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Energy expenditure of calorically restricted rats is higher than predicted from their altered body composition

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Abstract

Debate exists over the impact of caloric restriction (CR) on the level of energy expenditure. At the whole animal level, CR decreases metabolic rates but in parallel body mass also declines. The question arises whether the reduction in metabolism is greater, smaller or not different from the expectation based on body mass change alone. Answers to this question depend on how metabolic rate is normalized and it has recently been suggested that this issue can only be resolved through detailed morphological investigation. Added to this issue is the problem of how appropriate the resting energy expenditure is to characterize metabolic events relating to aging phenomena. We measured the daily energy demands of young and old rats under ad libitum (AD) food intake or 40% CR, using the doubly labeled water (DLW) method and made detailed morphological examination of individuals, including 21 different body components. Whole body energy demands of CR rats were lower than AD rats, but the extent of this difference was much less than expected from the degree of caloric restriction, consistent with other studies using the DLW method on CR animals. Using multiple regression and multivariate data reduction methods we built two empirical predictive models of the association between daily energy demands and body composition using the ad lib animals. We then predicted the expected energy expenditures of the CR animals based on their altered morphology and compared these predictions to the observed daily energy demands. Independent of how we constructed the prediction, young and old rats under CR expended 30 and 50% more energy, respectively, than the prediction from their altered body composition. This effect is consistent with recent intra-specific observations of positive associations between energy metabolism and lifespan and theoretical ideas about mechanisms underpinning the relationship between oxygen consumption and reactive oxygen species production in mitochondria.

Keywords: Caloric restriction; Aging; Metabolic rate; Doubly labeled water; Organ morphometrics

1. Introduction

The idea that an inverse relationship exists between metabolic rate and lifespan was originally suggested by Rubner (1908), who observed that across species the lifetime expenditure of energy per kg of tissue was approximately constant. Hypothetical mechanisms explaining this phenomenon have included Pearl (1928) suggestion that there is depletion over a lifetime of a vital cellular component at a rate proportional to metabolic rate, popularly known as the 'rate of living' theory. However, a more popular mechanistic hypothesis linking metabolism and aging phenomena was Harman's free radical theory of aging (Harman, 1956, 1969). This suggests that free radicals are fundamental to the aging process, and are produced continuously during oxidative phosphorylation at a relatively fixed proportion to the

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amount of oxygen consumed (Beckman and Ames, 1998; Barja, 2002a,b). While there is evidence supporting the suggestion that an increased metabolic rate is associated with increased oxidative damage (Greenberg et al., 2000; Selman et al., 2002), exercise protocols, for example, increase oxygen consumption but also extend mean lifespan (Holloszy and Smith, 1987). In addition, an elevated metabolic rate is positively associated with greater longevity (Speakman et al., 2004, 2003), in both dogs (*Canis familiaris*) and mice (*Mus* sp.). Moreover, it has recently been suggested that the dynamics of reactive oxygen species (ROS) production in mitochondria may actually favor lower production of ROS at higher metabolic rates (Brand, 2000; Nicholls, 2004; Speakman, 2004).

It is well established that a reduction in caloric intake, without malnutrition, significantly extends mean and maximum lifespan in a wide range of organisms (Masoro, 1995, 2000; Merry, 2002). However, the exact mechanism's involved have proved difficult to elucidate, although lifelong caloric restriction (CR) is associated with reduced production of reactive oxygen species and oxidative stress (Beckman and Ames, 1998; Gredilla et al., 2001; Barja, 2002a,b). While it has been suggested that the extension of lifespan and reduction in ROS observed during CR is, in part, linked to a lowered metabolic rate (Sacher, 1977; Greenberg, 1999) there is much confusion in the literature, with some studies reporting a decrease in metabolism following long-term CR (Blanc et al., 2003), although this may only be transient, in both rats (*Rattus* sp.) and primates (*Macaca mulatto*) (Masoro et al., 1982; Ramsey et al., 2000) and others reporting no effect (Masoro et al., 1982; McCarter et al., 1985; McCarter and Palmer, 1992).

Much of the confusion pertaining to the effect of CR on metabolism is because CR invariably leads to a reduction in total body mass (BM) and changes in body composition, both of which may impinge directly on metabolic rate (Speakman et al., 2002). Therefore, it is imperative to discern whether CR directly affects on metabolism per se or whether any metabolic effects are simply related to the reduction in BM and changes in the components that make up BM. Considerable debate exists in the literature on how to adequately correct for body mass differences following CR (Ramsey et al., 2000; Even et al., 2001; Van Voorhies, 2001; Speakman et al., 2002; Poehlman, 2003), and include dividing metabolic rate by BM, despite the intercept not being equal to zero, and by employing an intra-specific scaling factor, e.g. the Kleiber–Brody ($kg^{0.75}$) or surface-area ($kg^{0.67}$) mass exponent, despite the effects of CR being intra-specific. In addition, as CR alters body composition, primarily through a reduction in fat mass, several authors have corrected for differences in mass by dividing metabolic rate by lean mass or fat-free mass (McCarter et al., 1985; McCarter and Palmer, 1992). However, additional potential problems may be encountered by adopting several of the previously described methods in an attempt to control for differences in body mass or body composition. First, using lean mass/fat-free mass assumes erroneously that the contribution of fat tissue to energy demands is zero. Secondly, it assumes that organs and tissues, which differ significantly in specific metabolic rates (Krebs, 1950), change in proportion to BM or lean mass (Ramsey et al., 2000). However, following initiation of CR, metabolically active organs such as liver and gut decline rapidly, but fat mass, a tissue with a relatively lower metabolic rate declines less quickly (Speakman et al., 2002). It has been suggested that the only accurate method of controlling for significant differences in BM and body composition between groups of animals is to correct discrepancies as a function of organ mass (Greenberg, 1999; Greenberg and Boozer, 2000; Even et al., 2001). One final factor complicating this issue is that many studies have used resting metabolic rate or basal metabolic rate as an estimate of metabolism, which may not be the best representation of total energy expenditure over a lifetime in an organism (Speakman et al., 2002). This potential problem has been addressed in several studies through the use of the doubly labeled water (DLW) technique to measure daily rather than resting energy expenditure (Blanc et al., 2003; Speakman et al., 2003) or by performing continuous respirometry (McCarter and Palmer, 1992).

In the following study, we examined the effect of both a 2 month (short-term) and 22 month (long-term) 40% CR at 6 and 26 months of age, respectively, on total daily energy expenditure (DEE) using the doubly labeled water technique (Speakman, 1997, 1998). This technique allows an integrated measurement of an individual's total energy budget, i.e. the total energy expended on resting metabolic rate, heat increment of feeding, activity and thermoregulation. We sought to examine whether any differences in DEE following 40% CR could be explained simply by changes in body composition or whether CR has direct effects on metabolic rate.

2. Material and methods

2.1. Animals

Male ad libitum and caloric restricted Fischer 344 rats were purchased from the National Institute of Aging (NIA) (Indianapolis, IN, USA) at 5 (young) and 23 (old) months of age. Caloric restriction was started at 3.5 months of age (10% restriction), increased to 25% restriction at 3.75 months, and then maintained from 4 months onwards at 40% restriction under the feeding and housing protocols employed by the NIA appropriate for ad libitum (AD) and 40% caloric restricted rats. Individual rats were subsequently purchased at 5 or 25 months of age from the NIA and housed at The University of Florida Animal Care Services (Gainesville, FL, USA) again following the NIA guidelines, until the termination of the experiment at 6

and 26 month of age for the young and old rats, respectively. Therefore, the 'young' rats at 6 months were subject to a relatively short-term 40% CR regime of 2 months duration and the 'old' rats at 26 months of age were subject to a long-term CR regime of 22 months duration. With the exception the 3 weeks immediately prior to the termination of this experiment when the rats were housed at The University of Florida, all rats were maintained at the NIA for the majority of their lifespan and followed the NIA feeding regime appropriate for both 40% CR and for appropriate age-class. All animals were individually housed, maintained on food purchased directly from the NIA (AD-NIH31 diet; CR-NIH31/NIA Fortified diet), under a 12 h light:12 h dark photoperiod and an ambient temperature of ∼24 °C. This experiment received local institutional animal care and animal use committee approval.

2.2. Daily energy expenditure

Daily energy expenditure was estimated using the doubly labeled water technique as previously described (Speakman, 1997, 1998) at either 6 or 26 months of age. In brief, following one week to allow for acclimation to housing conditions at the University of Florida following purchase from the NIA, rats were weighed (0.01 g, Sartorius), and then an intraperitoneal injection of deuterium (enrichment = 4.63 at.%) and ^{18}oxygen (enrichment = 9.44 at.%) was administered, with the syringes containing the isotope were weighed to 0.0001 g (Ohaus Analytical Plus Balance) before and after injection to obtain the dose mass. After a period of 60 min following isotope injection, to allow isotope equilibration with the animal's water pool and during which the animals were returned to their original cages, an initial blood sample was collected by tail tipping (50 μl Vitrex pre-calibrated capillaries). All blood samples were immediately flame-sealed and stored until analysis. Final blood samples were collected exactly as above 24 h after the initial sample. In addition, background samples were collected to determine the naturally occurring enrichments of deuterium and oxygen. Analysis of samples using mass-spectrometry followed the methods previously described (Speakman, 1997, 1998).

2.3. Organ morphometric analysis

Individual rats were sacrificed for up to 5 days following DEE measurements. Every day, eight rats (two from each experimental group; 6 month AD, 6 month CR, 26 month AD and 26 month CR) were weighed and anaesthetized using an intraperitoneal injection of pentobarbital sodium solution (Abbot Laboratories, IL, USA; 5 mg/100 g body weight). A terminal blood sample was subsequently collected via cardiac puncture and immediately the heart, liver, brain, kidney, abdominal fat, gonadal fat, mesenteric fat, brown adipose tissue (BAT, dissected interscapular brown adipose tissue pad), pancreas, gonads, small intestine (SI), large intestine (LI), stomach, gastrocnemius, plantaris, soleus, extensor digitorum longus (EDL) and tibialis anterior (TA) were dissected out (always by the same operator), weighed to 0.0001 g (Ohaus Analytical Plus Balance, Ohaus Corp, Germany), and then immediately frozen in liquid nitrogen. In addition, the pelage, tail and remaining carcass were weighed and the length of the small and large intestine was measured to the nearest cm. The stomach, small intestine and large intestine were washed with saline to remove residual gut contents prior to being weighed as above. All samples were then stored at −80°C.

2.4. Uncoupling protein-1 (UCP-1) gene expression

Total RNA was extracted from brown adipose tissue using a guanidium isothiocyanate/phenol method (Chomczynski and Sacchi, 1987). The RNA were run on a 1.4% agarose gel, and then transferred to a positively charged nylon membrane (Roche Applied Science, UK) by vacuum blotting. The membrane was then cross-linked and hybridised overnight at 42° for UCP-1 mRNA using a 5 digoxigenin end-labelled, 32 mer oligonucleotide, 5 CGGACTTTGGCGGTGTC-CAGCGGGAAGGTGAT (Eurogentec Ltd., UK). Signals were detected by chemiluminescence using CDP-star as the substrate (Tropix, UK) followed by exposure to film (Trayhurn et al., 1995). Membranes were then stripped and hybridised for 18S rRNA 5 CGCCTGCTGCCTTCCT-TGGATGTGGTAGCCG. The signals were scanned and quantified by densitometry using Scion Image (Scion Corporation, MD, USA).

2.5. Statistical analysis

All values reported are mean ± standard deviation (S.D.), except where indicated and data were analyzed using Minitab (Minitab Inc., PA, USA, version 13) statistical software. All data was checked for normality and significance was indicated by p-values <0.05.

3. Results

3.1. Morphology

The mean body masses and the mean wet masses of the component tissues separated by age (6 or 26 month) and by treatment (ad libitum or caloric restricted) are shown in Table 1. The significance of the effects of age and treatment and the age by treatment interaction are shown in Table 2. There were major treatment effects ($p < .001$) on the wet masses of the heart, kidney, brown adipose tissue, large intestine, pancreas, tail, pelage and carcass. More minor treatment effects ($p < .05 > .001$) were evident in the liver, gonadal fat and three of the leg muscles (soleus, plantaris and extensor digitorum longus). In all these tissues, except brown adipose tissue, the wet masses were lower in the CR

Table 1
Mean body masses and wet organ masses dissected from young (6 month) and older (26 month old) rats fed either ad libitum (AD) or caloric restricted (CR)

	Age							
	6 month				26 month			
Treatment	AD ($N = 6$)		CR ($N = 6$)		AD ($N = 5$)		CR ($N = 7$)	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Tissue mass (g)								
BM	365.0	23.8	312.9	13.4	352.5	29.4	304.4	11.8
Heart	0.804	0.066	0.653	0.024	0.894	0.053	0.806	0.115
Liver	11.48	0.99	10.65	0.53	13.14	3.64	9.06	0.69
Kidney	2.20	0.16	1.90	0.11	2.91	0.37	2.21	0.86
Brain	1.95	0.055	1.91	0.04	2.06	0.08	2.06	0.067
Lungs	1.57	0.14	1.55	0.11	2.27	0.43	1.98	0.66
Gonads	9.74	1.76	8.78	0.89	8.21	1.61	7.48	1.43
Muscles								
Gast	3.41	0.26	2.96	0.19	2.57	0.24	2.51	0.11
Plant	0.66	0.066	0.60	0.035	0.52	0.02	0.53	0.025
Soleus	0.29	0.022	0.25	0.022	0.254	0.014	0.225	0.023
EDL	0.304	0.033	0.262	0.019	0.260	0.025	0.256	0.014
TA	1.165	0.067	1.005	0.077	0.903	0.085	0.927	0.056
Adipose tissue								
BAT	0.347	0.149	0.740	0.140	0.306	0.030	0.636	0.119
AF	8.48	0.987	7.47	0.639	7.36	3.407	5.53	0.977
GF	8.93	1.577	6.20	0.632	7.19	3.59	4.77	0.797
MF	6.73	0.477	5.83	0.809	7.67	2.84	5.62	0.77
GI tract								
Stomach	1.668	0.143	1.664	0.202	2.02	0.215	1.935	0.256
SI	5.639	0.472	5.291	0.201	6.292	1.166	6.07	0.417
LI	2.957	0.412	2.349	0.149	3.098	0.321	2.348	0.195
Pancreas	1.474	0.251	1.278	0.169	1.748	0.099	1.135	0.115
Tail	9.572	0.705	8.188	0.451	9.922	0.318	8.686	0.661
Pelage	73.22	4.96	62.35	2.46	74.38	11.87	57.28	2.99
Carcass	184.89	11.85	154.27	7.35	172.68	11.22	149.51	6.74

Body mass (BM), gastrocnemius (Gast), plantaris (Plant), extensor digitorum longus (EDL), tibialis anterior (TA), brown adipose tissue (BAT), abdominal fat (AF), gonadal fat (GF), mesenteric fat (MF), small intestine (SI) and large intestine (LI).

rats than their AD counterparts. The pattern for brown adipose tissue was exceptional in that the mass of this tissue was more than doubled in size in the CR animals at both ages (Tables 1 and 2). There was a major effect of the treatment on overall body mass ($F_{1,20} = 38.9$, $p < .001$). Since almost all the treatment effects on individual organs followed a similar pattern to the change in overall body mass these changes may have been only consequent of the overall mass effect. Once we included mass as a covariate in the GLM analysis the only treatment effects to remain highly significant ($p < .001$) were for the heart, kidney and brown adipose tissue, with minor but significant treatment effects on the soleus muscle and large intestine mass.

Age had fewer highly significant effects ($p < .001$) on the morphology than the CR treatment (Table 2). Effects were evident in kidney, brain, three of the dissected leg muscles and the stomach. More minor effects were observed in the heart, lungs, gonads, the other two leg muscles, abdominal and gonadal adipose tissue, the small intestine and carcass. Most of these effects remained, although at reduced significance, when body mass was included as a covariate. This indicated that the major effects of age were not simple consequences of an overall change in body mass.

3.2. Daily energy expenditure

There was no overall significant treatment effect on the total daily energy expenditure ($F = 0.41$, $p = 0.529$) but the effect of age almost reached significance ($F = 3.69$, $p = 0.068$) (Fig. 1). If the data are grouped by age then there was a significant treatment effect in the young animals, with the AD animals expending approximately 25% more energy than the CR animals ($F_{1,9} = 3.73$, $p < .05$). The absence of an overall treatment effect in the entire data set occurred because by the age of 26 months the difference between AD and CR animals had declined to only 2% and this was not significant ($F_{1,9} = 0.68$, $p > .05$) (Fig. 1). The convergence of the metabolic rates with age occurred exclusively because the AD animals reduced their metabolism, while that of the CR animals remained constant with age.

We selected the data pertaining to animals that were fed ad libitum ($n = 11$) and sought associations between the daily energy expenditure and body composition in this sub-

Table 2
Effects of age, treatment (Tt) and age × treatment interaction (In) on wet organ masses (refer to Table 1 for mean values) using generalized linear modelling

	Age	Tt	In	Mass	Age	Tt	In
Body mass		***		–		–	–
Heart	**	***			*	***	
Liver		**	*				*
Kidney	***	***			**	***	
Brain	***			*	**		
Lungs	**				**		
Gonads	*			**			
Muscles							
Gast	***	*	*	**	**		*
Plant	***		*	**	***		*
Soleus	**	**		*	**	*	
EDL	*	*			*		
TA	***		*	**	***		**
Adipose tissue							
BAT			***			***	
AF	*			**	*		
GF	*	**		*			
MF				**			
GI tract							
Stomach	***				***		
SI	**				**		
LI		***				**	
Pancreas		***	**				**
Tail		***		*	*		
Pelage		***		***			
Carcass	*	***		*	*		*

In the first three columns significances of the different effects are indicated when body mass was not included as a covariate in the analysis. The last four columns show the significance of the effect of mass itself and the treatment and age effects when mass was included as a covariate in the analysis. Gastrocnemius (Gast), plantaris (Plant), extensor digitorum longus (EDL), tibialis anterior (TA), brown adipose tissue (BAT), abdominal fat (AF), gonadal fat (GF), mesenteric fat (MF), small intestine (SI) and large intestine (LI). *$p < 05$, **$p < 0.01$ and ***$p < .001$.

group. Using a stepwise multiple regression technique (including both forward inclusion and backward elimination: MINITAB Inc., PA, USA) the best-fit equation utilised the masses of three of the morphological parameters (liver, EDL and mesenteric fat mass) and explained 94.5% of the

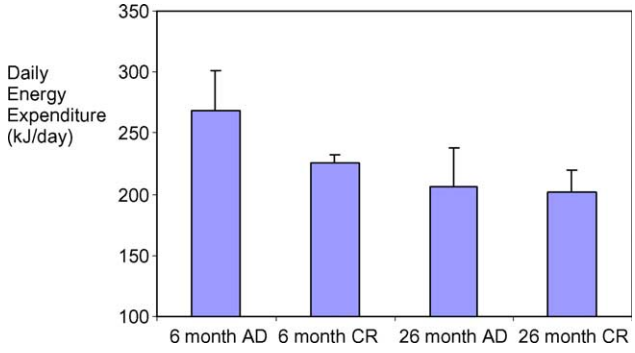

Fig. 1. Mean daily energy expenditures measured using the doubly labeled water method of rats aged 6 or 26 months fed ad libitum (AD) or 40% calorically restricted (CR). Error bars are standard errors. Sample sizes were 6, 6, 5 and 7, respectively.

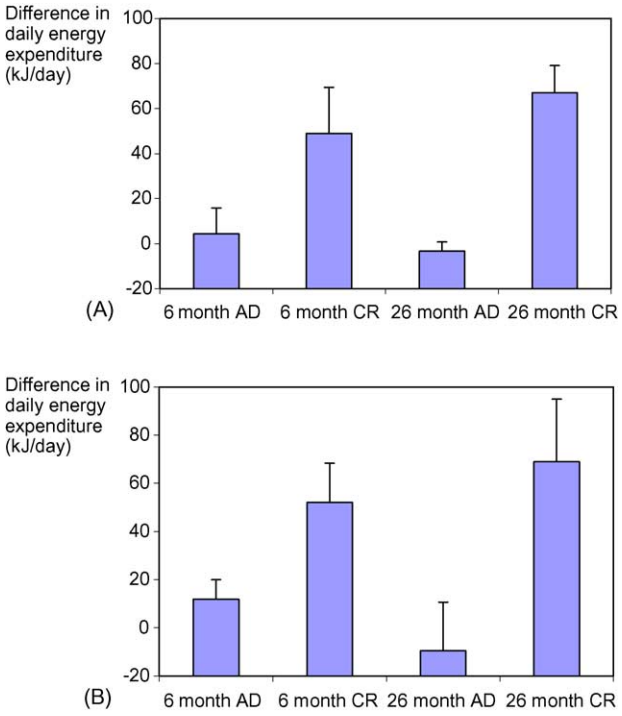

Fig. 2. Differences between the predicted daily energy expenditure based on predictive models from the morphology of the ad libitum (AD) fed rats and the actual observed daily energy expenditures. In (A) the predictive model was constructed by stepwise regression on the raw morphology data. In (B) the morphology was first re-described as a set of orthogonal principal components. In both cases the error bars are standard errors. Whatever analysis method was used to derive the prediction from morphology, caloric restricted (CR) rats had significantly elevated DEE relative to expectation at both ages.

observed variation in DEE across these individuals. The best-fit equation was

$$\log_e \mathrm{DEE\,(kJ/day)} = 5.717 + 1.407 \times \log_e(\mathrm{liver\ mass\ g}) + 2.342 \times \log_e(\mathrm{EDL\ mass\ g}) - 0.425 \log_e(\mathrm{mesenteric\ fat\ mass\ g})$$
$$(r^2 = 0.946,\ F_{3,7} = 39.76,\ p < .001) \quad (1)$$

We employed Eq. (1) to predict the expected daily energy expenditures of the CR and AD animals using the reported masses of these organs (Table 1). We then calculated the differences between the predicted DEE from the morphology and the actual observed DEE (Fig. 2A). The average differences for the AD individuals (4.3 kJ/day in the young animals and −3.4 kJ/day in the older animals) did not differ significantly from the prediction. This was expected because the data from these individuals had been used to construct the predictive equation. However, there was a strong treatment effect ($F_{1,20} = 16.68$, $p < .001$), with the young CR animals expending on average 49 kJ/day more, and the older CR animals expending 67.1 kJ/day more

Table 3
Correlations between the wet masses of the dissected organs in a sample of 11 rats fed ad libitum

	Hrt	Liv	Kid	Brn	Lung	Gon	Gast	Plant	Sol	EDL	TA	BAT	AF	GF	MF	Stom	SI	LI	Pane	Tail	Pel	Care
Liv	0.607																					
	0.048																					
Kid	0.831																					
	0.002																					
Brn																						
Lung	0.885	0.662	0.824					−0.694	−0.690			−0.734										
	0.001	0.026	0.002					0.018	0.019			0.010										
Gon																						
Gast			−0.732																			
			0.010																			
Plant			−0.636				0.898															
			0.035				0.001															
Sol				−0.604			0.777	0.800														
				0.049			0.005	0.003														
EDL							0.780	0.810	0.704													
							0.005	0.002	0.016													
TA			−0.707				0.960	0.914	0.762	0.830												
			0.015				0.001	0.001	0.006	0.002												
BAT																						
AF																						
GF												0.631										
												0.038										
MF							0.658															
							0.028															
Stom	0.802	0.665	0.676					−0.650	−0.612						0.903							
	0.003	0.002	0.022					0.030	0.045						0.001							
SI		0.821																				
		0.002																				
LI		0.723																				
		0.012																				
Panc	0.657						0.734															
	0.028						0.010															
Tail	0.630						0.685														0.851	
	0.038						0.020														0.001	
Pel						0.691								0.670	0.767							0.831
						0.019								0.024	0.006							0.002
Carc						0.960		0.752	0.770		0.726	0.668	0.749									
						0.001		0.008	0.006		0.011	0.025	0.008									

Only significant ($p < .05$) correlations are shown. Values show Pearson correlation coefficient (r) and below that the associated p-value. Highly significant correlations ($p < .001$) are shown in bold. Heart (Hrt), liver (Liv), kidney (Kid), gastrocnemius (Gast), plantaris (Plant), soleus (Sol), extensor digitorum longus (EDL), tibialis anterior (TA), brown adipose tissue (BAT), abdominal fat (AF), gonadal fat (GF), mesenteric fat (MF), stomach (Stom), small intestine (SI), large intestine (LI), pancreas (Panc), pelage (Pel) and carcass (Carc).

than predicted from their altered tissue morphology (see Tables 1 and 2).

It is important to recognize that the predictive regression equation is not a physiological model but a statistical description of the data. That is we do not imply by this model that the metabolism is only occurring in these three tissues. This is clearly the case because the effect of the mesenteric fat on DEE was negative, i.e. the greater fat the animals had, the lower their DEE. Such an effect might arise, for example, because mesenteric fat is well correlated with the other fat stores, and hence is a marker for total fatness, and fatter mice might be less active; thereby lowering their DEE. We recognize that such analyses are potentially compromised because the predictor variables included into the stepwise-multiple regression were not independent of each other (Table 3). Although the three predictor variables ultimately included in equation one were not significantly correlated to each other in the AD group, their ability to predict daily energy expenditures may hinge on their correlations to other predictors within the sample (Table 3). It is unlikely, for example, that the mass of the extensor digitorum longus muscle could itself be a major determinant of the daily energy demands, because this muscle is small, amounting to only around 0.3 g (Table 1), which is about 0.1% of the total body mass. This muscle probably entered the predictive equation as a marker for overall muscle mass, as it was correlated with all the other major muscle groups and the carcass weight (Table 3). If these correlations did not hold, or were altered, in the CR group, then a spurious prediction of expected metabolism might emerge for this group.

To overcome this effect we re-described the morphological data matrix for the AD animals using a principal components (PC) analysis, and extracted the first six principal components, which captured 93.2% of the original variance in the data. Eigenvectors for each of the original morphological traits included in the analysis for each PC are shown in Table 4. We calculated the scores for each individual for each of these PCs, and entered these as independent predictors of the daily energy expenditure. Since scores along principal components are by definition orthogonal, this procedure effectively overcomes the potential criticism that the derived equation from the original morphometric data included correlated traits. Scores on two of these PCs entered as significant predictors of DEE in a stepwise regression analysis, the best-fit equation explaining 84.9% of the observed variation in DEE of the AD individuals. The equation was

$$\log_e \mathrm{DEE}\,(\mathrm{kJ/day}) = 5.4307 + 0.1555\,\mathrm{PC3} + 0.1294\,\mathrm{PC4}$$
$$(r^2 = 0.849,\ F_{2,10} = 22.85,\ p < .001). \quad (2)$$

Inspection of the eigenvectors in Table 4 revealed that PC3 was dominated by the effects of the leg muscles and large intestine, while PC4 was dominated by the effects of BAT, the liver, brain, small intestine and gonadal fat. We used the coefficients relative to the original predictors to generate scores for all the data (AD and CR animals) along these two PCs and then inserted these scores into Eq. (2) to generate a prediction of the expected DEE. The pattern that emerged (Fig. 2B) was almost identical to that observed using the raw morphometric data (Fig. 2A) in that the predictions for the AD group matched closely the observed DEEs, but the CR animals expended more than predicted (by 52.2 kJ/day in the young animals and 69.0 kJ/day in the older animals). The treatment effect was still highly significant ($F_{1,20} = 8.87$, $p = .007$) showing the effect was robust to the method of analysis. CR animals expend more energy than anticipated from their morphology. Since the predicted DEEs for the young and old CR animals using Eq. (2) and their known morphology were 173.6 and 133.0 kJ/day, respectively, the extent to which the CR animals had greater DEE than anticipated was a large effect, amounting to 30.0% of the expectation in the young animals and 51.9% of the expectation in the older animals.

3.3. UCP-1 gene expression

One potential contributory factor to the elevated metabolism of the CR rats was the extent of uncoupling in the mitochondria in their brown adipose tissue, given that this was the only tissue that increased in mass under the CR treatment. However, there were no significant treatment ($F = 0.823$, $p > .05$), age ($F = 2.00$, $p > .05$) or interaction effects ($F = 0.294$, $p > .05$) on UCP-1 gene expression relative to expression of ribosomal RNA 18S by Northern blotting (data not shown).

Table 4
Eigenvectors for the source traits for the first six principal components extracted from a PCA analysis on the morphology of AD fed rats

Eigenanalysis of the correlation matrix						
Eigenvalue	9.1719	4.2339	2.7453	1.8308	1.4424	1.0749
Proportion	0.417	0.192	0.125	0.083	0.066	0.049
Cumulative	0.417	0.609	0.734	0.817	0.883	0.932

Variable	PC1	PC2	PC3	PC4	PC5	PC6
Hrt	−0.243	0.115	0.283	0.064	0.227	0.085
Liv	−0.223	0.127	0.169	**0.343**	**−0.310**	0.048
Kid	−0.269	0.023	0.115	−0.051	0.275	0.265
Brn	−0.161	**0.329**	−0.111	**−0.312**	0.096	−0.083
Lung	**−0.304**	0.060	0.124	−0.026	0.074	0.137
Gon	0.143	0.252	−0.244	0.289	−0.079	**0.440**
Gast	0.292	0.127	0.131	0.123	0.090	−0.064
Plant	0.272	0.099	0.260	0.137	−0.040	−0.127
Sol	0.228	−0.049	**0.372**	0.116	−0.032	0.156
EDL	0.243	0.061	0.280	−0.037	**0.322**	0.003
TA	0.290	0.102	0.179	0.064	0.094	−0.229
BAT	−0.026	0.010	−0.276	0.520	0.135	**−0.388**
AF	0.164	0.295	−0.257	0.118	−0.041	**0.381**
GF	0.203	0.052	0.183	**−0.346**	−0.251	−0.215
MF	0.024	**0.357**	−0.216	−0.254	−0.269	−0.271
Stom	−0.274	0.092	0.158	−0.144	−0.187	0.043
SI	−0.208	0.138	0.051	0.301	**−0.325**	−0.286
LI	−0.130	0.216	0.348	0.012	**−0.364**	0.129
Panc	−0.180	**0.308**	0.052	−0.038	**0.365**	−0.185
Tail	−0.147	0.350	0.105	0.168	0.258	−0.184
Pel	0.143	**0.379**	−0.212	−0.175	0.053	0.071
Carc	0.214	**0.317**	0.164	0.034	−0.032	0.125

Dominant factors influencing the PCs (eigenvectors >0.3 and <−0.3) are shown in bold. Heart (Hit), liver (Liv), kidney (Kid), gastrocnemius (Gast), plantaris (Plant), soleus (Sol), extensor digitorum longus (EDL), tibialis anterior (TA), brown adipose tissue (BAT), abdominal fat (AF), gonadal fat (GF), mesenteric fat (MF), stomach (Stom), small intestine (SI), large intestine (LI), pancreas (Panc), pelage (Pel) and carcass (Carc).

4. Discussion

There is broad agreement that the resting and total daily levels of oxygen consumption in rodents that are under prolonged caloric restriction are reduced when expressed on a whole animal basis (Ramsey et al., 2000; Speakman et al., 2002). That is the total level of energy expended on an individual level is lower. In our data, we also observed this effect in the animals at 6 months of age but not in the animals at 26 months of age (see below for potential explanations of this effect). The key problem that has emerged in the literature over the effects of caloric restriction on metabolism arises because rodents under protracted CR are also smaller (Greenberg and Boozer, 2000; Ramsey et al., 2000; Even et al., 2001). Hence, the question that has proved confusing to answer is whether the reduction in energy expenditure is greater, lower or not significantly different to that expected on the basis of the reduced body size. This problem opens up the

whole question of how to normalize for intra-specific body mass effects (Poehlman, 2003). It is has become increasingly apparent that utilization of simple per kg estimates, or estimates based on scaling exponents that have been established in inter-specific comparisons (such as 0.75 or 0.66), which generally indicate that energy demands in CR are reduced, are inappropriate for intra-specific normalization (Packard and Boardman, 1988, 1999; Ramsey et al., 2000; Speakman et al., 2002, 2003). On the other hand, the common clinical practice of expressing energy demands relative only to the lean tissue mass, which generally indicates no change in energy expenditure under CR (McCarter et al., 1985; McCarter and Palmer, 1992), opens up a whole series of different problems, namely the assumption of homogeneity of lean tissue and ignoring the contribution of fat tissue. As animals under CR have very marked different body compositions from those under ad libitum feeding (Poehlman, 2003), we examined whether through detailed morphological assessment in AD and CR rats, we could arrive at a satisfactory conclusion on whether energy demands under CR are altered (Greenberg, 1999; Greenberg and Boozer, 2000; Even et al., 2001).

To this complex debate over issues of normalization for body mass effects on measures of energy expenditure, which has complicated other areas of aging research in a similar manner (Van Voorhies and Ward, 1999; Braeckman et al., 2002; Van Voorhies, 2002a,b), there is the added question of how appropriate it is to use resting metabolic rate (RMR) to reflect the expenditure of energy relative to aging phenomena (Speakman et al., 2002). Animals expend energy doing many things in addition to the resting energy requirement, which typically accounts for about 30% of the total energy demands in most free-living animals (Speakman, 2000). Since energy demands are fuelled by a common mechanism in the mitochondria that generates ATP, it is unlikely that reactive oxygen species generated as a by-product of ATP generated to fuel RMR would have some special significance in the aging process. Consequently, if other components of the energy budget were variable between treatments it is quite feasible that trends in RMR might follow any pattern with CR (up, down or unaltered), but that these changes would be irrelevant to the total oxidative stress placed on the animals in question. Finally, the whole theoretical basis of what impact variations in energy demands might have on ROS production has been recently turned on its head by suggestions that elevated uncoupling that increases oxygen consumption might in fact reduce ROS production (Brand, 2000; Nicholls, 2004; Speakman, 2004).

Recognizing the potential limitations attached to using RMR as a proxy for total energy metabolism (Speakman et al., 2002), several recent studies have turned to isotope based methodologies such as the doubly labeled water method (Speakman, 1997) to measure total daily energy demands of animals under CR (Blanc et al., 2003). This is an extremely attractive alternative approach that we have used in the current study, combined with the suggestion that such measures might be best comprehended within the context of a detailed morphological examination (Even et al., 2001). It appears that the first result from our analysis of the doubly labeled water estimates of energy expenditure contradicts the first law of thermodynamics in that we found no significant treatment effect on the rates of total daily energy expenditure, despite the fact the animals under CR were fed 40% less food relative to the AD rats. Closer investigation of the data, however, revealed that the CR rats did expend less energy at 6 months of age, but not at 26 months of age. We suggest several explanations for these observations. When the first measures at 6 months of age are considered there was a difference in expenditure that amounted to about 25% (270 V, 220 kJ/day). This was less than the expected 40% based on the gross caloric intake, although it is feasible that the CR animals may have increased their energy absorption efficiency to extract more energy from the restricted food levels they were provided with. Blanc et al. (2003) also found the difference in total daily energy expenditure between AD and CR groups of Rhesus Macaques (*Macaca mulatto*) measured by the DLW technique (at 17%), was also much narrower than that expected from the level of caloric restriction (40%). The extent of this discrepancy with the DLW estimate giving a difference about half of that expected from gross food intake was very similar to our own observations in rats at 6 months of age. A similar discrepancy, however, was not observed by DeLany et al. (1999) when studying total daily energy expenditures of monkeys that were dietary restricted by weight clamping for 10 years, compared with controls that were not weight clamped. This difference may reflect the various forms of dietary control employed across the different studies.

However, by 26 months of age the difference in expenditure in our AD and CR animals had narrowed to only 2%, despite the nominal 40% restriction in gross food intake. To understand this discrepancy, it is important to recognize that the extent of restriction was a 40% reduction relative to the ad libitum food intake, as suggested by the NIA and employed for the 3-week duration that the rats were maintained at The University of Florida. However, the AD animals were free to choose their daily food intake throughout the experiment. Therefore, it is conceivable that an age-related decline in ad libitum food intake occurred, resulting in a convergence of food intakes of the 26 month old AD and CR animals have been suggested by other authors (Ramsey et al., 1997). Fischer 344 rats do exhibit declines in food intake as they get older (Blanton et al., 1998), but it has been suggested that this does not occur until after 27 months of age (Turturro et al., 1999). However, while this is slightly later than the aged rats in our study (∼26 months), the significance of Turturro et al. (1999) findings are debatable as no measure of variability around their calculated mean food intake was reported. In addition, this age-associated decline in food intake appears highly variable, with different cohorts showing age-related declines

in body mass and food intake at different times between 24.5 and 29 months of age (Horwitz et al., 2002; Coppola et al., 2004). Indeed senescent terminal weight loss has been reported to commence from 21 months of age in AD Fischer 344 rats (Black et al., 2003), although only 50% of the rats in Black et al. (2003) study showed an associated decline in food intake, with the other 50% actually showing an increase in food intake at the time of body mass decrease. Hence, the daily energy expenditures of our AD and CR rats were probably so close because the food intake of the ad libitum group by that age had declined. Supporting this interpretation the CR animals, which had been fed the same ration throughout, had the same level of expenditure at both 6 and 26 months as would be anticipated (Fig. 1), and the convergence was entirely attributable to a decline in expenditure of the AD group. Overall this convergence meant that the treatment effect on daily energy demands was not significant. It should be pointed out that the rats in this study (Table 1) were considerably lighter than other data published for Fischer 344 rats (Yu et al., 1985; Turturro et al., 1999) and the 17% difference in BM between 6 month ad lib and CR rats is less than may be expected following such a 40% restriction regime. As mentioned previously, the rats were purchased directly from the NIA and were maintained at The University of Florida only to allow for suitable acclimation following transport to Florida and for the duration of this experiment (approximately 3 weeks in total). Therefore, all individuals were maintained, under the appropriate NIA guidelines for feeding and maintenance for no more than 3 weeks. In addition, BM did not change significantly in any group from arrival from NIA to termination of this experiment and all animals were in good condition throughout the project. In addition, we have no lifespan data available for these particular animals due to our experimental end-point and lifespan data is unavailable for age-matched littermates of these animals, although lifespan data has been previously published from NIA derived rodent colonies (Turturro et al., 1999). We contacted NIA to enquire if individual data on survival was available for this cohort and it was not. We (Selman et al., 2003) have previously purchased Fischer 344 rats at 4 months of age from the NIA and kept these under exactly the same housing conditions for 8 weeks and these animals showed very similar BM profiles to other published data (Yu et al., 1985; Turturro et al., 1999). However, our experimental design would certainly have been improved if we had studied an intermediate group of rats around 16–18 months of age, i.e. prior to the marked increase in mortality rate associated with advancing age, particularly as median lifespan of ad libitum male Fischer 344 rats appears to be 23–24 months (Yu et al., 1985; Turturro et al., 1999; Black et al., 2003). Indeed, the longest-lived decile of ad libitum Fischer 344 rats generally appears to be around 27 months of age (Yu et al., 1985). It is certainly quite possible that the 26 month rats in our study were senescent and had various pathologies associated with old age (see, for example, Maeda et al., 1985), and while no gross pathologies were observed on dissection, they may well have been detected had we performed a more rigorous and precise histopathological examination post-mortem. Therefore, it is conceivable that the old 26 month AD rats may not, in hindsight, have given us the most reliable information on the lifelong differences in metabolic rate between AD and CR rats.

Despite this convergence of intakes over the entire duration of the experiment, and the lighter than expected BM in all four groups, there were profound differences in the lifetime amounts of energy consumed by the two groups reflected in their altered body compositions. When we generated a model to predict energy expenditure from morphological variation in the ad lib fed animals, the CR animals had levels of expenditure significantly greater than predicted, after taking into account their altered morphology. The outcome was robust to potential inadequacies in derivation of the model using the raw morphology data in multiple regression procedures that assume the predictor variables are independent, when morphological variation is demonstrably not so. Using a detailed morphological examination in an attempt to control for differences observed in body composition following CR (Even et al., 2001; Poehlman, 2003) and using the doubly labeled water technique to determine total energy expenditure (Speakman et al., 2002). We suggest that rats under 40% CR have a significantly higher metabolic rate when taking in to account their altered body composition. The shift in body composition in combination with the elevated metabolism meant that the rats aged 26 months under CR had an overall increased metabolic intensity per gram of tissue. This is also consistent with the suggestion that CR rats have a greater food intake per gram throughout most of their lifespan compared to AD controls (Masoro, 2003).

Perhaps, the most striking aspect of the morphological variation we uncovered was the hypertrophic reaction of interscapular brown adipose tissue to the CR treatment. In fact, BAT mass was twice that of the appropriate AD age matched controls at both time points. Given the putative role of uncoupling phenomena in the generation of ROS (Brand, 2000) we suspected that the elevated mass of BAT might also be accompanied by increases in levels of UCP-1, that could act to reduce membrane potential, simultaneously elevating oxygen consumption and reducing ROS production. This potential linkage has been shown experimentally in rats (Lambert and Merry, 2004), where a decrease in liver ROS production following CR was attributed to decreased protomotive force, which was subsequently shown to be reversed by insulin treatment. In our study, we did not observe any associated treatment or age effects at the level of UCP-1 gene expression measured by Northern blotting. However, we (Selman, Gredilla and Leeuwenburgh unpublished data) previously observed, in a separate cohort of 6 month old Fischer 344 male rats under the same 40% CR paradigm, that 40% CR significantly increased UCP-1 protein levels as determined by Western blotting. CR rats have lower average daily body temperatures than AD

controls (Duffy et al., 1989) and the rats in our study were maintained (24 °C), at approximately the lower limit of the thermoneutral zone (25 °C) of AD Fischer 344 rats (Tocco-Bradley et al., 1985). The greater than anticipated metabolism of the CR rats, combined with BAT hypertrophy, our previous observation of elevated UCP-1 protein levels and the significantly higher state four respiration in BAT mitochondria of CR rats (Lambert et al., 2004) strongly suggests that there is a shift in the thermoneutral zone in CR rats, such that the CR animals were under mild thermoregulatory stress at our housing temperature (24 °C). We did not characterize the complete thermoregulatory responses of our rats and are not aware that this comparison has been performed previously for CR and AD Fisher 344 rats. However, the potential difference in thermoregulatory status adds an additional dimension to the complexity of the debate about comparability of determinations of metabolism in AD and CR animals.

Since increased expenditure is associated with increased longevity in other intra-specific models (Flurkey et al., 2002; Lin et al., 2002; Speakman et al., 2004, 2003), the demonstration here of a similar response in CR animals points to a common mechanism, that is also compatible with modern thoughts on mitochondrial function and ROS generation. In summary, we suggest that detailed organ morphometrics can give insights in to how best to control for altered body composition following CR, as previously suggested (Greenberg and Boozer, 2000; Even et al., 2001; Poehlman, 2003), but obviously it use is restricted in many long-term studies due to being terminal in nature. Therefore, it is conceivable that DLW measurements in conjunction with state-of-the-art imaging techniques, particularly employing cohorts of animals that were not so aged as the ones we studied, will allow both in vivo tissue oxygen consumption rates and organ morphology measurements, to help answer how CR affects metabolic rate. Understanding tissue level variations in energy expenditure, the molecular basis of such variability and how this alters with age will be key steps forwards in furthering our understanding in this area.

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