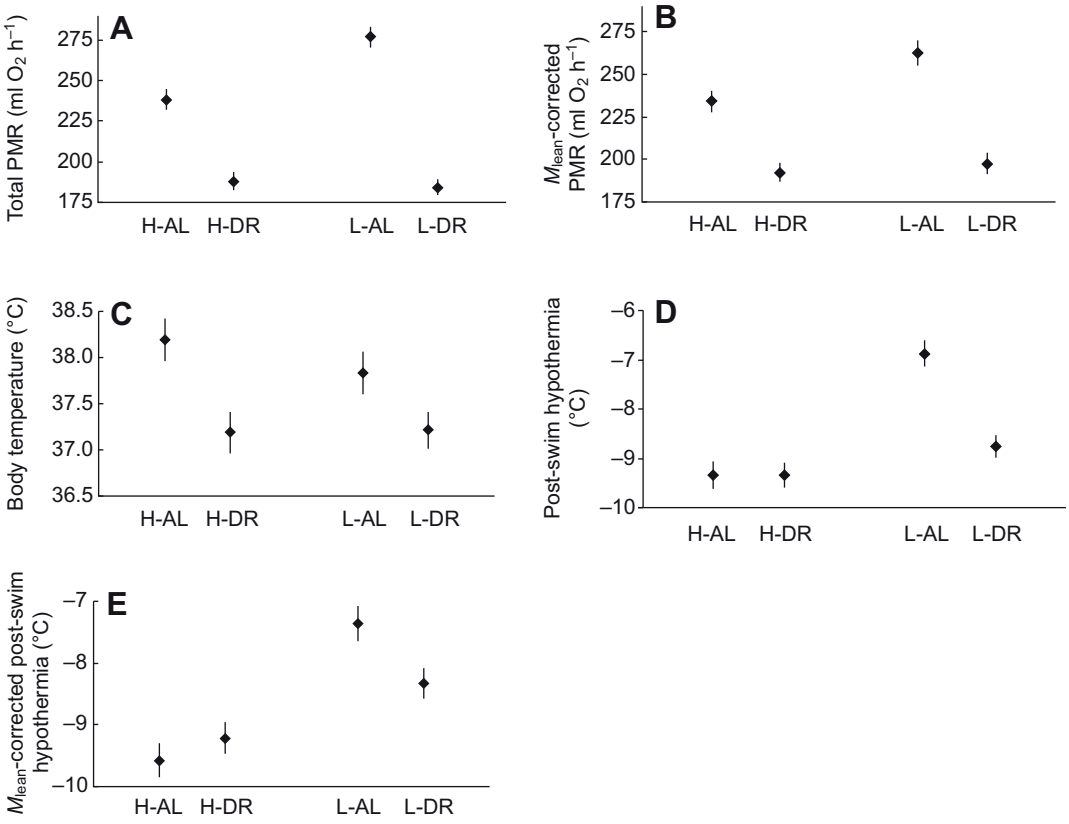

Fig. 5. Total peak metabolic rate (PMR) (A), M_{lean}-corrected PMR (B; LS means), core body temperature (C), post-swim hypothermia (D) and M_{lean}-corrected post-swim hypothermia (E; LS means) of mice in four experimental groups after completion of the present experiment. For further details, see Fig. 1.

Table 5. Composition of total cell membrane phospholipids from liver and kidney in mice from four experimental groups and summary of ANOVA results

	Experimental group				Feeding regimen		Line type	
	H-AL	H-DR	L-AL	L-DR	*F*	*P*	*F*	*P*
Liver								
SFA	48.1±0.3	46.6±0.3	48.2±0.3	46.0±0.3	35.67	<0.0001	0.47	0.5
MUFA	11.4±0.4	15.3±0.3	10.8±0.4	15.7±0.3	164	<0.0001	0.01	0.9
PUFA	40.5±0.3	38.1±0.3	40.9±0.3	38.2±0.3	58.07	<0.0001	0.59	0.44
UI	160.2±1.1	153.9±1.1	161.5±1.1	156.7±1.0	24.95	<0.0001	3.64	0.06
Length	18.0±0.1	17.9±0.1	18.0±0.1	17.9±0.1	36.79	<0.0001	0.79	0.38
Kidney								
SFA	47.5±0.5	48.3±0.4	46.9±0.5	47.7±0.4	2.76	0.1	1.68	0.2
MUFA	8.4±0.2	10.2±0.2	8.2±0.2	10.6±0.2	112	<0.0001	0.24	0.63
PUFA	44.1±0.4	41.6±0.4	44.9±0.4	41.8±0.4	52.2	<0.0001	1.53	0.22
UI	189.1±2.1	175.3±2.1	195.4±2.1	176.5±1.9	64.7	<0.0001	2.99	0.088
Length	18.5±0.1	18.3±0.1	18.5±0.1	18.3±0.1	79.4	<0.0001	3.16	0.079

H-AL, high-basal metabolic rate (BMR) mice fed *ad libitum*; H-DR, high-BMR mice subject to dietary restriction (DR); L-AL, low-BMR mice fed *ad libitum*; L-DR, low-BMR mice subject to DR.

Data are means ± s.e.m. Units for saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) are percentage of total FA; units for unsaturation index (UI) are the average number of double bonds per 100 FA chains; units for length are the average number of carbon atoms in the FA chain.

d.f.=1,84 for liver and 1,83 for kidney.

reduction of BMR and PMR. This indicates that both BMR and PMR are largely underlined by different metabolic pathways, and that they are independently affected by DR treatment (see also Rikke and Johnson, 2007).

Indeed, except for the heart, changes in the masses of other metabolically active internal organs are likely to contribute to changes in BMR, rather than PMR (Książek et al., 2004; Gębczyński and Konarzewski, 2011). We showed earlier that swim-elicited PMR involves not only an increased MR due to locomotor activity, but also a significant component of thermogenic capacity related to a higher mass of brown adipose tissue in L-BMR mice (Książek et al., 2004; Gębczyński, 2008). There are two lines of evidence that suggest that reduction of thermogenic capacity is associated with the DR-elicited changes of PMR. First, DR caused a significant decrease of T measured prior to swimming (Table 4, Fig. 5C). Second, the effect of DR on ΔT_{swim} was stronger in L-BMR than H-BMR mice (Table 4, Fig. 5D; although this can be partly explained by differences in body mass, Fig. 5E); this pattern was also found in PMR (Fig. 5A,B). Taken together, our results indicate that DR reduced thermogenic capacity, but only in L-BMR mice that had a higher initial level of this parameter than H-BMR mice.

Effect of DR on susceptibility to oxidative stress

Although a higher level of BMR may be associated with more effective antioxidative protection (e.g. Speakman et al., 2002), we did not find a significant effect of either line type or DR on antioxidative capacity of blood serum. The lack of a beneficial effect of DR on antioxidative mechanisms has been reported in many other studies (see Masoro, 2002; Masoro, 2005). However, DR presumably increases the resistance to oxidative stress through decreased UI in cell membrane lipids (Merry, 2002; Hulbert, 2005; Hulbert et al., 2007). In our experiment, DR reduced the UI of mice from both line types by 3–4% in liver and 7–10% in kidneys (Table 5). These values are comparable to those reported by Faulks et al. (Faulks et al., 2006) for mice subjected to identical DR regimens, and are probably sufficient to exert a beneficial effect on lifespan (Hulbert et al., 2006). However, our results provide no support for the association between variation in MR and mechanisms underlying protection against oxidative stress (antioxidant capacity of blood serum or DR-mediated changes in susceptibility of cell membranes for oxidative stress).

Conclusions

To the best of our knowledge, this study offers the first experimental analysis of the link between DR-induced changes in BMR and PMR, and differences in their initial levels within a given pool of studied animals. We found that long-term DR had a stronger effect on BMR, PMR and thermogenic capacity in animals characterized by their higher initial levels. These results have three important implications for studies of the effect of DR on metabolism and ageing. First, they suggest that the initial level of BMR can affect how DR modulates lifespan. Second, initial differences in MR can significantly affect the responses to DR and should be controlled for, because they can confound the interpretation of results. Third, even within a given set of experimental animals, DR can affect different measures of metabolism in different ways (e.g. BMR and PMR in our case), and thus the choice of studied metabolic traits may affect the final conclusions of the experiment.

LIST OF SYMBOLS AND ABBREVIATIONS

BMR	basal metabolic rate
DR	dietary restriction
FA	fatty acid
H-BMR, L-BMR	mice selected for high and low BMR, respectively
M_b	body mass
M_{lean}	lean body mass
MR	metabolic rate
MUFA	monounsaturated fatty acid
PMR	peak metabolic rate
PUFA	polyunsaturated fatty acid
SFA	saturated fatty acid
T	pre-swim core body temperature
UI	unsaturation index
ΔT_{swim}	post-swim hypothermia

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REFERENCES

Anderson, R. M. and Weindruch, R. (2010). Metabolic reprogramming, caloric restriction and aging. *Trends Endocrinol. Metab.* **21**, 134-141.
Barja, G. (2007). Mitochondrial oxygen consumption and reactive oxygen species production are independently modulated: implications for aging studies. *Rejuvenation Res.* **10**, 215-224.
Brzęk, P., Bielawska, K., Książek, A. and Konarzewski, M. (2007). Anatomic and molecular correlates of divergent selection for basal metabolic rate in laboratory mice. *Physiol. Biochem. Zool.* **80**, 491-499.
Chappell, M. A., Rezende, E. L. and Hammond, K. A. (2003). Age and aerobic performance in deer mice. *J. Exp. Biol.* **206**, 1221-1231.
Faulks, S. C., Turner, N., Else, P. L. and Hulbert, A. J. (2006). Calorie restriction in mice: effects on body composition, daily activity, metabolic rate, mitochondrial reactive oxygen species production, and membrane fatty acid composition. *J. Gerontol. A Biol. Sci. Med. Sci.* **61**, 781-794.
Ferguson, M., Sohal, B. H., Forster, M. J. and Sohal, R. S. (2007). Effect of long-term caloric restriction on oxygen consumption and body temperature in two different strains of mice. *Mech. Ageing Dev.* **128**, 539-545.
Finkel, T. and Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature* **408**, 239-247.
Folch, J., Lees, M. and Sloane-Stanley, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**, 497-509.
Garland, T., Jr and Rose, M. R. (2009). *Experimental Evolution: Concepts, Methods, and Applications of Selection Experiments*. Berkeley, CA: University of California Press.
Gębczyński, A. K. (2008). Nonshivering thermogenesis capacity *versus* basal metabolic rate in laboratory mice. *J. Therm. Biol.* **33**, 250-254.
Gębczyński, A. K. and Konarzewski, M. (2009a). Locomotor activity of mice divergently selected for basal metabolic rate: a test of hypotheses on the evolution of endothermy. *J. Evol. Biol.* **22**, 1212-1220.
Gębczyński, A. K. and Konarzewski, M. (2009b). Metabolic correlates of selection on aerobic capacity in laboratory mice: a test of the model for the evolution of endothermy. *J. Exp. Biol.* **212**, 2872-2878.
Gębczyński, A. K. and Konarzewski, M. (2011). Effects of oxygen availability on maximum aerobic performance in *Mus musculus* selected for basal metabolic rate or aerobic capacity. *J. Exp. Biol.* **214**, 1714-1720.
Hempenstall, S., Picchio, L., Mitchell, S. E., Speakman, J. R. and Selman, C. (2010). The impact of acute caloric restriction on the metabolic phenotype in male C57BL/6 and DBA/2 mice. *Mech. Ageing Dev.* **131**, 111-118.
Hulbert, A. J. (2005). On the importance of fatty acid composition of membranes for aging. *J. Theor. Biol.* **234**, 277-288.
Hulbert, A. J. (2007). Membrane fatty acids as pacemakers of animal metabolism. *Lipids* **42**, 811-819.
Hulbert, A. J., Faulks, S. C., Harper, J. M., Miller, R. A. and Buffenstein, R. (2006). Extended longevity of wild-derived mice is associated with peroxidation-resistant membranes. *Mech. Ageing Dev.* **127**, 653-657.
Hulbert, A. J., Pamplona, R., Buffenstein, R. and Buttemer, W. A. (2007). Life and death: metabolic rate, membrane composition, and life span of animals. *Physiol. Rev.* **87**, 1175-1213.
Keys, A., Brozek, J., Henschel, A., Mickelsen, O. and Taylor, H. L. (1950). *The Biology of Human Starvation.* Minneapolis, MN: University of Minnesota Press.
Konarzewski, M. and Diamond, J. (1995). Evolution of basal metabolic rate and organ masses in laboratory mice. *Evolution* **49**, 1239-1248.
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.
Liao, C. Y., Rikke, B. A., Johnson, T. E., Diaz, V. and Nelson, J. F. (2010). Genetic variation in the murine lifespan response to dietary restriction: from life extension to life shortening. *Aging Cell* **9**, 92-95.
Masoro, E. J. (2002). *Caloric Restriction: A Key to Understanding and Modulating Aging.* Amsterdam: Elsevier.
Masoro, E. J. (2005). Overview of caloric restriction and ageing. *Mech. Ageing Dev.* **126**, 913-922.
McCarter, R. J. and Palmer, J. (1992). Energy metabolism and aging: a lifelong study of Fischer 344 rats. *Am. J. Physiol.* **263**, E448-E452.
McCarter, R. J., Masoro, E. J. and Yu, B. P. (1985). Does food restriction retard aging by reducing the metabolic rate? *Am. J. Physiol.* **248**, E488-E490.
Merry, B. J. (2002). Molecular mechanisms linking calorie restriction and longevity. *Int. J. Biochem. Cell Biol.* **34**, 1340-1354.
Moe, B., Angelier, F., Bech, C. and Chastel, O. (2007). Is basal metabolic rate influenced by age in a long-lived seabird, the snow petrel? *J. Exp. Biol.* **210**, 3407-3414.
Moe, B., Rønning, B., Verhulst, S. and Bech, C. (2009). Metabolic ageing in individual zebra finches. *Biol. Lett.* **5**, 86-89.
O'Connor, T. P., Lee, A., Jarvis, J. U. M. and Buffenstein, R. (2002). Prolonged longevity in naked mole-rats: age-related changes in metabolism, body composition and gastrointestinal function. *Comp. Biochem. Physiol.* **133A**, 835-842.
Ramsey, J. J. and Hagopian, K. (2006). Energy expenditure and restriction of energy intake: could energy restriction alter energy expenditure in companion animals? *J. Nutr.* **136**, 1958S-1966S.
Rikke, B. A. and Johnson, T. E. (2007). Physiological genetics of dietary restriction: uncoupling the body temperature and body weight responses. *Am. J. Physiol.* **293**, R1522-R1527.
Rikke, B. A., Yerg, J. E., III, Battaglia, M. E., Nagy, T. R., Allison, D. B. and Johnson, T. E. (2003). Strain variation in the response of body temperature to dietary restriction. *Mech. Ageing Dev.* **124**, 663-678.
Rikke, B. A., Liao, C. Y., McQueen, M. B., Nelson, J. F. and Johnson, T. E. (2010). Genetic dissection of dietary restriction in mice supports the metabolic efficiency model of life extension. *Exp. Gerontol.* **45**, 691-701.
Ruggiero, C., Metter, E. J., Melenovsky, V., Cherubini, A., Najjar, S. S., Ble, A., Senin, U., Longo, D. L. and Ferrucci, L. (2008). High basal metabolic rate is a risk factor for mortality: the Baltimore Longitudinal Study of Aging. *J. Gerontol. A Biol. Sci. Med. Sci.* **63**, 698-706.
Sacher, G. A. (1977). Life table modification and life prolongation. In *Handbook of the Biology of Aging* (ed. C. E. Finch and L. Hayflick), pp. 582-638. New York: Van Nostrand-Reinhold.
Sawicka-Kapusta, K. (1975). Fat extraction in Soxhlet apparatus. In *Methods for Ecological Energetics* (ed. W. Grodzinski, R. Z. Klekowski and A. Duncan), pp. 288-293. Oxford: Blackwell Scientific Publications.
Selman, C., Phillips, T., Staib, J. L., Duncan, J. S., Leeuwenburgh, C. and Speakman, J. R. (2005). Energy expenditure of calorically restricted rats is higher than predicted from their altered body composition. *Mech. Ageing Dev.* **126**, 783-793.
Sinclair, D. A. (2005). Toward a unified theory of caloric restriction and longevity regulation. *Mech. Ageing Dev.* **126**, 987-1002.
Sohal, R. S., Ferguson, M., Sohal, B. H. and Forster, M. J. (2009). Life span extension in mice by food restriction depends on an energy imbalance. *J. Nutr.* **139**, 533-539.
Speakman, J. R. (1999). The cost of living: field metabolic rates of small mammals. *Adv. Ecol. Res* **30**, 177-297.
Speakman, J. R., Selman, C., McLaren, J. S. and Harper, E. J. (2002). Living fast, dying when? The link between aging and energetics. *J. Nutr.* **132**, **Suppl. 2**, 1583S-1597S.
Speakman, J. R., van Acker, A. and Harper, E. J. (2003). Age-related changes in the metabolism and body composition of three dog breeds and their relationship to life expectancy. *Aging Cell* **2**, 265-275.
Speakman, J. R., Talbot, D. A., Selman, C., Snart, S., McLaren, J. S., Redman, P., Krol, E., Jackson, D. M., Johnson, M. S. and Brand, M. D. (2004). Uncoupled and surviving: individual mice with high metabolism have greater mitochondrial uncoupling and live longer. *Aging Cell* **3**, 87-95.
Vaanholt, L. M., Daan, S., Schubert, K. A. and Visser, G. H. (2009). Metabolism and aging: effects of cold exposure on metabolic rate, body composition, and longevity in mice. *Physiol. Biochem. Zool.* **82**, 314-324.
Weindruch, R. and Walford, R. L. (1988). *The Retardation of Aging and Disease by Dietary Restriction*. Springfield, IL: Charles C. Thomas Publisher Ltd.
Weiss, E. P., Racette, S. B., Villareal, D. T., Fontana, L., Steger-May, K., Schechtman, K. B., Klein, S., Ehsani, A. A., Holloszy, J. O. and Washington University School of Medicine CALERIE Group (2007). Lower extremity muscle size and strength and aerobic capacity decrease with caloric restriction but not with exercise-induced weight loss. *J. Appl. Physiol.* **102**, 634-640.