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Uncoupled and surviving: individual mice with high metabolism have greater mitochondrial uncoupling and live longer

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Summary

Two theories of how energy metabolism should be associated with longevity, both mediated via free-radical production, make completely contrary predictions. The 'rate of living-free-radical theory' (Pearl, 1928; Harman, 1956; Sohal, 2002) suggests a negative association, the 'uncoupling to survive' hypothesis (Brand, 2000) suggests the correlation should be positive. Existing empirical data on this issue is contradictory and extremely confused (Rubner, 1908; Yan & Sohal, 2000; Ragland & Sohal, 1975; Daan *et al*., 1996; Wolf & Schmid-Hempel, 1989]. We sought associations between longevity and individual variations in energy metabolism in a cohort of outbred mice. We found a positive association between metabolic intensity (kJ daily food assimilation expressed as g/body mass) and lifespan, but no relationships of lifespan to body mass, fat mass or lean body mass. Mice in the upper quartile of metabolic intensities had greater resting oxygen consumption by 17% and lived 36% longer than mice in the lowest intensity quartile. Mitochondria isolated from the skeletal muscle of mice in the upper quartile had higher proton conductance than mitochondria from mice from the lowest quartile. The higher conductance was caused by higher levels of endogenous activators of proton leak through the adenine nucleotide translocase and uncoupling protein-3. Individuals with high metabolism were therefore more uncoupled, had greater resting and total daily energy expenditures and survived longest – supporting the 'uncoupling to survive' hypothesis.

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Introduction

There are two contrasting ideas about how differences in energy metabolism might be associated with changes in longevity, both mediated via differences in free-radical production. The 'rate of living – free-radical damage' theory (Pearl, 1928; Harman, 1956; Sohal, 2002) suggests that higher rates of metabolism should be negatively linked to lifespan. The mechanistic basis of this hypothesis is that higher rates of oxidative metabolism result from a greater flux of electrons through the electron transport chain, with a consequent increase in production of reactive oxygen species (ROS) – which is generally assumed to form a fixed percentage (quoted anywhere between 0.1 and 4%) of total oxygen consumption (Nicholls & Ferguson, 2002; Melov *et al*., 2001). Production of ROS during oxidative phosphorylation occurs at two sites in the electron transport chain – at complex I and at complex III (Brand, 2000). It has been recently noted that production of ROS at complex III depends critically on the time that a reduced ubisemiquinone (Q-) exists at the p-site (the side of complex III adjacent to the positively charged side of the mitochondrial inner membrane), where it is capable of donating an electron to an oxygen atom. This duration depends on the potential across the mitochondrial inner membrane, which retards movement of electrons from the p to the n site (on the negative side of the membrane). In theory a high membrane potential prolongs the lifetime of Q- at the p-site, increasing the rate of ROS production. Two factors, however, may decrease the membrane potential, thereby shortening the lifetime of Q- at p. First, if adenosine di-phosphate (ADP) is nonlimiting in mitochondria (state 3 respiration), the flux of H + via the FoF1-ATPase lowers the membrane potential (Brand, 1990; Nicholls, 2004). Second, the potential is also dependent on the flow of protons across the inner membrane via various proton leak pathways, including the adenine nucleotide translocase (Samartsev *et al*., 1997) and the uncoupling proteins (UCP1-3) (Klingenberg *et al*., 2001; Garlid *et al*., 2001; Klingenberg, 1999). Since greater uncoupling should lead to lower ROS and longer lifespans this has been called the 'uncoupling to survive' hypothesis (Brand, 2000). Both state 3 respiration and higher uncoupling lead to greater oxygen consumption, hence this 'uncoupling to survive' hypothesis predicts a completely contrary, positive, association between rates of energy metabolism and longevity.

Unfortunately, the empirical data on this issue is extremely confused. One hundred years ago, Rubner (Rubner, 1908) observed that larger species combine slower metabolic rates with longer lives in a manner that results in the product of these two traits being constant across species: a so-called 'life history invariant'. However, this interspecific comparison neglects the evident covariation in these traits due to body mass. Species that are larger tend to also live longer and also have lower metabolic rates per gram of body tissue. An association between low metabolism and extended lifepsans may thus arise only by virtue of the effects on both of body size differences between different species. To this very obvious problem is added the less well recognized difficulty of treating each species as an independent statistical entity in the regression analysis. Because species are the products of an evolutionary process, all mammals share to differing extents their evolutionary history, and thus do not represent independent data. Statistical methods have been developed in the last 2 decades that overcome the problem of phylogenetic lack of independence and some studies have suggested that the association of high metabolic rates with short lives disappears once this factor and the covariation due to body mass are taken into account (Harvey *et al*., 1989; Promislow & Harvey, 1990). Adding to the confusion, however, other analyses indicate that the association is preserved even when these factors are accounted for (Speakman *et al*., 2002).

At the intraspecific level many experimental manipulations of animals have indicated that reducing their metabolic rates by, for example, restricting their activity (Yan & Sohal, 2000), or in exotherms lowering the ambient temperature (Ragland & Sohal, 1975), leads to extensions in lifespan. Complementary studies have shown that increasing metabolism shortens lifespan (Daan *et al*., 1996; Wolf & Schmid-Hempel, 1989). Conversely, several studies show that increasing metabolism by cold exposure and exercise has no effect, or even increases lifespan (Holloszy & Smith, 1986). Some studies indicate that mutants of *Caenorhabditis elegans* that have extended lifespans only do so by virtue of reduced metabolism (Van Voorhies & Ward, 1999; Van Voorhies, 2002), but this is hotly contested by others suggesting no differences occur between the mutants and wild types (Braeckman *et al*., 2002; Braeckman *et al*., 2002). Similarly, the energy expenditures of calorically restricted, long-lived animals have been a matter of continued debate (Ramsey *et al*., 2000; McCarter & Palmer, 1992) as is the involvement of mitochondria in these effects (Merry, 2004). Comparisons across different breeds of dog indicate a positive association (Speakman *et al*., 2003), and dwarf strains of mice, with higher metabolic rates (per gram) (Liang *et al*., 2003) generally also live longer (Brown-Borg *et al*., 1996), but again in these comparisons body mass is a complicating covariable. Across different strains of *Drosophila* there is apparently no association between metabolic rate and lifespan (Promislow & Haselkorn, 2002). Moreover, studies of the transition from fast state 3 to slow state 4 respiration in mitochondria have not detected the predicted increase in ROS production (Barja, 1999).

Individual animals within a single species or strain vary widely in their energy metabolism. The association between metabolic rate and longevity at this level has seldom been addressed. We aimed to investigate the association between metabolic intensity and longevity across individuals from a single strain of mice. Having established an association we then characterized different groups of the same strain of mice for their energy metabolism, and compared the upper and lower quartiles for the extent of mitochondrial uncoupling.

Results

We initially studied a cohort of 42 female MF1 mice, which were purchased from a commercial breeder at the age of 6 weeks, and housed individually. The MF1 strain is an outbred strain and we requested when the animals were purchased that the sample should include no siblings. Between the ages 6 and 13 months we measured several parameters related to energy balance in the mice and then monitored them until they died.

The lifespans of the individual mice varied from 244 days to 988 days and were normally distributed (Anderson-Darling test $A^2 = 0.351$, $P = 0.455$) around a mean of 567.5 days (se = 25.5, $n = 42$). Sentinel animals submitted for analysis during the course of this experiment revealed the colony was clear of infections. Lifespan was not related to body mass ($F_{1,40} = 0.62$, $P = 0.437$), body temperature ($F_{1,40} = 0.22$, $P = 0.625$), body fat content ($P = 0.755$) or body lean tissue content ($P = 0.958$) measured between 6 and 13 months of age. Daily food intake and dry mass assimilation efficiency were both normally distributed (Anderson-Darling test $A^2 = 0.441$, $P = 0.227$ and $A^2 = 0.25$, $P = 0.772$, respectively) with means of 6.03 g food per day (se = 0.101) and 81.24% (se = 0.37%), respectively. There was a significant positive relationship between daily food intake and body mass (daily food intake (g) = 2.55 + 0.0824 Body mass (g) $F_{1,40} = 5.55$, $P = 0.024$, $r^2 = 0.122$), and between daily food intake and assimilation efficiency (Assimilation [%] = 72.98 + 1.36 Food intake (g), $F_{1,40} = 7.00$, $P = 0.013$, $r^2 = 0.200$). Once the animals were mature (6 months of age) they increased in body mass only very slowly. Making limiting assumptions that all the mass increase was fat and comparing this to the measured ingested energy we calculated that on average 99.4% of the measured daily energy intake was expended on metabolism. Consequently there was a positive link between daily energy expenditure (DEE) and body mass.

Methods for normalizing the effects of body mass on metabolism have been a source of contention for many years [e.g., Packard & Boardman,1988). We therefore removed the effect of body mass on energy demands in three different ways. First, by calculating the residual to the fitted relation of mass to metabolism, second, by calculating the metabolic intensity (kJ daily food energy · g body mass^{-1}) and third by calculating metabolic rate relative to the lean body mass (kJ · g lean body mass^{-1}). For any given level of energy expenditure there was a wide range of lifespans. Nevertheless, across all the individuals, lifespan was significantly positively related to the residual daily energy expenditure accounting for the effects of body mass (Fig. 1a: Lifespan (days) = 567.5 + 7.40 DEE residual, $F_{1,40} = 7.69$, $P = 0.008$, $r^2 = 0.161$) the metabolic

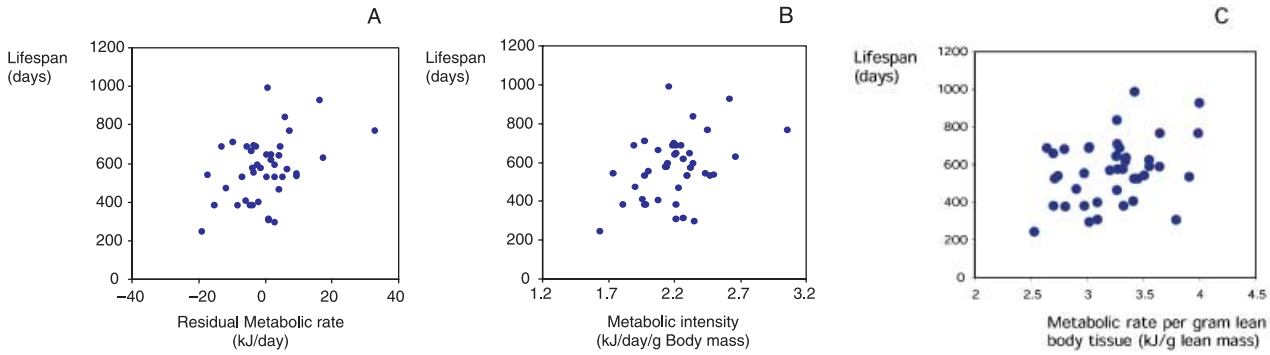

Fig. 1 Relationships between individual lifespans of 42 female MF1 mice and different expressions of daily energy metabolism measured when the individual mice were 6 months of age (a) residual daily energy expenditure (DEE) – removing the significant effect of body mass on DEE (b) metabolic intensity (DEE/body mass) and (c) metabolism per gram of lean body tissue (DEE lean^{-1} body mass). All three relationships were significant (see text for statistics).

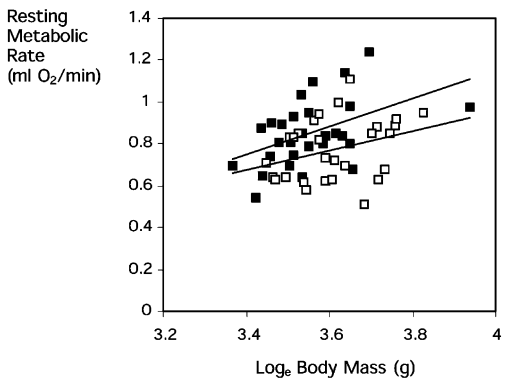

Fig. 2 Resting metabolic rate as a function of body mass for individual mice in the upper (closed symbols) and lower (open symbols) quartiles of energy intensity (daily food energy assimilation per gram of body mass). Both body mass and group of origin had significant effects (see text for statistics). Lines are fitted regression curves.

intensity (kJ · g body mass–1) (Fig. 1b: Lifespan (days) = –4.1 + 270.4 Metabolic intensity, $F_{1,40} = 7.68$, $P = 0.008$, $r^2 = 0.161$) and metabolism per gram lean body mass (Fig. 1c: Lifespan (days) = 76.9 + 157.2 Metabolism · g lean body mass^{-1}, $F_{1,40} = 5.11$, $P = 0.026$, $r^2 = 0.113$). Although the explained variation in lifespan was low, at 11–16% depending on the normalization process, the differences between individuals with high metabolism and those with low metabolism were profound. Individuals from the lowest quartile of metabolic intensities lived on average 472 days (se = 46 days, $n = 10$) while those from the highest quartile had 30.2% higher metabolic intensity and lived on average 641 days (se = 58, $n = 10$), which is 36% longer [(highest lowest^{-1} – 1) × 100]. In this cohort of mice, high metabolic intensity was linked with living longer.

In two further cohorts of 60 and 56 mice, respectively, we measured food intake at 6 months of age and selected the upper and lower quartiles for their metabolic intensity (food energy assimilated per gram body tissue) – hereafter referred to as high and low metabolism mice, respectively. In these upper and lower quartiles we measured resting metabolic rate (RMR) at thermoneutrality (30 °C) by indirect calorimetry. Resting metabolism was significantly related to body mass ($F_{1,53} = 10.3$, $P < 0.002$) and the group of origin ($F_{1,53} = 7.81$, $P < 0.007$), with high metabolism mice having mass-adjusted resting metabolic rates that were on average 17% higher than the low metabolism group [Fig. 2].

In both these cohorts, the proton conductance across the inner membrane of mitochondria isolated from skeletal muscle of high metabolism mice was greater than that of low metabolism mice. The combined data is shown in Fig. 3(a). In all the experiments in Fig. 3 bovine serum albumin (BSA) was absent, so endogenous fatty acids and any other endogenous activators would have been present. Inhibiting the adenine nucleotide translocase (ANT) with carboxyatractylate (CAT) lowered the proton conductance of mitochondria from both groups and abolished the difference between the high and low metabolism groups (Fig. 3b), showing that the difference was mediated by the ANT. Addition of GDP to inhibit UCP3 did not affect the proton conductance of the mitochondria from the low metabolism group, but did lower the proton conductance of the high metabolism group (Fig. 3c), abolishing the difference observed in Fig. 3(a), and showing that UCP3 also contributed to the difference in proton conductance of mitochondria from the two groups in the absence of BSA.

To establish whether the differences between high and low metabolism groups were caused by intrinsic differences in the mitochondria or by differences in their activation state, the experiments in Fig. 3 were repeated with the concentration of activatory fatty acids clamped by addition of 0.3% BSA and 300 μM palmitate. Under these conditions, the difference in proton conductance seen in Fig. 3(a) disappeared (Fig. 4a), suggesting that the difference in Fig. 3(a) was caused by a higher level of endogenous fatty acids in the high metabolism group. With clamped fatty acid concentrations, the ANT-mediated (CAT-sensitive) component of the proton conductance was present but identical in both groups (Fig. 4b), again suggesting that the difference seen in Fig. 3(b) was caused by higher levels of endogenous activatory fatty acids in the high metabolism group. In the presence of BSA and palmitate, GDP inhibition of UCP3 had no effect on the proton conductance of either

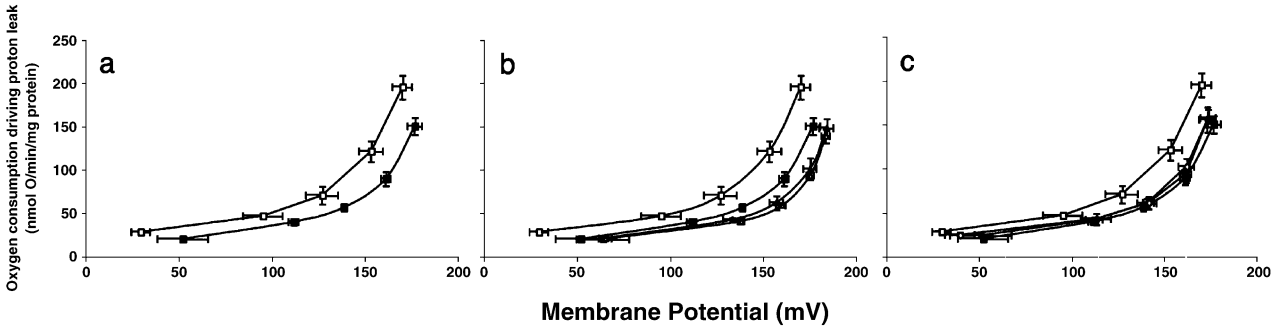

Fig. 3 Proton conductance kinetics in the absence of bovine serum albumin (BSA) of skeletal muscle mitochondria isolated from high and low metabolism mice. Oxygen consumption driving the proton leak is shown as a function of its driving force (membrane potential) with succinate as a substrate and potential varied with sequential additions of malonate as described under 'methods'. (a). High metabolism mice have more uncoupled mitochondria. Open squares: high group; closed squares: low group; (b) Carboxyatractylate (CAT) reduces the proton conductance of both groups of mitochondria and eliminates the difference between them. Open triangles: high group plus 1.2 μM CAT; closed triangles: low group plus 1.2 μM CAT. (c) Inhibition of UCP3 reduces the proton conductance from the high group only. Open circles: high group plus 1 mM GDP; closed circles: low group plus 1 mM GDP. Data are means ± s.e.m. of four independent experiments each performed in duplicate.

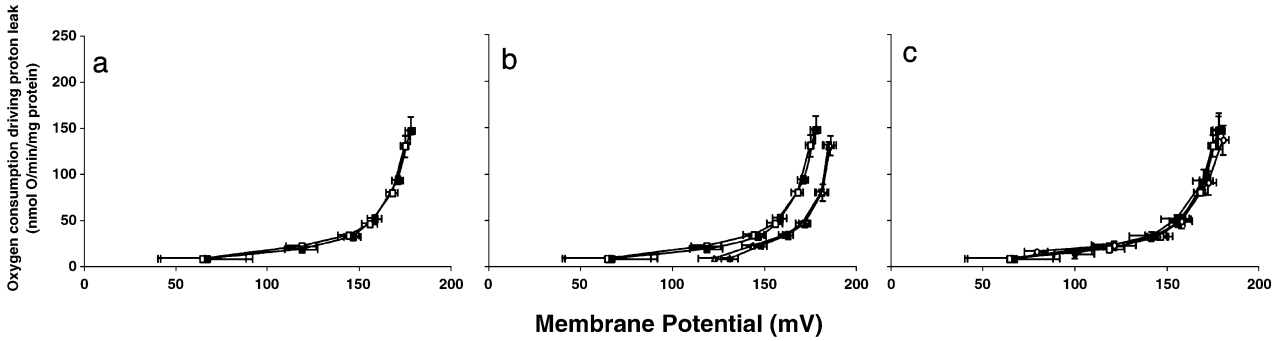

Fig. 4 Proton conductance kinetics in the presence of BSA and palmitate of skeletal muscle mitochondria isolated from high and low metabolism mice. The medium contained 0.3% (w/v) BSA and 300 μM palmitate to clamp the fatty acid concentration to the same value for both mitochondrial populations. (a) No difference in proton conductance between high and low metabolism mice in the presence of BSA and fatty acid. Open squares: high group; closed squares: low group; (b) Equal palmitate-dependent ANT-mediated proton conductance of mitochondria from both groups of mice. Open triangles: high group plus 1.2 μM CAT; closed triangles: low group plus 1.2 μM CAT. (c) Inhibition of UCP3 is without effect. Open circles: high group plus 1 mM GDP; closed circles: low group plus 1 mM GDP. Data are means ± s.e.m. of four independent experiments each performed in duplicate.

group (Fig. 4c). Thus, BSA removed some endogenous activator of UCP3 that was present only in mitochondria from the high metabolism mice (Fig. 3c), but (unlike the situation in Fig. 4b) addition of palmitate could not substitute for this activator. The identity of this endogenous UCP3 activator is unknown, but it might be related to known *in vitro* activators such as retinoic acid (Echtay *et al*., 2003).

Discussion

There is considerable confusion in the literature regarding the expected association between rates of energy metabolism, rates of free radical production and the consequent rates of aging and longevity (reviewed in 14,21). This theoretical confusion has been compounded by the fact that empirical data adduced to support or refute different positions has been drawn from a mix of intraspecific, interspecific and interclass comparisons. Hence it has been often noted that across different mammalian species those that live longest have the lowest mass specific rates of metabolism (Pearl, 1928; Rubner, 1908). The confounding effects of body mass and phylogeny when comparing species as diverse as mice and elephants are apparent – yet agreement on the consequences of statistically removing such influences has been elusive. Some studies indicate that statistically removing the effects of mass and phylogeny also removes the association (Harvey *et al*., 1989; Promislow & Harvey, 1990), but in other reviews the associations are preserved (Speakman *et al*., 2002). The difference between these results perhaps revolves around the methods utilized to express rates of energy metabolism, with the two studies finding no association (Harvey *et al*., 1989; Promislow & Harvey, 1990) relying on resting metabolic rate as a proxy for total daily metabolism, but the study finding a significant negative association (Speakman *et al*., 2002) being based on direct measures of daily energy demands. Whatever the interspecific comparisons within mammals ultimately show it is generally agreed that the interclass comparison, of birds to mammals, reveals that birds combine elevated rates of daily energy metabolism (Nagy *et al*., 1999) with increased lifespans (Holmes *et al*., 2001). Making comparisons however, at the interspecific and intraclass levels are confounded by many

other traits that differ systematically between species and between classes.

Theoretically the effects of variations in metabolic rate might be expected to be more profound within species where these confounding factors are kept to a minimum. Nevertheless, similar confusion over the association between energy metabolism and lifespan reigns in intraspecies studies – with several showing a negative link (Yan & Sohal, 2000; Ragland & Sohal, 1975; Daan *et al*., 1996; Wolf & Schmid-Hempel, 1989; Van Voorhies & Ward, 1999; Van Voorhies, 2002), others finding no such association (Holloszy & Smith, 1986; Braeckman *et al*., 2002; Braeckman *et al*., 2002; Promislow & Haselkorn, 2002), and yet further studies indicating the association may be positive (Speakman *et al*., 2003; Liang *et al*., 2003; Brown-Borg *et al*., 1996). The present study [Fig. 1] clearly indicated that there was a positive association between the habitual levels of energy expenditure across a cohort of individual mice and their longevity. There has been much discussion in the literature about the methods used to normalize levels of energy expenditure and these problems of normalization appear to underlie at least some of the confusion regarding the association between energetics and aging (Braeckman *et al*., 2002; Braeckman *et al*., 2002; McCarter & Palmer, 1992). Fortunately in this instance the association persisted whatever method we used to normalize the expression of energy metabolism for individual differences in body mass. There can be no doubt therefore over whether the association is an artefact of the manner in which metabolic rates were expressed. The magnitude of the effect was also impressive with a 36% difference in lifespans between the animals with the lowest 25% and highest 25% of metabolic rates. This is equivalent to an age difference in humans from 75 to 102 years.

Given the importance of this effect it is worth exploring whether the effect we have found might stem from an artefact due to some necessary assumptions that we made during the calculation of energy expenditures in the animals in question. In deriving the estimated daily energy expenditures of these animals we made three important assumptions: first, that the energy content of the faeces that was only measured as an average across all the individual mice was an appropriate value in all the individuals. Second, we assumed that the energy lost in urine as urea amounted to only 3% of the total daily energy intake. Finally, we assumed that the change in body weight of the individuals over the 30 day period consisted completely of fat tissue. We will discuss the potential impacts of each of these assumptions on our calculations. It is not only possible, but highly likely that some variation could occur from mouse to mouse in fecal energy content. However, it has been known since at least the 1930s that the variation in fecal energy content is small compared to the variation in mass of faeces produced, which is the dominant factor influencing assimilation efficiency. In other studies, we have measured individual fecal energy contents and the range from mouse to mouse is less than 5% of the mean. However, because the fecal output is only 18.8% of intake, the maximum possible impact on the estimate of total assimilated energy contents of, say, assuming that all mice produce faeces with the maximal energy content, is only 5% of 18.8% (i.e. about 0.9%). By using the mean in the calculations the maximum error introduced by the assumption is 0.45%.

The value we assumed of 3% for the energy lost in urine is a typical one used in construction of mouse energy budgets where animals eat diets that are high in carbohydrates and low in protein (around 12% by weight). Again individual variation in this trait is small, and since the mice were measured at 6 months of age, they were measured before the onset of any complications that might arise due to age-related onset of diabetes, although we found no evidence of the disease, even in old age. The absolute maximum error introduced by this assumption is probably in the region of 2%, i.e. individuals could potentially vary from 1 to 5% in the energy exported as urea in urine.

Over the 30 days of measurements the changes in body mass of the mice were trivially small compared to the total mass of food they consumed. By assuming that all the mass change was fat, we made an assumption of the maximal contribution that such mass changes could make to the total energy utilization, using which the contribution was only 0.6%. It is very likely that the change in mass was not all fat. However, if we assume it was actually all water, and completely calorie free, the impact on the estimate of total energy utilization would be only 0.6%.

Assuming the worst case scenario that all three factors combined in the same direction in any given individual and hence their effects did not cancel each other out, the maximum error that could be introduced into an individual value would be 3.0%. To test the impact of such uncertainty in our measurements we randomly varied all the data by plus or minus 3.0% and recalculated the regressions and differences between the upper and lower 25 centile groups. In none of these simulations was statistical significance lost in the relation of energy demands to lifespan, and, because random errors in the individuals cancelled, the differences between the top and bottom 25 centile groups were almost completely unaffected. Finally, if we make the very unlikely assumption that all animals have a 3% error (itself a very small possibility), and by chance all the top 25% of animals had their energy demands overestimated by 3.0%, and the bottom 25% had theirs underestimated by 3.0%, the gap between them would of course narrow by 6.0%. Even ignoring the low chance that all animals have the maximal possible error, this scenario would only occur with a probability of $1/2^{20}$ (= 0.000000953) and even this improbable effect is minor relative to the observed 30.3% difference. We can be confident therefore that these assumptions in the constructed energy budgets make no impact on the validity of the data we have presented.

Together, the data in Figs 3 and 4 show that skeletal muscle mitochondria isolated from high metabolism mice were more uncoupled than those from low metabolism mice. The higher proton conductance was caused by higher concentrations of endogenous activators: fatty acids, which activated the proton conductance of ANT, and an unidentified activator of UCP3. We speculate that the activation of uncoupling was also present *in*

vivo in the high metabolism mice, and explains their phenotype. There was a puzzling nonadditivity between the two inhibitors. We have previously seen (and commented on) the same puzzling nonadditivity in systems where we know that both ANT and UCP are activated (Promislow & Harvey, 1990), so there is no reason to believe that the nonadditivity compromises our interpretations of the inhibitor effects in the current study.

An additional potential problem was that we studied the properties of mitochondria *in vitro*, but these properties may not necessarily translate completely into the behaviour of mitochondria *in vivo*. For example, with respect to fatty acids, the processes used to isolate mitochondria from muscle may markedly alter fatty acid concentrations. Also, the amount of superoxide, which is an established activator of UCP3, could be markedly different between the live animal and an *in vitro* preparation. The same is true for endogenous inhibitors of UCP3, i.e. the purine nucleotides. However, it is not yet technically possible to accurately measure the conductance of mitochondria *in vivo*. What we can say is that the conductance properties of the mitochondria we isolate are significantly different between the two groups, that this difference can be explained by different levels of modulators such as fatty acids, superoxide, alkenals or nucleotides *in vitro*, and that it must reflect some *in vivo* difference between the animals that the mitochondria were taken from. Our suggestion that it reflects differences in the activation of ANT and UCP3 *in vivo* in the muscles of more metabolically active mice is the simplest and most direct interpretation.

One alternative explanation of the differences we observed in the proton leak curves is that these were an artefact of the normalization process during which everything was expressed relative to the amount of matrix protein. If the amounts of total mitochondrial protein differed greatly between the groups this might introduce an artefactual alteration in the leak curves during the normalization process. However, if matrix protein did differ, then all of the conductance curves would shift to the same extent independent of the treatments we applied. This was clearly not the case: the curves in the presence of CAT or GDP or BSA overlay each other, while those without additions do not. This suggests that any differences in matrix protein were not a problem.

Another assumption we made was that the mitochondria extracted from the muscle were representative of mitochondria from elsewhere in the body or that muscle mitochondria play a dominant role in the aging process. Our reason for choosing skeletal muscle for these measurements was that skeletal muscle is responsible for a large proportion of metabolic rate in mice, so is the most likely site where small mitochondrial differences might cause the observed differences in metabolic rate. The assumption that these mitochondria reflect mitochondria in other tissues is reasonable, given that all mitochondria contain ANT, and that activatory fatty acids are unlikely to be confined to the mitochondria of skeletal muscle.

Sentinel animals from our colony did not reveal any indication that these animals were infected and consequently there was no evidence that the differences in the lifespans were a result of differential susceptibility to infection. We did not perform routine autopsy work on the animals in the first cohort, but in a few cases where there was obvious pathology the animals died (or were terminated on ethical grounds) because of abdominal tumour development. Cancer would be a consistent pathology associated with accumulated oxidative damage. We feel confident therefore that our study is not complicated by responses to infectious disease.

The work we performed on mitochondria extracted from the second and third cohorts of mice, in combination with the first cohort [Fig. 1] where we showed that mice with higher metabolic intensities lived longest, provide greater support for the 'uncoupling to survive' hypothesis than for the 'rate of living-free-radical damage' hypothesis, at the level of individual phenotypic differences in metabolic intensity. Since we used an outbred strain kept in constant environmental conditions, presumably these phenotypic differences have a genetic component at their origin; this conclusion is supported by the fact that the same association between longevity and metabolic intensity is also observed across inbred strains (Storer *et al*., 1967).

Methods

Female mice (strain MF1) aged 6 weeks were purchased from a commercial breeder (Harlan, Bicester, Oxon, UK Ltd) and housed singly in shoebox cages measuring 12(h) × 12(w) × 40(h) cm, containing sawdust and paper bedding. Food and water were provided *ad libitum*. Room temperature was maintained at 22 °C (± 2°C) and the photoperiod was maintained at 12 L:12D, lights on at 09.00 hours. Between 6 and 13 months of age we measured a series of parameters relating to individual aspects of energy balance. Mice were then monitored daily until they died and relationships were sought between the parameters measured during the period from 6 to 13 months and their lifespans.

Food intake

Mice were fed on a dry pelleted feed (CRM pellets, SDS diets, BP Nutrition Ltd): 88.5% dry matter and an energy content measured by bomb calorimetry of 18.36 kJ g^{-1} dry mass. In the first cohort of mice we measured daily food intake over two periods of 30 days commencing at age 6 months and at age 12 months. Each day, between 14.00 and 17.00 hours, the food was weighed (Sartorius balance, 2 dp) and intake evaluated by the difference from the previous day's measurement. We made searches through the sawdust for any large pieces of food taken from the hoppers but not ingested. In another study of the same strain we made thorough searches of the sawdust and established that uneaten food accounted for less than 2% of the mass missing from the hoppers (Krol & Speakman, 1999). There was a strong correlation between the mean daily food intake measured during the first session and that measured during the second session, indicating consistent individual variation in the intake over time. Dry food intake (g) was expressed as the average daily intake over the first 30 days measurement at 6 months of age.

Assimilation efficiency

Apparent dry mass assimilation efficiency was measured over two 3-day periods, simultaneous to the measurements of food intake when the mice were 6 months old. At the start of the measurement period the cages were cleaned and new sawdust provided. At the end of the period the cage contents were removed and the faeces manually sorted from the sawdust and bedding. The faeces were dried (60 °C for 14 days) and the apparent dry mass assimilation efficiency was expressed as the difference between the dry mass of faeces produced and the dry mass of food ingested divided by the dry mass of food ingested. There was a strong correlation between the replicate measurements of each individual and therefore we took the average assimilation efficiency across the two measurements.

Body mass

Body mass was measured daily between 14.00 and 17.00 hours (Sartorius balance, 2 dp) during the two periods of 30 days when food intake was also measured daily. The measurements of body mass made at 6 months and at 12 months were well correlated indicating consistent individual changes over time. We expressed body mass as the mean of the values at 6 months of age, and growth as the difference in mass between the start and end of this measurement period.

Daily energy expenditure

The energy content in a sample of faeces averaged 16.9 kJ g^{-1} dry mass. Apparent energy assimilation was estimated as the difference between energy intake and elimination in the faeces averaged over the two 3-day periods. We predicted the daily production of faeces for each of the 30 days using the known food intakes for each day and the individual estimates of assimilation efficiency derived over the two 3-day periods of monitoring. We then multiplied the food intake by the known energy content of the food and multiplied the predicted faecal production by the known energy content of the faeces. The difference was the apparent energy absorption. We did not measure urinary energy losses in these animals but assumed that urinary energy loss amounted to 3% of the apparent energy intake (Krol & Speakman, 1999). We applied this correction to the apparent energy absorption to obtain the apparent metabolisable energy intake. Making a limiting assumption that the mass accumulated over the 30 day monitoring period at age 6 months was exclusively fat (39.6 kJ g^{-1}), and subtracting this from the total apparent metabolisable energy intake, we estimated the minimum daily energy expenditure.

Body composition

Body composition (fat and lean tissue contents) was determined at 9 months of age using a live animal total body electrical conductivity (TOBEC) meter (Jagmar Ltd, Wroclaw, Poland) with a dedicated small animal measuring chamber. Individuals had their body masses and body temperatures measured by a deep (3–4 cm) rectal probe, and were then placed in the measurement chamber. Ten measurements of total body conductivity were made at approximately 10 s intervals and the lowest and highest values were rejected before the mean value was calculated. The mean values were inserted into a validation equation derived from a comparison of TOBEC measurements to the actual lean mass calculated following ether extraction of a sample group of mice (n = 35) of the same strain (Mitchell, Hambly and Speakman, unpublished data). The lean mass was therefore measured indirectly using TOBEC and the fat mass inferred from the difference between the predicted lean mass and the body mass.

Resting metabolic rate

We measured resting metabolism in the thermoneutral zone using procedures previously described for this strain of mice (Johnson *et al.*, 2001).

Mitochondrial extraction

Isolation of skeletal muscle mitochondria was performed as previously described (Cadenas & Brand, 2000) with all steps carried out at 4 °C. We chose skeletal muscle because, while it has a low rate of metabolism, it comprises a large proportion of total body mass and therefore contributes significantly to total metabolism. It also contains UCP3, which may be involved in control of ROS production. Animals were killed by stunning, followed by cervical dislocation. Tissue was pooled from four animals for each mitochondrial preparation. Skeletal muscle from the hindlimbs was dissected and placed in ice cold CP-1 medium containing: 100 mM KCl; 50 mM Tris and 2 mM EGTA, pH 7.4 at 4 °C. Muscle tissue was shredded, rinsed with CP-1 medium, stirred for 4 min in CP-2 medium (100 mM KCl, 50 mM Tris, 2 mM EGTA, 1 mM ATP, 5 mM $MgCl_2$, 0.5% (w/v) BSA, 210 units per 100 mL protease (subtilsin) pH 7.4 at 4 (°C) and homogenized in CP-2 medium. The homogenate was stirred in CP-2 for a further 6 min, then mitochondria were isolated using differential centrifugation. Protein concentration was determined using the biuret method (Gornall *et al.*, 1949).

Measurement of mitochondrial proton conductance

Respiration rate and membrane potential were measured simultaneously using electrodes sensitive to oxygen and the potential-dependent probe triphenylmethyl phosphonium cation (TPMP +) (Brand *et al.*, 1995). Mitochondria (0.35 mg of protein/mL) were incubated at 37 °C in assay medium (120 mM KCl, 5 mM KH_2PO_4, 3 mM Hepes, 1 mM EGTA, 1 mM $MgCl_2$, pH 7.2) containing 5 μM rotenone, 43 ng mL^{-1} nigericin and 1 μg/mL oligomycin, and assumed to contain 406 nmol O/mL at 37°C (Reynafarje *et al.*, 1985). When required, 0.3% (w/v) BSA and 300 μM palmitate were added. Although BSA was included in

the isolation buffer, fatty acids are continuously generated by endogenous phospholipases and hence further treatment with BSA is required in the incubation medium to remove these potential activators. Respiration and potential were inhibited progressively through successive steady states by additions of malonate up to 10 mM using 4 mM succinate as substrate. At the end of each run, 1 μM FCCP was added to dissipate the membrane potential and release all TPMP + back into the medium, allowing for correction of any small electrode drift. The TPMP + binding correction factor was taken as 0.35 μl/mg of protein-1 for skeletal muscle (Rolfe *et al*., 1994). Where indicated GDP and CAT were added at 1 mM and 1.2 μM, respectively.

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