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Intra-specific variation in resting metabolic rate in MF1 mice is not associated with membrane lipid desaturation in the liver

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Abstract

The 'membrane pacemaker' hypothesis provides a putative mechanistic linkage between variations in energy metabolism, rates of ageing and lifespan across different species. Within species we have found positive associations between longevity and metabolism, which contrast the inter-specific trends. It is of interest to know therefore how levels of lipid desaturation in membranes are linked to variation in metabolism between individuals within species. We explored this problem by extracting membrane fatty acids from the livers of mice that varied in their metabolic rate, in a strain (MF1) where we have previously demonstrated a positive association between metabolism and lifespan. We measured resting metabolic rate (RMR) in 60 mice, each measured on three occasions, and measured their body compositions using dual energy X-ray absorptiometry (DXA). We selected 28 individuals that exhibited a wide variation in their mean resting metabolic rates (RMR) and extracted membrane lipids from the livers of these mice post mortem and analysed them for the patterns of contribution of different fatty acids. We then sought associations between the levels of membrane desaturation and the individual variability in RMR, using the proportional contributions of each fatty acid as predictors in a stepwise regression or by re-describing the variation in fatty acyl lipids using a PCA analysis and then seeking associations between scores on the derived components and RMR. We used whole animal RMRs and also RMR with the effects of body composition (fat free mass) removed. The level of individual variation in RMR was consistent with our previous observations. There was a significant positive association ($p = 0.019$) between the proportion of palmitic acid (16:0) in the membranes and RMR, which was strengthened ($p = 0.014$) when we adjusted RMR for differences in fat free mass. The proportion of palmitic acid (16:0) explained 20.9% of the individual variation in residual RMR. There was no association between RMR or mass adjusted RMR and the proportional representation of any other fatty acid, including 22:6 (DHA) predicted by the membrane pacemaker hypothesis to be of particular significance. High levels of saturated fatty acids in the membranes of mice with high rates of metabolism may contribute to their greater longevity, but the mechanism tying together increased membrane saturation with elevated RMR remains unclear.

Keywords: Membrane pacemaker; RMR; Lifespan; Ageing; Metabolism

1. Introduction

The idea that ageing should be linked in some manner to the rate of energy utilisation is intuitively attractive and has been under investigation for almost the past 100 years, since the seminal observations of Rubner (1908) that the product of resting energy expenditure and maximal lifespan in a sample of five mammal species was invariant. This invariance implies that energy metabolism and lifespan are negatively associated—mammal species with high rates of metabolism live short lives. Increases in the database over the past 100 years have served only to reinforce the original idea in mammals (Calder, 1984; Peters, 1983) and in birds (Prinzinger, 2005). However, recent analyses have cast doubt on the veracity of the relationships (Speakman et al., 2002). If the effect of body mass on both lifespan and RMR is removed then the relationships between them is substantially weakened (Speakman, 2005a). Moreover, if daily rather than resting energy demands are used, then the product of energy expenditure and maximal lifespan is no longer constant, but indicates those animals with higher rates of

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metabolism have tissues that burn through more energy during their lives than animals with lower rates of metabolism: opposite the direction of the presumed association (Speakman, 2005a). This relationship is similar to interclass observations of endotherms, which suggest that birds—which have higher rates of metabolism than mammals (Bennett and Harvey, 1987; McKechnie and Wolf, 2004; Reynolds and Lee, 1996) live longer (Barja, 1998; Carey and Judge, 2000; Holmes and Austad, 1995a,b; Holmes et al., 2001; Lindstedt and Calder, 1976; Ricklefs and Scheuerlein, 2001), and interordinal comparisons suggesting marsupials combine shorter lives with slower metabolic rates (Austad and Fischer, 1991).

In contrast to the plethora of inter-specific, and interclass, comparisons, studies of links between energy metabolism and lifespan at the level of the individual have been only rarely performed. In a cohort of MF1 mice, however, we have previously shown that those individuals with higher rates of metabolism lived longer lives (Speakman et al., 2004a). This relationship was true if the energy expenditure was expressed per whole animal, per gram of tissue, or if the effects of mass were removed statistically by analysis of covariance (ANCOVA). This phenomenon may be more widely significant since in humans, centenarians have higher rates of metabolism than anticipated (Rizzo et al., 2004), and it is consistent with the observations that species and classes with high rates of metabolism utilise more energy per gram of tissue during their lifetimes (Speakman, 2005a). A positive association between lifespan and metabolic rate has also been observed in yeast (Lin et al., 2002), photoperiodic mutant hamsters (Oklejewicz and Daan, 2002; Oklejewicz et al., 1997) and dogs (Speakman et al., 2003).

The mechanism underpinning this linkage remains uncertain. We have shown that mice with the top 25% of RMRs had higher levels of mitochondrial proton leak and higher activation of uncoupling protein 3 (UCP-3) and the adenine nucleotide translocase (ANT) in their mitochondria compared to mice with the bottom 25% of RMRs (Speakman et al., 2004a). These patterns are consistent with the hypothesis that production of radical oxygen species by mitochondria should be sensitive to the level of mitochondrial uncoupling (Brand, 2000; Brookes, 2005; Castiella et al., 2001; Nicholls and Ferguson, 2002; Speakman, 2004). The link of UCP expression to lifespan has been confirmed recently by transgenic overexpression of UCP2 in the brains of Drosophila (Fridell et al., 2005).

One factor that may confound these observations however is the level of membrane lipid desaturation. It has been suggested that membrane lipid desaturation is a key factor driving the level of membrane proton leakage (Brookes et al., 1998; Hulbert et al., 2002b; Porter et al., 1996). Experimentally increasing DHA content of the liver in mice elevates proton leak (Stillwell et al., 1997). In addition membrane lipids may radically affect the behaviour of protein complexes embedded in the membranes, notably the activity of the Na-K ATPase (Else and Wu, 1999; Turner et al., 2003, 2005; Wu et al., 2001, 2004) and also components of the cytochrome system (Hulbert et al., 2006). These twin effects of membrane fatty acyl desaturation on key proteins and proton leakage may drive variation in resting metabolic rate between species (Hulbert et al., 1991, 2002b) and has been called the 'membrane pacemaker' hypothesis for variation in resting metabolism (Hulbert and Else, 1999, 2000, 2005; Hulbert, 2007). The same hypothesis provides a potential link between metabolism and ageing phenomena (Hulbert, 2003, 2005; Pamplona et al., 2000) because membrane fatty acyl chain desaturation is also correlated with the propensity for oxidative damage and with lifespan (Pamplona et al., 2002; Portero-Otin et al., 2001). This association may occur because fatty acids differ in their susceptibility to oxidative damage: single-bonded carbons located between the doubled-bonded carbons in fatty acyl chains are most susceptible to damage by radical oxygen species (ROS) (Halliwell and Gutteridge, 1999). However, the linkage implied by this association is the opposite to that we empirically observed in the MF1 mice.

The correlations between membrane desaturation and both metabolism and lifespan have been developed by making inter-specific comparisons that largely ignore covariances of both traits with body size and the potentially confounding effects of phylogeny (see Promislow (1993) and Speakman (2005b) for discussions of these problems). Analyses removing these artefacts suggest the associations between membrane desaturation and metabolism may be less robust than previously considered (Valencak and Ruf, 2007). In the current paper we aimed to explore the association between individual variability in membrane desaturation and RMR in the MF1 strain of mouse to examine if the useful insights generated by this hypothesis across species are replicated within species.

2. Experimental procedures

2.1. Animals

Mice were maintained in accordance with the UK Home Office Animals (Scientific procedures) Act 1986. Male MF1 outbred mice (*Mus musculus*) were purchased from Harlan UK Ltd. The mice were 9–11 weeks old during the present study ($n = 60$). During the measurements mice were housed individually in cages (48 cm × 15 cm × 13 cm) with enrichment provided (red plastic housing, wood shavings and paper bedding) in a temperature-controlled environment with a 12-h light/dark cycle with lights on at 7 a.m., with *ad libitum* access to chow (pelleted rat and mouse breeder and grower diet, Special Diet Services, BP Nutrition, UK) and water.

2.2. Dual-energy X-ray absorptiometry

Body composition was assessed using a dual energy X-ray absorptiomtery (DXA) machine (Lunar PIXImus mouse densitometer, GE Medical Systems, UK). Mice were placed under isofluorane inhalational anaesthetic during X-ray scanning, the duration of which was approximately 3.25 min. The head region was excluded from analysis following the manufacturer's recommendations and was in any case obscured by the mask used to administer the anaesthetic. We used an individual calibration for this particular machine as recommended by Johnston et al. (2005). Fat-free mass values were derived from fat percentages produced by the PIXImus.

2.3. Resting metabolic rate

Resting metabolic rate (RMR) was measured as in Selman et al. (2001) using sampling criteria as outlined in Hayes et al. (1992). Briefly, RMR was quantified as the rate of oxygen consumption at thermoneutral temperature for mice (30 °C) during the light-phase using an open-flow respirometry system.

Oxygen consumption was measured using a paramagnetic oxygen analyser and infra-red CO_2 analyser (Xentra, 1400, Servomex, UK). A standard flow rate (600–800 ml/min) was pumped (Charles Austen Pumps, UK) through the system and regulated by a mass flow controller (Dwyer flow controller). Each mouse spent approximately 3 h being measured, during which they were unrestricted in a cylindrical Plexiglass chamber (9 cm diameter by 35 cm in length). This was repeated two more times on separate days to obtain triplicate RMRs to calculate an average RMR estimate (units in VO_2 ml min^{-1}) and also VCO_2. Respiratory quotient (RQ) was determined from the ratio of the rates of oxygen consumption and carbon dioxide production.

2.4. Dissection

Following completion of the third RMR measurement, mice were sacrificed by carbon dioxide inhalation. Tissues were promptly removed into cryotubes and frozen in liquid nitrogen, blood was also additionally collected via cardiac puncture. Tissues collected included liver, heart, brain, brown adipose tissue, white adipose tissue, and skeletal muscle (gastrocnemius and soleus). Once dissection was completed the tissues were removed from liquid nitrogen and stored at −80 °C. Care was taken to keep the bench-top and tools sterile by cleaning with 70% ethanol between each dissection.

2.5. Membrane fatty acid composition

All solvents and chemicals used in the extraction and preparation of fatty acid methyl esters (FAMEs) were all obtained from VWR, Poole, Dorset. Fatty acids were extracted and converted to methyl esters using procedures based on methods described by Hulbert et al. (2002b). Samples of liver (approximately 200 mg) were homogenised (Ultra Turrax, IKA Werke; GmbH & Co KG; D-179219; Staufen; Germany) for 1min with 4mls of chloroform/methanol (2:1, v/v) The homogeniser was rinsed with a further 2× 1 ml of chloroform/methanol (2:1, v/v). All solvents used throughout the sample preparation process contained 0.01% butylated hydroxytoluene (BHT) as an antioxidant. The homogenate was rotated for a least 3–4 h to ensure maximum extraction of lipids in a tube roller (Denley Instruments, Billinghurst, West Sussex). The mixture was shaken for 15–20 s following the addition of 1.5 ml of 2 M HCl before centrifugation at 1000 rpm for 10 min. The upper layer was removed by aspiration and discarded. A small quantity of sodium hydrosulphite was added to the chloroform (remaining lower phase), shaken and then filtered into a clean tube. Chloroform was evaporated off under Nitrogen then 5 ml hexane was added to the dry tube. This was transferred to a silica Sep-Pak cartridge (Waters, Elsrtee, England) and triglycerides eluted with 3× 5 ml ethyl acetate and phospholipids eluted using 6× 2.5 ml methanol.

Extracted fatty acids were converted to fatty acid methyl esters (FAME) by direct trans-esterification (Lepage and Roy, 1986). Briefly 2 ml of methanol:-toluene (4:1, v/v) was added. To this 200 μl of acetyl chloride was added very slowly whilst vortexing. The mixture was then heated at 100 °C for 1 h on a heating block. This was followed by rapid cooling in cold water and the slow addition of 5 ml of potassium carbonate (6% solution). After vortexing the mixture was centrifuged for 10 min at 300 rpm at 4 °C to separate out the upper toluene phase which was removed and stored at −20 °C until GC analysis.

FAME were separated and quantitated using a gas chromatograph (Model 6890, Agilent Technologies UK Ltd., Stockport, UK) equipped with a flame-ionization detector, auto-injection port and a 50-m fused silica capillary column (0.25 mm i.d.) coated with 0.2 μm film of cyanopropyl polysiloxane (CP-SIL 88; Varian Analytical Instruments, Walton-on-Thames, Surrey, UK). Total FAME profile in a 1 μl sample at a split ratio of 15:1 was determined using a temperature gradient programme (initial temperature 80 °C for 1 min; increased at a rate of 25 °C/min to 160 °C which was held for 3 min; increased at a rate of 1 °C/min to 190 °C; increased at a rate of 10 °C/min to 230 °C held for 30 min) and helium as the carrier gas operated at constant pressure (14 psi) and flow rate of 0.6 ml/min. Injector and detector temperatures were maintained at 250 and 270 °C, respectively. Peaks were routinely identified by comparison of retention times with authentic FAME standards The calibration standard used was a 37 component mix of fatty acid methyl esters, supplied by Supelco, Poole, Dorset.

As mechanical extraction might conceivably cause greater degradation to unsaturated fats than saturated fats (A. Hulbert, personal communication) we made a comparison of the fatty acid compositions of the livers of four individual mice of the same strain using both mechanical and hand homogenisation of pieces of liver (Fig. 1). There was no significant difference in the profile of fatty acids extracted using the different methods across the four individuals (paired *t*-test—$t = 1.0$, $p > .05$).

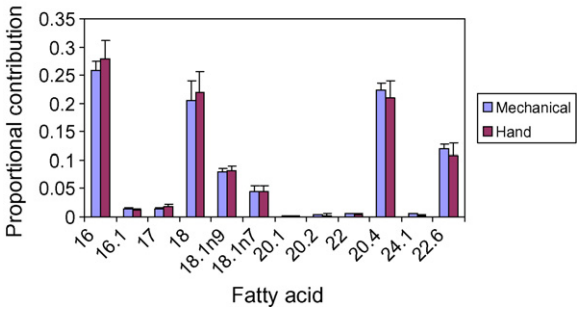

Fig. 1. Proportional contribution of different fatty acids in the liver membranes of MF1 mice evaluated using mechanical or hand extraction. There was no significant difference between the different extraction methods.

3. Results

3.1. Resting metabolic rate

The mean resting metabolic rate (resting oxygen consumption) averaged across the three repeated measurements in each individual averaged 0.74 ml O_2/min (minimum 0.56, maximum 1.00, S.D. = 0.09, $n = 60$). The coefficient of variation across the three repeated measurements for each individual, averaged across all individuals was 8.01%. The mean respiratory quotient (RQ) across all individuals was 0.82 (minimum 0.70, maximum 0.95, S.D. = 0.04, $n = 60$). The variation between individuals in RMR was much greater than the variation within individuals ($F = 3.99$, $p < .001$). The within individual variance contributed about 20% to the total variance across all measurements (total mean squares = 0.258, within mean square = 0.0517). The between individual variation in mean resting metabolic rate was related to the fat free mass,

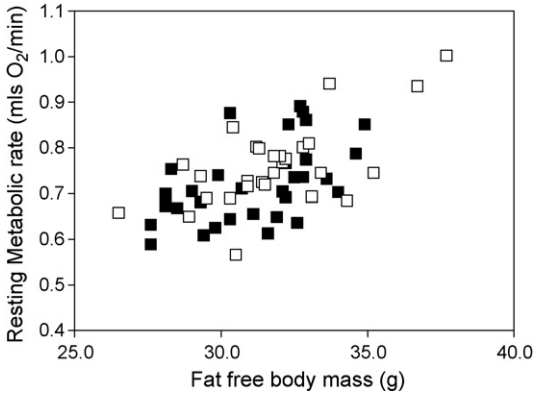

Fig. 2. Resting metabolic rates (ml O_2 consumed per minute) of MF1 mice plotted against fat free body mass measured by DXA (g). The total sample was 60, the 28 individuals chosen for analysis of membrane fatty acid contents are shown as open symbols (statistics for whole sample: mean $VO_2 = -0.0036 + 0.0236$FFM (g), $F = 6.36$, $p = 0.019$ and for selected subset mean $VO_2 = -0.0183 + 0.0245$FFM (g), $F = 17.89$, $p < .001$).

Table 1
The mean, median, truncated mean (top and bottom 5% of values removed), standard deviation and standard errors of the proportions of the major membrane fatty acids in the livers of 28 MF1 mice

FA	*N*	Mean	Median	TrMean	S.D.	S.E. mean
12:0	28	0.03472	0.03575	0.03477	0.00756	0.00143
16:0	28	0.25391	0.25357	0.25410	0.00956	0.00181
16:1 $n-7$	28	0.01581	0.01646	0.01617	0.00394	0.00075
18:0	28	0.15035	0.15046	0.15018	0.01183	0.00223
18:1 $n-9$	28	0.07993	0.07968	0.07978	0.00716	0.00135
18:1 $n-7$	28	0.03405	0.03459	0.03375	0.00694	0.00131
18:2 $n-6$	28	0.15538	0.15639	0.15546	0.00539	0.00102
20:2	28	0.00378	0.00385	0.00376	0.00047	0.00009
20:3 $n-6$	28	0.02533	0.02612	0.02533	0.00279	0.00053
20:4 $n-6$	28	0.16974	0.17032	0.16985	0.01030	0.00195
20:5 $n-3$	28	0.00246	0.00232	0.00243	0.00090	0.00017
22:6 $n-3$	28	0.07453	0.07385	0.07452	0.00879	0.00166
Unsat index	28	166.25	169.25	166.22	0.74	0.14

which explained 34.8% of the variation (Fig. 2: mean $VO_2 = -0.0036 + 0.0236$FFM (g), $F = 6.36$, $p = 0.019$). The effect of fat mass was not significant ($p = 0.314$). There was a high residual variation in the metabolic rate once fat-free mass had been accounted for. The same patterns were observed if oxygen consumption was converted to energy expenditure using the individual specific RQ. The pattern observed in the entire sample was repeated in the subset of 28 animals selected for analysis of fatty acid composition (mean $VO_2 = -0.0183 + 0.0245$FFM (g), $F = 17.89$, $p < .001$).

3.2. Membrane fatty acid composition

The mean levels of the major fatty acids extracted from the membranes in the liver are presented in Table 1. The commonest unsaturated fatty acid comprising on average 16.9% of the total was aracadonic acid (20:4) and the commonest saturated fatty acid (25.4% of the total) was palmitic acid (16:0). On the whole, polyunsaturated fatty acids (PUFAs) comprised 43.1% of the total, and mono-unsaturated fatty acids 12.9%. The unsaturation index (Hulbert et al., 2006), which reflects the number of double bonds in an average of 100 fatty acids, averaged 166. The pattern of variation in fatty acid composition of membranes from the liver was broadly similar across other tissues (Fig. 3, $n = 4$ individuals of the same strain). However there were some minor differences, in particular the levels of 22:6 (DHA) were higher in the brain and muscle tissues than in either the liver or kidney, whereas levels of 20:4 (arachidonic acid) were higher in the liver and kidney. Overall these tissue differences were statistically significant (ANOVA, $F = 2.66$, $p = 0.033$).

We sought associations between the proportional contributions of the individual fatty acids and the resting metabolic rate—either removing or ignoring the impact of body size on RMR. The proportions of the total membrane fatty acids represented by each component were entered as predictors into a stepwise regression model. Two factors entered the model as significant—the proportion of the total fatty acid represented by palmitic acid (16:0) which had a positive effect ($t = 2.62$, $p = 0.015$) and the unsaturation index which also had a positive effect ($t = 2.08$, $p = 0.048$). The overall equation

$$\text{RMR} = -1.13 + 4.47(16:0\text{p}) + 0.458(\text{unsat index})$$

explained 24.8% of the variation in average RMR ($F = 4.13$, $p = 0.028$).

When we considered the variation in RMR with the effect of fat free mass removed there was a highly significant correlation between the residual RMR and the proportion of 16:0 fatty acid

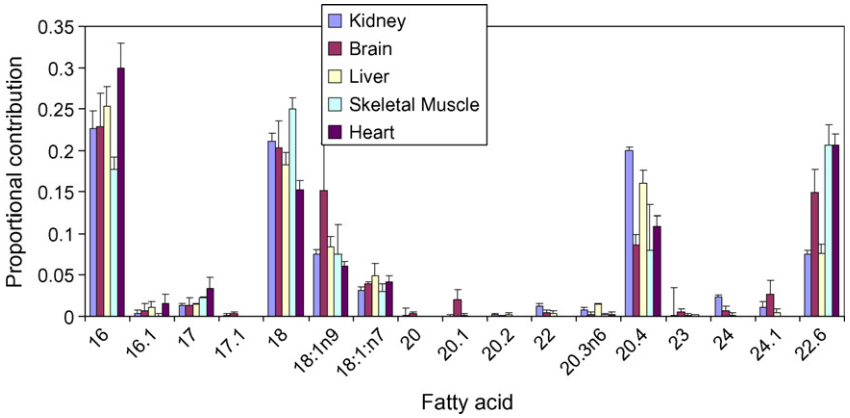

Fig. 3. Membrane fatty acid compositions from five different tissues extracted from four individual MF1 mice. The variation in membrane fatty acid composition was broadly similar across all tissues although the small tissue differences did reach statistical significance (ANOVA: $p = 0.033$).

Table 2
Pearson correlation coefficients and their associated p values between both RMR and mass adjusted RMR and the proportion of different fatty acids in the membranes extracted from livers of MF1 mice ($n = 28$)

	RMR	Mass adjusted RMR
16:0	0.343	0.569
	0.019	0.014*
18:1	−0.134	−0.127
	0.495	0.545
22:6	0.221	0.05
	0.259	0.814

* $p < .05$ adjusted for multiple comparisons. The number of comparisons was restricted to avoid the erosion of significance levels by multiple testing.

in the membranes (Table 2). In a stepwise multiple regression only the effect of 16:0 was significant, although an effect of DHA (22:6) approached significance ($p = 0.069$). The regression equation

$$\text{resid RMR} = -1.01 + 3.99\ \ 16:0\text{p}$$

explained 20.9% of the residual variation in RMR ($F = 8.96$, $p = 0.014$) (Fig. 2). The fact that this relationship to 16:0 palmitate was strengthened when we excluded the effect of fat-free mass indicated that the original relationship was not an artifact of failing to remove the fat free mass effect. On the other hand the disappearance of the effect of unsaturation index indicated that it was only an artifact of not controlling for fat-free mass (Fig. 4).

The membrane pacemaker hypothesis emphasizes the relative levels of poly-unsaturated to monounsaturated fatty acids as the driver for energy metabolism in particular the levels

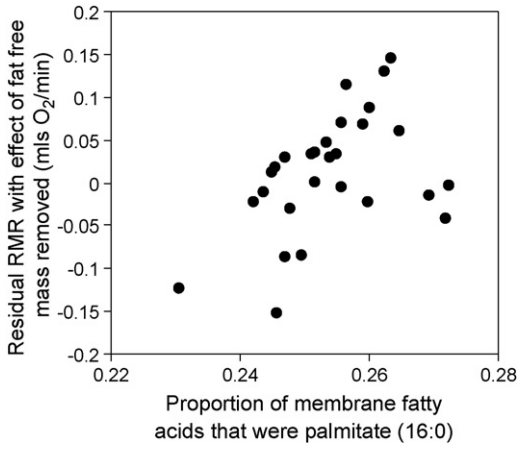

Fig. 4. Residual variation in resting metabolic rate, with the effect of fat free mass removed plotted against the proportion of liver membrane fatty acids that were palmitate (16:0). The positive relationship was significant ($p = 0.014$).

of DHA (22:6) (Hulbert and Else, 1999, 2000, 2005). The most abundant monounsaturated fatty acid in the tissues of our mice was 18:1 (oleic acid). The proportional contributions of neither of these fatty acids entered as predictors of RMR or mass adjusted RMR when each was considered as an independent predictor variable (Table 2 and Fig. 5). The same patterns were observed if RMR was expressed as energy rather than oxygen consumption using the individual specific estimates of RQ. We did not test ratios of fatty acids because the number of potential combinations becomes very large—such that with a limited sample of 28 animals it would be impossible to maintain statistical significance with multiple testing.

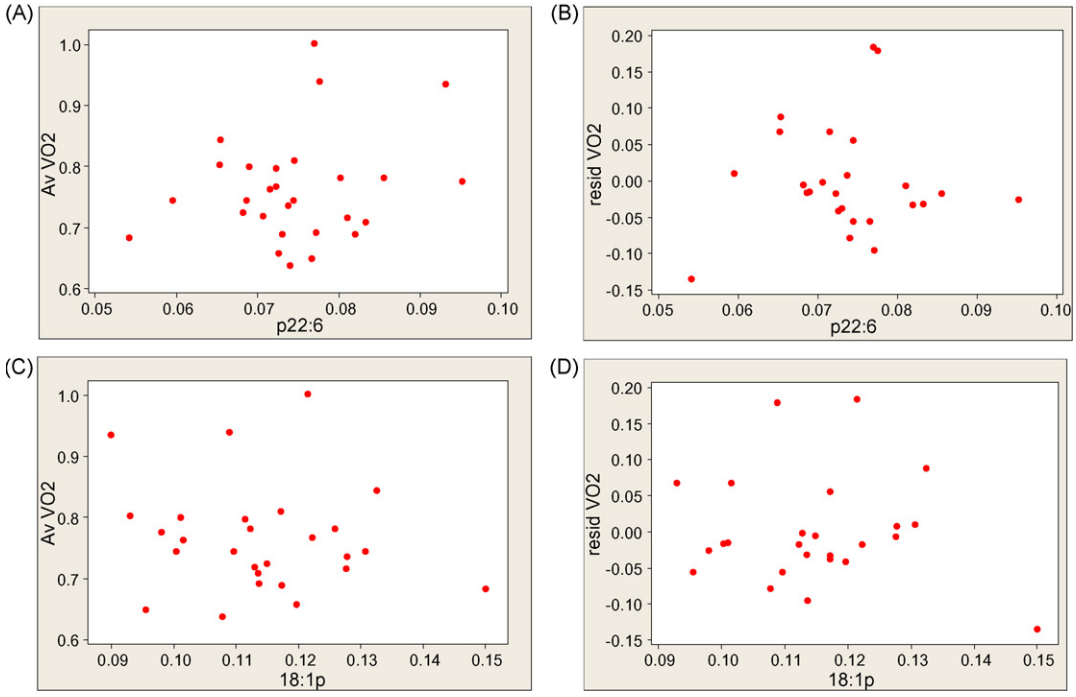

Fig. 5. Variation in resting metabolic rate (VO_2) and residual resting metabolic rate (res VO_2) with the effects of fat free mass removed plotted against the proportion of membrane fatty acids that were DHA (22:6) (plots A and B, respectively) and the proportion of membrane fatty acids that were oleic acid (18:1 trans) (plots C and D, respectively) in the membranes of the liver. The 'membrane pacemaker' hypothesis suggests that these fatty acids should be significantly linked to energy metabolism, but in the MF1 mouse they were not.

Table 3
Correlation matrix between the different proportional contributions of fatty acids extracted from membranes from the livers of MF1 mice

	12p	16p	16:1p	18p	18:1(9)	18:1(11)	18:2p	20:2p	20:3p	20:4p	20:5p	22:6p
16p	−0.320											
16:1p	−0.095	−0.090										
18p	0.234	0.081	−0.259									
18:1(9)	−0.127	−0.115	0.520	−0.396								
18:1(7)	−0.011	−0.355	0.354	−0.540	0.808							
18:2p	−0.194	0.266	−0.011	−0.207	−0.080	−0.272						
20:2p	0.148	−0.009	−0.275	−0.310	0.147	0.356	0.240					
20:3p	−0.299	−0.228	0.497	−0.685	0.635	0.716	−0.094	0.166				
20:4p	−0.312	−0.280	−0.489	−0.133	−0.438	−0.274	0.013	−0.086	−0.169			
20:5p	−0.072	−0.265	0.493	−0.472	0.249	0.334	0.108	0.016	0.683	−0.277		
22:6p	−0.094	−0.242	−0.237	−0.204	−0.592	−0.279	−0.182	−0.132	−0.019	0.452	0.236	
Unsat in	−0.324	−0.381	−0.223	−0.463	−0.388	−0.094	−0.011	−0.042	0.190	0.749	0.249	0.852

The levels of different fatty acids were significantly correlated with each other across the individuals. A matrix of correlations is shown in Table 3. An effect of one fatty acid like palmitic acid (16:0) detailed above might arise therefore because of a complex interaction of effects dependent on the inter-correlations of the different fatty acids. To capture this pattern of variability, the pattern of variation was re-described using principal components analysis (PCA). This was performed in two ways. First, using the raw fatty acid data (area under peak), and second, using the data on proportions of each fatty acid in the total. Using the raw data the first five principal components (PCs) from this analysis contained 89% of the original variation (Table 4B). Scores on these PCs were used as predictors of RMR and fat-free mass adjusted RMR. None of the scores on any PC was significantly related to either RMR or fat-free mass adjusted RMR. Using the proportions, the first five PCs captured 85.3% of the variation (Table 4A) but again none of the scores on these five PCs were significantly related to the RMR or fat-free mass adjusted RMR. These data suggest the association between RMR and levels of palmitic acid were not due to complex interactions between it and other fatty acids in the membranes. Expressing RMR as energy rather than oxygen consumption did not affect the absence of significant relationships.

An alternative approach to assessing the role of fatty acid composition on RMR is to pool different classes of fatty acid together (such as the %saturates, %monounsaturates, %polyunsaturates, $\%n-3$ polyunsaturates and $\%n-6$ polyunsaturates) and seek a relationship between representation of these broader composite classes with RMR. This is not as strong an analysis as that performed using principal component analysis (above) because there still remains some interdependence between the derived traits which is eliminated in the PCA. Nevertheless when we used these pooled classes there was no significant relationship between fat-free mass adjusted RMR and any of the composite classes (%saturates: $F = 0.02$, $p = 0.903$, %monounsaturates, $F = 0.14$, $p = 0.714$, %polyunsaturates, $F = 0.23$, $p = 0.639$, $\%n-3$ polyunsaturates, $F = 0.06$, $p = 0.814$, $\%n-6$ polyunsaturates, $F = 0.25$, $p = 0.620$). In a stepwise multiple regression none of the composite traits entered as significant predictors.

Table 4
Eigenvectors for the first five principal components relative to the original scores in a PCA analysis to redescribe the variation in membrane fatty acids

(A) Raw data					
Eigenvalue	7.7010	1.7376	0.8548	0.7091	0.4199
Proportion	0.642	0.145	0.071	0.059	0.035
Cumulative	0.642	0.787	0.858	0.917	0.952
	Variable				
	PC1	PC2	PC3	PC4	PC5
12:0	−0.043	−0.497	−0.764	0.028	−0.333
16:0	−0.339	−0.141	0.202	−0.080	−0.073
16:1	−0.228	0.395	−0.133	−0.504	−0.483
18:0	−0.286	−0.329	0.253	−0.285	−0.115
18:1 (9)	−0.333	0.165	0.120	0.146	−0.319
18:1 (7)	−0.261	0.352	−0.066	0.527	−0.054
18:2	−0.346	−0.137	0.084	−0.069	−0.071
20:2	−0.293	−0.148	−0.096	0.513	−0.006
20:3	−0.338	0.202	−0.074	0.066	0.057
20:4	−0.331	−0.218	0.145	−0.022	0.126
20:5	−0.222	0.380	−0.477	−0.236	0.447
22:6	−0.303	−0.209	−0.063	−0.172	0.55
(B) Proportions data					
Eigenvalue	3.8923	2.0679	1.6690	1.4777	1.1320
Proportion	0.324	0.172	0.139	0.123	0.094
Cumulative	0.324	0.497	0.636	0.759	0.853
	Variable				
	PC1	PC2	PC3	PC4	PC5
12:0	−0.065	−0.209	0.503	−0.261	−0.482
16:0	−0.119	−0.296	−0.524	0.137	0.044
16:1	0.335	−0.135	0.034	0.466	−0.124
18:0	−0.330	−0.342	0.253	0.160	0.044
18:1 (9)	0.428	−0.234	0.002	−0.062	0.288
18:1 (7)	0.436	−0.010	0.164	−0.257	0.230
18:2p	−0.022	−0.037	−0.581	−0.101	−0.404
20:2p	0.114	−0.012	−0.158	−0.703	−0.204
20:3p	0.452	0.201	−0.050	0.049	0.062
20:4p	−0.211	0.492	−0.064	−0.139	0.369
20:5p	0.327	0.244	−0.003	0.241	−0.478
22:6p	−0.135	0.579	0.107	0.128	−0.197

(A) Using the raw fatty acid compositions; (B) using the proportional contribution of each fatty acid.

4. Discussion

The pattern of variation in RMR was consistent with our previous observations in this strain (Speakman et al., 2004a) and also with the patterns of variation in RMR in other small mammal species (Jackson et al., 2001; Labocha et al., 2004) and humans (Johnstone et al., 2005; Weyer et al., 2000). As with the present study, a significant portion of the individual variation in RMR in these previous studies can be accounted for by differences in fat free-mass (FFM), but there was still a large residual variability between individuals once the effects of FFM were removed. The nature of this variation is enigmatic (Speakman et al., 2004b). Although some studies have linked intra-specific variation in RMR of mice to differences in the size of the liver (e.g. Selman et al., 2001) we have performed several studies now for MF1 mice where we have sought associations between the variation in RMR and detailed aspects of body composition (Johnson et al., 2001; Speakman and Johnson, 2000; Krol et al., 2003) and in none of these cases was variability in the sizes of particular organs an explanation of the residual variance in energy metabolism. In humans several studies have indicated links with hormone levels, e.g. thyroxine (Johnstone et al., 2005) and genetic factors (Astrup et al., 1999), but these generally explain only small components of the variation once overall body composition has been accounted for. In some cases the links to hormones, such as leptin levels, disappear entirely when differences due to body composition are removed (Johnstone et al., 2005). The within individual variation reported here at a coefficient of variation of 8% was almost identical to the repeatability reported previously (Speakman and Krol, 2005) for RMR measurements on voles (*Microtus agrestis*). Given our previous observations that variation in organ size was not an important factor influencing residual variation in RMR in this strain of mouse we have assumed that the mass specific rate of metabolism in the livers of mice with high metabolic rates was greater than that in the livers of mice with low metabolic rates and these differences were reflected in the observed variation in whole animal metabolic rates of these animals.

The pattern of variation in membrane fatty acids was consistent with the patterns previously observed in mice and rats, with the most abundant saturates being palmitic (16:0) and stearic (18:0) acids and the most abundant PUFAs being linoleic (18:2) and arachidonic (20:4) acids (Hulbert et al., 2006). The overall level of PUFAs at 44% was similar to the level reported previously for liver tissue from mice of 51% (Couture and Hulbert, 1995) and that reported generally for mammals, which vary between 39 and 70% (Valencak et al., 2004). The desaturation index reported here was however lower than has been previously reported in other strains of mice. The cause of this difference is uncertain but it is unlikely to be a consequence of differential degradation of unsaturated fatty acids during extraction resulting from our use of a mechanical extractor rather than performing the homogenisations by hand (Fig. 1). Presumably the difference is a strain effect. If so significant strain differences in unsaturation index may provide a valuable resource for further testing the membrane pacemaker hypothesis and links between membrane saturation and ageing phenomena at the intra-specific level. Valencak et al. (2004) explored the covariation in levels of membrane fatty acids in muscle tissues of the brown hare (*Lepus europeaus*) using a PCA analysis similar to that performed here. The patterns observed in mice did not match the patterns detected in the hares. In particular in mice there were no consistent loadings on any PC that grouped together either the saturated or mono-unsaturated fatty acids (Table 4). Clearly covariation in the patterns of membrane fatty acids must reflect individual patterns of the activities of desaturase enzymes that interact with dietary intakes of fatty acids in complex species specific and probably tissue specific ways—hence the absence of a close correspondence between the patterns in the livers of mice and muscles of hares is not surprising.

Previous studies of the role of membrane lipid desaturation on RMR and on lifespan have been made at the level of inter-specific comparisons or interclass comparisons (Hulbert, 2003, 2005; Hulbert and Else, 1999, 2000, 2005; Pamplona et al., 2000). The present study is the first to attempt to tie together the patterns of inter-individual variations in RMR within a species to the same patterns of variation in membrane lipid desaturation. Despite the fact that RMR was highly variable between individuals, and there was also variation in lipid desaturation in the liver between individuals, there was no indication that these patterns were correlated. Most notably there was no association between RMR (or mass adjusted RMR) and either the proportion of DHA (22:6) or oleic acid (18:1) in the liver membranes (Table 2 and Fig. 5). DHA level has been previously identified as key component of the membrane pacemaker hypothesis (Hulbert and Else, 1999, 2000, 2005). Our analysis was performed using both whole body RMR and fat-free mass adjusted RMR as well as using raw lipid contributions, scores along a PC analysis and proportional representation of different functional groupings of fatty acids with the same results. This analysis indicates that, at least as far as liver membranes are concerned, at the level of the individual within a species, the membrane pacemaker hypothesis does not explain the variance in RMR (or residual RMR). We chose liver for these analyses because it is a large organ with a high metabolic rate that has been speculated to contribute a high proportion to the total RMR. It remains to be established whether the absence of a correlation between desaturation of membranes in the liver and energy metabolism shown here is repeated in other tissues. However, we did show a broad correlation between the membrane fatty acid composition of the liver and the membrane fatty acid compositions of other tissues suggesting that the liver may be generally representative of the membrane fatty acid composition throughout the body as has been assumed in other studies.

The absence of an effect of desaturated lipids in these data may occur because the extent of variation that is evident within a given species is very small when compared with the enormous differences between species across the class Mammalia (Couture and Hulbert, 1995; Hulbert and Else, 1999, 2000, 2005), or the differences between representatives of different major vertebrate classes (Hulbert et al., 2006). Hence the

membrane pacemaker theory may explain these much broader patterns of variation, but at the level of the individual within a species other factors become much more important. However, as noted in the introduction, the membrane pacemaker idea has been developed largely ignoring confounding influences of body size and phylogeny on relatively small data sets. The problems with such analyses have been previously highlighted (Promislow, 1993; Speakman, 2005b). A more detailed and sophisticated analysis of 42 mammalian species taking account of these factors did not support the hypothesis of a link between membrane desaturation and basal metabolism (Valencak and Ruf, 2007) suggesting that even at the inter-specific level the importance of membrane desaturation on metabolism may have been over-emphasised.

Finally, one of the factors that may be linked to RMR suggested by the current study was the proportion of fatty acyl chains in the membrane that are 16:0 palmitate (Fig. 3). The reasons for this association are unclear and the trend is opposite that suggested by the membrane pacemaker hypothesis. However, the association between variation in 16:0 and RMR was strong and large (Fig. 2). A difference in the proportion of 16:0 palmitate from 0.23 to 0.27 was correlated with a difference in residual RMR from around −0.12 to +0.12 ml/min. At the mean RMR of 0.76 O_2 ml/min this difference is equivalent to a difference of 35% between the individuals with low and high levels of palmitate. This was about the same difference between the upper and lower 25 centiles of metabolism of mice that differed in their lifespans by 36% (Speakman et al., 2004a). The higher levels of the saturated fatty acid in the membranes of the mice with high metabolic rates would be consistent with these mice being protected from oxidative stress because saturated fats are less prone to oxidation—which predominantly affects PUFAs (Pamplona et al., 2002). Birds, which live longer than mammals of equivalent body mass, and have higher metabolic rates, also have higher levels of membrane saturation (Brand et al., 2003; Hulbert et al., 2002a; Portero-Otin et al., 2001). The mechanism linking greater saturation to elevated RMR however is currently unclear.

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