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Published in:
Physiological and Biochemical Zoology

DOI:
10.1086/589727

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2009

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Vaanholt, L. M., Daan, S., Schubert, K. A., & Visser, G. H. (2009). Metabolism and Aging: Effects of Cold Exposure on Metabolic Rate, Body Composition, and Longevity in Mice. *Physiological and Biochemical Zoology*, *82*(4), 314-324. https://doi.org/10.1086/589727

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Metabolism and Aging: Effects of Cold Exposure on Metabolic Rate, Body Composition, and Longevity in Mice

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Accepted 4/28/2008; Electronically Published 12/30/2008

ABSTRACT

The proposition that increased energy expenditure shortens life has a long history. The rate-of-living theory (Pearl 1928) states that life span and average mass-specific metabolic rate are inversely proportional. Originally based on interspecific allometric comparisons between species of mammals, the theory was later rejected on the basis of comparisons between taxa (e.g., birds have higher metabolic rates than mammals of the same size and yet live longer). It has rarely been experimentally tested within species. Here, we investigated the effects of increased energy expenditure, induced by cold exposure, on longevity in mice. Longevity was measured in groups of 60 male mice maintained at either 22°C (WW) or 10°C (CC) throughout adult life. Forty additional mice were maintained at both of these temperatures to determine metabolic rate (by stable isotope turnover, gas exchange, and food intake) as well as the mass of body and organs of subsets of animals at four different ages. Because energy expenditure might affect longevity by either accumulating damage or by instantaneously affecting mortality rate, we included a third group of mice exposed to 10°C early in life and to 22°C afterward (CW). Exposure to cold increased mean daily energy expenditure by ca. 48% (from 47.8 kJ d^{-1} in WW to 70.6 kJ d^{-1} in CC mice, with CW intermediate at 59.9 kJ d^{-1}). However, we observed no significant differences in median life span among the groups (WW, 832 d; CC, 834 d; CW, 751 d). CC mice had reduced body mass (lifetime mean 30.7 g) compared with WW mice (33.8 g), and hence their lifetime energy potential (LEP) per gram whole-body mass had an even larger excess than per individual. Greenberg (1999) has pointed out that the size of the energetically costly organs, rather than that of the whole body, may be relevant for the rate-of-living idea. We therefore expressed LEP also in terms of energy expenditure per gram dry lean mass or per gram "metabolic" organ mass (i.e., heart, liver, kidneys, and brain). No matter how it was expressed, LEP in CC mice significantly exceeded that of WW mice. This result demonstrates that increased energy expenditure does not shorten life span and adds evidence to the intraspecific refutation of the rate-of-living theory. We suggest that increased energy expenditure has both positive and negative effects on different factors determining life span and that the relationship between energy turnover and longevity is fundamentally nonmonotonic.

Introduction

Since the beginning of the twentieth century, there has been much debate about the influence of energy expenditure on life span. The rate-of-living theory proposed in 1928 by Pearl (1928) states that there is a negative relationship between energy expenditure and life span. The mechanism linking metabolic rate and aging may be the inevitable production of free radicals during oxygen consumption as proposed by the so-called free-radical theory of aging postulated half a century ago by Harman (Harman 1956; Beckman and Ames 1998). Free radicals (or reactive oxygen species [ROS]) can cause damage to macromolecules, which could eventually result in cell death (see Beckman and Ames 1998 for review).

Whereas the free-radical theory has gained much support in recent years, the rate-of-living theory has been discounted by many researchers based on interspecific comparisons and the lack of effects on lifetime energy expenditure in calorically restricted animals. Interspecific comparisons within the subgroups of birds and mammals do show that larger animals, with lower rates of energy expenditure (per gram body mass), generally live longer (Speakman et al. 2002; Speakman 2005*a*). This association does not hold across taxa. Birds spend up to three times more energy per day and yet live longer than mammals with similar body mass. Marsupials should live longer than eutherian mammals based on their metabolic rates, but they do not. Such counterexamples do not preclude the possibility that for the individual animal, increased energy expenditure shortens life. This relationship cannot be studied by interspecific comparisons and therefore requires experimental studies.

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Physiological and Biochemical Zoology 82(4):314–324. 2009. © 2009 by The University of Chicago. All rights reserved. 1522-2152/2009/8204-8034$15.00
DOI: 10.1086/589727

Experiments addressing the effect of energy expenditure on mortality have manipulated workload (honeybees; Wolf and Schmid-Hempel 1989) and family size (kestrels; Daan et al. 1996) in the field. In more controlled laboratory conditions, caloric restriction (CR) has long been established as a manipulation that increases mean and maximum life span in rodents, as first shown by McCay et al. (1935). Sacher (1977) proposed that CR extends life span by decreasing metabolic rate. However, most studies investigating these effects have not shown significant reductions in lifetime energy expenditure in CR animals (Masoro et al. 1982; McCarter and Palmer 1992; Ramsey et al. 1996). Interpretation of these results is confounded by the fact that metabolic rate is usually expressed per gram body mass or lean mass, whereas the relative sizes of organs that are most metabolically active are not the same for CR animals and those that are fed ad lib. (Greenberg 1999; Greenberg and Boozer 2000). Speakman (Speakman et al. 2002; Speakman 2005*a*) has pointed out that this is not the only problem that occurs when testing the rate-of-living theory. The primary problems are as follows. (1) The measures generally used to estimate life span (maximum life span) and energy expenditure (basal metabolic rate) do not reflect the parameters relevant to test the proposition. (2) Using a single measure of metabolic rate in the lifetime of an animal might not be sufficient to make an accurate estimate of lifetime energy potential (LEP). (3) The need to scale energy metabolism to body mass is complicated due to the fact that not all parts of the body use the same amount of energy. (4) Interspecific comparisons are complicated by the fact that animals from different species may reflect adaptive or genetic differences in free-radical production or differences in defense and repair mechanisms.

The rate-of-living theory needs to be tested within species by manipulating energy expenditure in different ways. Temperature reductions have been used to decrease metabolism in poikilotherms (drosophila: Miquel et al. 1976; nematodes: Van Voorhies and Ward 1999) and heterotherms (hamsters: Lyman et al. 1981), leading to extensions of life span. In this study, we investigated the effects of cold-induced elevations in energy turnover rate on life span in a homeothermic mammal, paying attention to Speakman's considerations (Speakman et al. 2002; Speakman 2005*a*). We manipulated energy metabolism by exposing mice to low (10°C) or high (22°C) temperature throughout their adult lives and studied the relationship between energy metabolism and life span. For an endothermic animal such as the mouse, cold exposure is a very powerful tool to establish a strong increase in energy expenditure due to an increase in heat production (Barnett 1965). Long-term cold exposure will lead to acclimation such as changing the thickness of fur, changing body mass, and/or changing activity level (see Barnett 1965). Despite these adjustments, energy expenditure is dramatically increased in acclimated cold-exposed mice compared with control mice (Barnett 1965). Metabolic rate was assessed by stable isotope turnover, gas exchange, and food intake in subsets of animals at four different ages. In addition, body mass and composition were determined at the same ages. Because energy expenditure might affect longevity by either accumulating wear of tissues or by instantaneously affecting mortality rate, we included a third group of mice exposed to 10°C early in life and to 22°C afterward. This aimed to test the basic proposition implicit in both the rate-of-living and free-radical theories that the presumed effects of energy turnover accumulate during life. In accordance with these theories, we expect that animals exposed to cold (10°C), which have high lifetime energy expenditure, will have reduced life span compared with animals kept at 22°C, which have lower lifetime energy expenditures. In addition, animals that are kept in the cold early in life and at 22°C later are expected to have intermediate lifetime energy expenditure compared with the cold and warm mice and to have an intermediate life span.

Material and Methods

Animals and Housing

Male C57BL/6JOlaHsd mice were obtained from Harlan Netherlands (Horst) at 4 wk of age. Animals were housed in groups of three at 22°C during the second month of life to keep developmental conditions equal between the groups. At 2 mo of age, when animals had reached adulthood and sexual maturity, they were caged individually and housed at different ambient temperatures. At this time, all mice were randomly allocated to three experimental groups of 100 mice each. Group WW was housed at 22°C (warm) throughout adult life. Group CC was housed at 10°C (cold) from the age of 2 mo onward. The third group (CW) was housed at 10°C from age 2 mo until 15 mo and at 22°C from age 15 mo onward. Animals were housed in Macrolon type II cages (UNO Roest Vast Staal, Zevenaar) with Hemparade (HempFlax, Oude Pekela) as bedding material and EnviroDry (BMI, Helmond) as nesting material. Mice had ad lib. food (Standard rodent chow, RMH-B, Hopefarms, Woerden) and water and were on a 12L : 12D cycle throughout their lives. Body mass of all animals was measured once a month, and cages were cleaned every 2 wk.

Each experimental group of 100 mice was randomly split into two subgroups of 60 and 40 mice. The subset of 60 animals (life-span subgroup) was left undisturbed throughout life (except for cage cleaning and monthly measurements of body mass); time of spontaneous death was noted to construct survival curves. Of the other 40 animals (test subgroup, see below), we used eight mice at each of four ages (3, 11, 19, and 27 mo) to measure food intake and metabolic rate, killing them to collect tissues and determine body composition. At the later ages, after some mortality had occurred, sample sizes were smaller than eight in some of the groups (see samples sizes given below; Tables 2, 3). All procedures concerning animal care and treatment were in accordance with the regulations of the ethical committee for the use of experimental animals of the University of Groningen (license DEC 2777).

Test Subgroup

Food intake and metabolic rate were measured in part of the test subgroup ($n = 6–8$) at the ages 3, 11, 19, and 27 mo. Food

intake (g d^{-1}) was measured over a period of 3 d. To express food intake in metabolizable energy intake, we corrected for changes in wet food mass on a control tray in each room. A food sample was taken and dried to constant weight over 4 h in an oven at 103°C (ISO 6496–1983[E]) to determine dry mass of the food.

Daily energy expenditure (DEE, kJ d^{-1}) was measured for each mouse with the doubly labeled water (DLW) technique. Before each trial, the mouse was weighed to the nearest 0.1 g and injected with about 0.1 g DLW (^2H and ^{18}O concentrations of the mixture 37.6% and 58.7%, respectively). The dose was quantified by weighing the syringe before and after administration to the nearest 0.0001 g. After 1 h for equilibration, the tail tip was punctured, and an initial blood sample was collected and stored in three glass capillary tubes each filled with about 15 μL blood. These capillaries were immediately flame sealed with a propane torch. The mouse was then put back in its cage and weighed again after 48 h, when a final blood sample was collected. In each sampling period, we also collected blood samples from four mice that had not been injected with DLW, to assess natural abundances of ^2H and ^{18}O in the body-water pools.

Determinations of $^2H/^1H$ and $^{18}O/^{16}O$ isotope ratios of the blood samples were performed at the Groningen Centre for Isotope Research using a SIRA 10 isotope-ratio mass spectrometer following Visser and Schekkerman (1999). In brief, each capillary was microdistilled in a vacuum line. The $^{18}O/^{16}O$ isotope ratios were measured in CO_2 gas, which was allowed to equilibrate with the water sample for 48 h at 25°C. The $^2H/^1H$ ratios were assessed from H_2 gas, which was produced after passing the water sample over a hot uranium oven. With each batch, we also analyzed a sample of the diluted dose and at least three internal laboratory water standards with different enrichments. These standards were also stored in flame-sealed capillaries and were calibrated against International Atomic Energy Agency standards. All isotope analyses were run in triplicate. The rate of CO_2 production (rCO_2 moles d^{-1}) for each animal was calculated with Speakman's (1997) equation:

$$rCO_2 = \frac{N}{2.078 \times (k_o - k_d) - 0.0062 \times N \times k_d},$$

where N represents the size of the body-water pool (moles) and k_o (d^{-1}) and k_d (d^{-1}) are the fractional turnover rates of ^{18}O and ^2H, respectively, calculated using the age-specific background concentrations and the individual-specific initial and final ^{18}O and ^2H concentrations. The body-water volume for each animal was derived from the carcass analyses. Finally, the rate of CO_2 production was converted to energy expenditure assuming a molar volume of 22.4 L mol^{-1} and an energetic equivalent per liter CO_2 based on respiratory quotient (RQ) measurements in our respirometry setup (on average, 22 kJ L^{-1} CO_2; Gessaman and Nagy 1988).

Three days after the DLW measurements, the same mice were moved to our eight-channel open-flow respirometry system and CO_2 production ($\dot{V}CO_2$, L h^{-1}) and O_2 consumption ($\dot{V}O_2$, L h^{-1}) were measured along with ambient temperature and activity (passive infrared, PIR; see Vaanholt et al. 2007 for detailed description). Mice in the eight respirometry chambers were measured in sequence throughout the measurement period. Data for each mouse were collected over a 1-min interval every 10 min and automatically stored on a computer. All mice were measured for 24 h at an ambient temperature of 22°C with ad lib. food and some apple for water. Body mass was measured at the start and end of the measurement. Body mass remained fairly stable during the respirometry measurements; mean weight gain was 0.23 ± 0.83 g. Mice housed in the cold were subsequently measured for 24 h at 10°C. This enabled us to determine whether changes had occurred in metabolic rate between the groups (when measured at a similar temperature) and to determine the resting metabolic rate (RMR) under experimental conditions. Metabolic rate (MR, kJ h^{-1}) was calculated using the equation $MR = 16.18 \times \dot{V}O_2 + 5.02 \times \dot{V}CO_2$ (Romijn and Lokhorst 1961; Gessaman and Nagy 1988). With this equation, we could accurately estimate the heat production of mice under different nutritional states. RMR (kJ d^{-1}) was defined as the lowest value of heat production calculated as the running mean over .5 h (i.e., four measurements of 1 min per mouse) when the animal was inactive (based on PIR measurements) and in a postabsorptive state (RQ = 0.7).

After the respirometry measurements, animals were weighed and killed using CO_2 followed by decapitation. Trunk blood was collected in prechilled tubes with anticoagulant (EDTA, 20 μL/mL) within 90 s from initial disturbance to the final drop of blood. Samples were spun down at 2,600 *g* for 15 min at 4°C. Plasma was aspirated and stored at −80°C until later analysis for hormone levels. Corticosterone levels were determined in duplicate with commercial radioimmunoassay kits (Linco Research, Nuclilab). Heart, liver, kidneys, intestines, stomach, lung, brain, testes, hind-limb muscles, brown adipose tissue (BAT), white adipose tissue (WAT), and skin were then dissected out and each weighed to 0.0001 g. Subsamples of heart, liver, kidney, hind-limb muscle, BAT, and WAT were immediately frozen at −80°C. The gut fill of stomach and intestines was removed, and the samples were weighed again. Tissues were stored at −20°C. Water content was determined by drying for 4 h to constant weight in an oven at 103°C (ISO protocol 6496-1983[E]). Fat was extracted using a soxhlet and petroleum ether (Boom), and samples were dried again for 2 h at 103°C. With the assumption that subsamples were representative of the whole tissue, dry lean masses of the organs from which subsamples had been taken were calculated using the leftover pieces. In six healthy animals (Hsd:ICR[CD-1] mice), water and fat content of hind-limb muscle, WAT, and BAT was determined. Water content was 70% ± 7%, 31% ± 6%, and 9% ± 3% in muscle, WAT, and BAT, respectively, and fat content was 12% ± 8%, 90% ± 4%, and 59% ± 7%, respectively. For the estimation of the dry lean mass of the remainder of the carcass, we calculated the dry lean mass of leg muscle, BAT, and WAT assuming these water and fat contents.

Data Analysis

We performed statistical comparisons using general linear models (GLM) applied in SPSS for Windows (ver. 14.0). We tested for effects of group, age, and interactions between group and age. Body mass was added to the models as a covariate where appropriate (i.e., body composition, food intake, and metabolic rates). The LifeTables option in the survival analysis in SPSS for Windows was used to create life tables, and differences between groups were analyzed using the Kaplan-Meijer test. In addition, the WinModest program developed by Pletcher (1999) was used to fit several models to the mortality data. For a detailed description of the procedure see "Results." The significance level was set at $P < 0.05$, and all tests were two tailed.

Results

Survival

As shown in Figure 1*A*, survival curves had similar shapes for the three experimental groups. Both mean and median age at death were virtually identical for mice exposed to 10°C (CC) and 22°C (WW) throughout their life (see Table 1). In mice that were exposed to cold only early in life (CW), median age at death was 82 d less than in groups CC and WW (Table 1). This may be due to increased mortality in the CW group between 600 and 800 d of age (Fig. 1*A*). Maximal life span was similar in all groups (Table 1). Overall and pairwise comparisons of the survival curves using Kaplan-Meijer survival analysis showed no significant differences in survival between the groups ($P > 0.05$).

Instantaneous mortality rates (μx) were calculated for each group over intervals of 100 d using the formula $\mu x = \ln(1 - N_e/N_b)$ (see Krebs 1994), where N_e and N_b are the numbers of animals at the end and the beginning of the interval, respectively (Fig. 1*B*). We tested Gompertz and logistic models of increasing mortality with age for goodness of fit to these data using the WinModest program developed by Pletcher (1999). The Gompertz model is determined by two parameters, one estimating the initial mortality rate (A) and one that represents the rate of exponential increase with age (b). The logistic model contains an additional parameter (s) that represents the deceleration in the increase in mortality at the end of life (if $s = 0$, logistic = Gompertz). Both, the Gompertz and logistic model were also tested with a constant additive term for early adulthood mortality (Makeham term, *c*).

Maximum log likelihoods were used to determine the model that best described our data, and the WinModest program calculated the associated probabilities. The Gompertz model was never rejected in any comparison ($P > 0.1$). The better fit of the Gompertz model than the logistic model means that increasing age-specific mortality did not decelerate or plateau at the end of life. The better fit of Gompertz alone without a constant early adult Makeham term means that mortality rates began increasing in early adulthood. Once models were fitted, a likelihood ratio test was used to test for significant variation in the Gompertz parameters among the groups. For each pair of treatment groups, we compared likelihood ratio tests of models assuming common A, common b, or common A and b to models that independently estimated both A and b. There were no differences in mortality rates between WW and CC mice ($\chi^2 = 1.1$, $P = 0.58$). Comparing WW with CC mice showed the highest likelihood for the model where both parameters were estimated, but this model did not fit significantly better than the model with common A and b ($\chi^2 = 5.7$, $P = 0.057$).

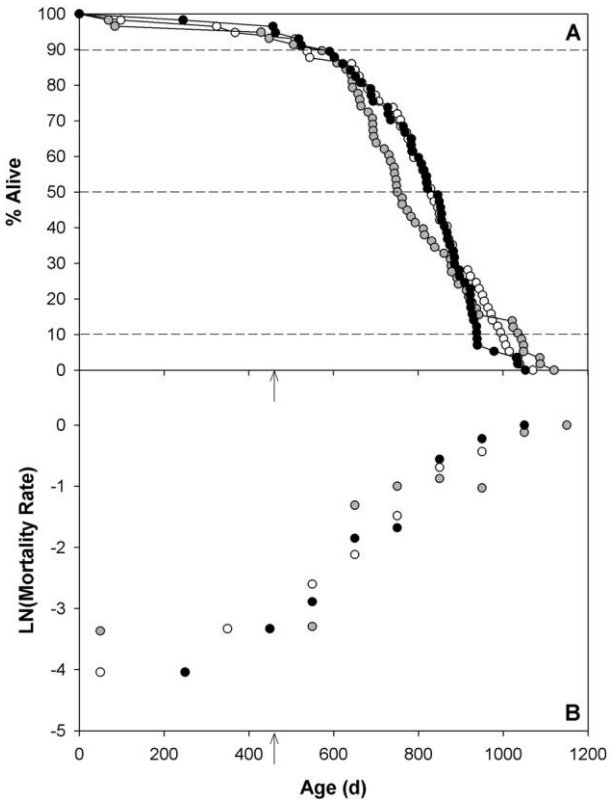

Figure 1. Effect of cold exposure on survival (*A*) and instantaneous mortality rates for 100-d intervals (*B*; see text for formula) in male mice housed at 22°C (WW, *white circles*), 10°C (CC, *black circles*), and 10°C early in life (up to 15 mo, *gray circles*; the arrow indicates when the temperature was switched) and at 22°C later on (CW). In the survival curve (*A*) each data point represents a single mouse. Sample size was 60 per group.

Body Mass

In all groups, the development of body mass was characterized by a strong increase early in life followed by a slower increase later in life and then by a decrease at the end of life (see Fig. 2). When we compared the different experimental groups, several patterns emerged. Initially, CC mice had a mean increase in body mass similar to WW mice. Then, after approximately 300 d, CC mice did not increase in body mass further, whereas WW mice kept increasing in body mass up until about 500 d of age. When the temperature was switched from 10° to 22°C, CW mice immediately increased their body mass and reached a plateau at a level intermediate between the CC and WW group. For statistical analyses, average body mass was calculated

Table 1: Survival data

Group	*n*	Mean Age at Death (d)	SEM	Median Life Span (d)	SEM	90% (d)	Maximum Age at Death (d)
WW	57	801	25	832	21	1,002	1,071
CC	57	798	21	834	19	939	1,053
CW	58	768	27	751	19	1,035	1,121

Note. Mean age at death (±SEM), median life span (50%), 90th percentile, and maximum age at death of mice exposed to various ambient temperatures are shown in days.

over three intervals (0–250 d, 251–500 d, and 501–750 d). We tested for significant differences between groups at different ages using one-way ANOVA. At all ages, the WW group had a significantly higher mean body mass than the CW and CC groups. In the oldest age group (501–750 d), the CW group had significantly higher mean body mass than the CC group ($P < 0.05$).

Body Composition

At four different ages, a subset of mice from each of the three treatment groups was killed, and body composition was determined (Tables 2, 3). Statistically significant variation among groups was assessed with general linear models including group, age, and group × age as fixed factors. Body mass was always included as a covariate. Therefore, significance of model terms refers to changes in the proportion of body components or organs relative to body mass. Post hoc Tukey tests were performed to test which groups differed from each other. As shown in Figure 2, body mass of CC mice was decreased compared with WW mice (GLM, effect of group: $F_{2,75} = 5.8$, $P < 0.001$). Analysis of body composition showed that this was mainly due to a reduction in fat mass (Table 2 for absolute values; GLM, effect of group: $F_{2,74} = 2.0$, $P = 0.06$; post hoc comparison between WW and CC: $P < 0.01$). In contrast, dry lean mass was slightly increased in CC mice compared with WW mice ($F_{2,74} = 6.3$, $P = 0.003$; post hoc comparison between WW and CC: $P < 0.01$). All variables showed a significant increase with age ($P < 0.001$).

Several changes in organ mass occurred in mice exposed to cold compared with WW mice. Differences between groups were most pronounced at 19 mo of age, and organ weights of mice at this age are shown in Table 3. Also, this age is just before the rise in mortality. Statistical analysis on all mice (GLM) showed that cold-exposed mice had significantly increased heart and kidney weights, while skin mass was significantly decreased (Table 3). Liver, brain, stomach, intestines, and lung masses did not differ significantly between CC and WW mice. As expected, body composition of CW mice was similar to CC mice at 3 and 11 mo of age, and at 19 and 27 mo of age it became more similar to WW mice (data only shown for 19 mo; Table 3).

In the models applied, we also evaluated effects of age on organ masses. All organ masses, except for liver and brain, showed a significant increase with age (GLM with body mass as a covariate: $P < 0.001$). For heart and kidney mass, a significant interaction between group and age was also found ($P < 0.01$), indicating that heart and kidney mass increased more rapidly with age in CC mice than in WW mice (data not shown).

Corticosterone Levels

Basal levels of plasma corticosterone from trunk blood were measured at 3, 11, 19, and 27 mo in animals from each experimental treatment group (Table 4). Corticosterone levels did not differ statistically between groups and were not affected by age (GLM, $P > 0.05$).

Food Intake and Energy Expenditure

Cold exposure significantly affected food intake, RMR, and DEE (Fig. 3; Table 5). Mean food intake was significantly increased in group CC compared with group WW at 11, 19, and 26 mo of age (post hoc tests: $P < 0.05$; Table 5).

RMR (kJ h^{-1}) of cold-exposed mice was measured at two ambient temperatures: 10°C (RMR_{EXP}) and 22°C ($RMR_{22°C}$). In the WW group, RMR was only measured at 22°C. There were

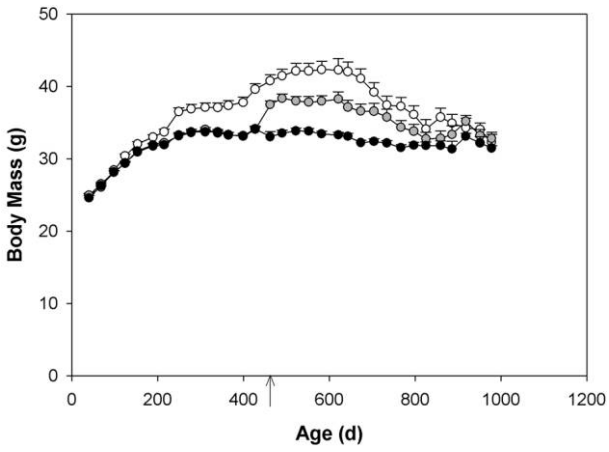

Figure 2. Development of body mass in male mice exposed to warm (WW, *white circles*) or cold (CC, *black circles*) ambient temperature throughout life and in mice exposed to cold only early in life and at warm temperatures later on (CW, *gray circles*; the arrow indicates when the temperature was switched). Sample size was 60 per group at the start of the experiment.

Table 2: Body composition of mice housed at different ambient temperatures at various ages

Group	3 mo[a]	11 mo[b]	19 mo[c]	27 mo[d]
WW:				
Body mass	27.4 (.4)	37.4 (1.9)	35.8 (.5)	34.7 (3.2)
Fat free mass	22.6 (.3)	24.2 (.4)	25.5 (.6)	27.1 (.9)
Dry lean mass	6.1 (.1)	7.0 (.1)	7.3 (.1)	6.9 (.3)
Fat mass	4.8 (.5)	13.2 (2.0)	10.3 (.9)	7.6 (2.8)
CC:				
Body mass	28.1 (.5)	32.6 (.8)	32.5 (.8)	29.7 (.9)
Fat free mass	23.5 (.4)	24.2 (.3)	27.0 (.6)	26.5 (1.1)
Dry lean mass	6.4 (.1)	6.7 (.1)	7.2 (.1)	6.6 (.2)
Fat mass	4.6 (.2)	8.4 (.9)	5.5 (.6)	3.2 (.6)
CW:				
Body mass	28.1 (.4)	32.5 (.8)	37.3 (2.3)	30.2 (1.3)
Fat free mass	23.8 (.3)	25.0 (.4)	26.7 (.3)	25.8 (.6)
Dry lean mass	6.4 (.1)	6.9 (.1)	7.5 (.3)	6.4 (.2)
Fat mass	4.3 (.2)	7.5 (.7)	10.5 (2.1)	4.4 (1.2)

Note. Mean (SEM) weights (g) of various components of the body are shown for mice housed at 22°C (WW), 10°C (CC), and 10°C until the age of 15 mo and 22°C thereafter (CW). Measurements were made at four ages: 3, 11, 19 and 27 mo.

[a] $n = 8$ for all groups.

[b] $n = 8$ for all groups.

[c] $n = 7$ for WW and CC mice; $n = 6$ for CW mice.

[d] $n = 6$ for WW and CC mice; $n = 7$ for CW mice.

no significant differences in mean RMR between CC and WW mice at 22°C (Table 5). In all statistical tests, body mass was added as a covariate, and statistical results thus refer to changes in energy expenditure corrected for effects of body mass. Under experimental conditions, mean RMR_{EXP} (10°C for CC mice and 22°C for WW mice) was increased by approximately 60% in CC mice compared with WW mice (Fig. 3). Age affected RMR_{EXP} in both groups, but RMR decreased slightly faster with age in the cold mice (as shown by a significant interaction effect; see Table 5). Mean DEE, measured in the home cage of the animals (CC at 10°C and WW at 22°C), was markedly increased in CC mice compared with WW mice and decreased significantly with age in both groups. Animals in the CW group had similar food intake, RMR, and DEE as CC mice at 3 and 11 mo of age and as WW mice at 19 and 27 mo of age.

Lifetime Energy Potential

Traditionally, LEP (kJ) has been estimated on the basis of measurements of RMR and maximum life span. Life, however, is not spent solely in the resting state, and LEP should be measured as the product of DEE and life span. RMR might be used as an estimator of DEE but only if DEE and RMR have a fixed ratio. In most animals, that is obviously not the case. In the mice in this study, RMR was on average 92% of DEE in WW mice and 86% in CC mice. Although small, these differences call for the use of DEE to calculate LEP as a more accurate estimate of the LEP. Maximum life span represents only a single event in each group and is therefore subject to large variance and is highly dependent on the sample size that is used. Using the median or 90% mortality yields a more reliable measure of life span (see also Speakman et al. 2002).

Taking these considerations into account, we estimated LEP based on measurements on DEE (with DLW) and the age at which 50% (data not shown) or 90% of the animals had died and corrected these for different measures of body composition: body mass, dry lean mass, and organ mass (heart, liver, kidney, and brain). To incorporate changes in DEE that occurred with age, we calculated the average DEE per group based on measurements at four ages throughout life (3, 11, 19, and 27 mo; see Table 6). LEP was calculated based on median or 90% survival was considerably higher in CC mice than in WW and CW mice, and this increase in lifetime energy expenditure remained apparent when values were expressed relative to body mass, dry lean mass, or organ mass (Table 6).

Discussion

Despite a 48% increase in overall DEE and a 64% increase in mass-specific energy expenditure throughout adult life, mice in the cold lived just as long on average as mice in warm temperatures. These results strengthen the existing doubts about the rate-of-living theory, which states that increased mass-specific metabolic rate reduces life span (Rubner 1908; Pearl 1928). It has been pointed out by Greenberg (1999) that a more precise formulation of the theory should take changes in body mass and composition into account. In Table 6, we have therefore calculated the LEP for all groups based on measurements of DEE and the median (50%) or maximum (90%) life span in four different ways: per animal (LEP), per gram body mass (LEP_{BM}), per gram dry lean mass (LEP_{DL}), and per gram mass of metabolically highly active organs (heart, liver, kidneys, brain; LEP_{OM}). Rubner (1908) originally suggested that LEP_{BM} (calculated using measures of food intake and maximum life span, 100%) was size invariant in interspecific comparison.

Table 3: Fresh organ masses (g) of mice at 19 mo of age

Organ	GLM	WW	CC	CW
Heart	$P = .001$	.19 (.01)[a]	.28 (.03)[b]	.21 (.01)[ab]
Liver	NS	1.73 (.08)	1.74 (.07)	1.78 (.13)
Kidney	$P = .001$	.52 (.01)[a]	.59 (.01)[b]	.57 (.01)[b]
Brain	NS	.48 (.01)	.46 (.01)	.49 (.01)
Stomach	NS	.18 (.01)	.19 (.01)	.20 (.01)
Intestines	NS	1.84 (.09)	1.87 (.11)	1.99 (.10)
Lung	NS	.21 (.02)	.23 (.01)	.23 (.02)
Skin	$P = .003$	5.2 (.2)[a]	3.9 (.1)[b]	5.2 (.4)[b]
Rest	$P = .02$	22.9 (.3)[a]	20.1 (.6)[b]	24.3 (1.7)[ab]

Note. Mean (SEM) weights of organs of 19-mo-old mice housed at 22°C (WW), 10°C (CC), and 10°C until the age of 15 mo and 22°C thereafter (CW). Where statistical tests (GLM including group and age as fixed factors and body mass as a covariate) showed significant differences between groups (see table under GLM), letters are used to indicate which groups significantly differed from each other within each row; that is, groups labeled with a superscript "a" differed from groups labeled with a superscript "b" but did not differ from similarly labeled groups in post hoc Tukey tests.

Table 4: Basal plasma corticosterone (mg/mL) of CC, WW, and CW mice at different ages

Group	3 mo	11 mo	19 mo	27 mo
WW	77 (14)	50 (9)	62 (11)	46 (10)
CC	51 (13)	75 (22)	*157 (26)*	54 (24)
CW	60 (15)	75 (22)	76 (12)	54 (18)

Note. Mean (SEM) basal corticosterone levels of mice housed at 22°C (WW), 10°C (CC), and 10°C until the age of 15 mo and 22°C thereafter (CW). Italics indicate that the group was slightly disturbed before blood sampling, and values do not represent basal levels of corticosterone. Measurements for all groups at 19 mo were therefore omitted from statistical analysis. No significant effects of group or age were found when tested using GLM.

In our intraspecific experiment manipulating energy metabolism, LEP_{BM} clearly increased in the cold due to an increase in energy turnover without reducing longevity. Expressing metabolic rate in any of the other measures relative to dry lean mass or metabolic (organ) mass did not change this conclusion.

Studies investigating the effects of cold exposure on life span in rats have yielded conflicting results. Kibler and Johnson (1961; Johnson et al. 1963; Kibler et al. 1963) have shown that rats kept at 9°C continuously had shorter life spans than rats at 28°C, and these studies have been cited as providing experimental support for the rate-of-living theory. Holloszy and Smith (1986) argued that the continuous cold exposure in Johnson et al.'s (1963) study was a chronic stressor. The rats might thereby have suffered from deleterious effects on health and longevity (Paré 1965) mediated by chronic elevation of stress hormones (e.g., corticosteroids) unrelated to the increase in energy expenditure. In our study, we applied a protocol similar to that of Kibler et al. (1963) and did not find differences in corticosterone levels (Table 4) or in life spans between cold-exposed or control mice. Apparently, cold exposure was not a chronic stressor, or at least it did not result in increased levels of corticosterone. As in Holloszy and Smith's (1986) study of rats, we found a strong increase in energy expenditure in cold exposure with no effect on life span. In another study investigating the relationship between energy metabolism and longevity, we found that artificial selection for high activity (increased voluntary wheel running) increased lifetime energy expenditure (~20%) but had no effect on life span (L. M. Vaanholt, S. Daan, T. Garland Jr., and G. H. Visser, unpublished results; see also Bronikowski et al. 2006). These findings join a growing body of evidence suggesting that in mammals, lifetime energy expenditure per se does not underlie the determination of life span (Holloszy and Smith 1986, 1987; Speakman et al. 2003, 2004; Navarro et al. 2004).

In apparent contrast to our present conclusions, metabolic rate is known to play an important role in aging via the production of ROS (Harman 1956; Beckman and Ames 1998). ROS are produced in mitochondria during oxidative phosphorylation and can cause damage to lipids, DNA, and proteins, which may eventually result in cell death. If ROS were increased in our mice in the cold due to increased energy turnover rates, how did they protect themselves against this increase in oxidative stress? Animals have developed several defense systems against ROS and the damage they cause, for example, antioxidant enzymes to scavenge ROS and protein turnover to remove and replace damaged proteins. The rate of radical production during oxidative phosphorylation depends highly on the speed of the process affected, in particular, by the supply

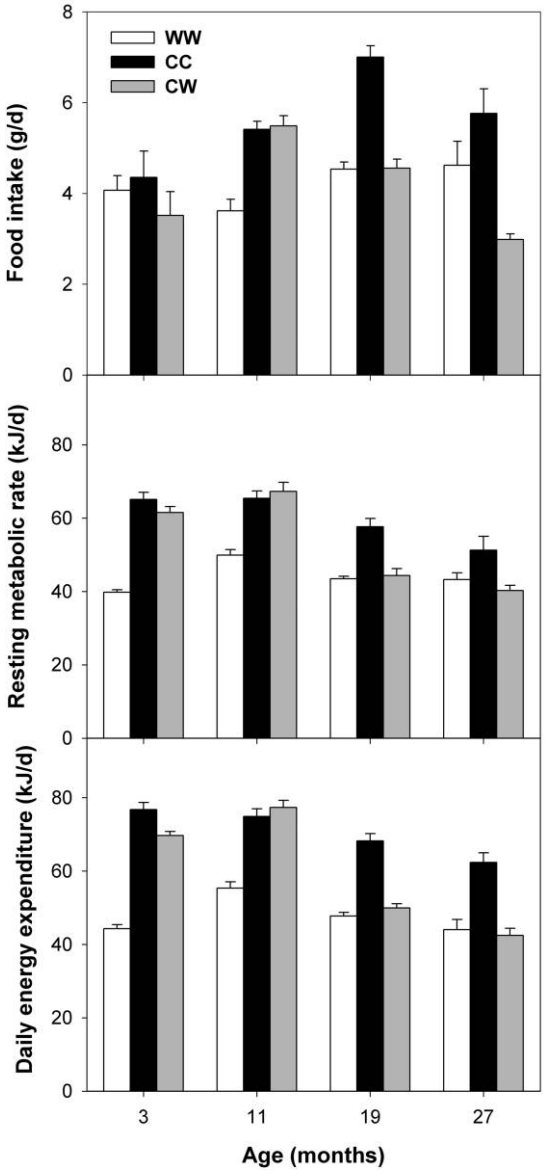

Figure 3. Food intake, resting metabolic rate, and daily energy expenditure in subsets of mice exposed to cold (10°C, *black bars*) or warm (22°C, *white bars*) environments and sampled at different ages (3, 11, 19, and 27 mo). Gray bars represent mice that were housed at cold temperatures up to 15 mo and at warm temperatures afterward. Sample size was 6–8 in all groups (see Table 2).

Table 5: GLM testing for effects of group and age on food intake and energy metabolism

	Group			Age			Group × Age			
Trait	df	*F*	*P*	df	*F*	*P*	*F*	*P*	Covariate	*P*
Food intake (g d^{-1})	2, 73	17.8	<.001	3, 64	7.7	<.001	4.0	<.001	BM	NS
$RMR_{22°C}$ (kJ d^{-1})	2, 74	1.7	NS	3, 74	16.4	<.001	1.2	NS	BM	.001
RMR_{EXP} (kJ d^{-1})	2, 74	77.6	<.001	3, 74	48.2	<.001	12.0	<.001	BM	.002
DEE (kJ d^{-1})	2, 75	140.9	<.001	3, 75	68.0	<.001	16.8	<.001	BM	NS

Note. GLM were applied to data on metabolic rates to test for differences between groups and effects of age. Group, age, and group × age were added to the models as fixed factors, and body mass was added as a covariate. *F* and *P* values are shown for all fixed factors, and *P* values are shown for the covariate. Absolute values were used in the analysis. Total sample size was 88 animals: 8, 8, 7, and 7 mice in the WW group; 8, 8, 7, and 6 in the CC group; and 8, 8, 6, and 7, in the CW group at 3, 11, 19, and 27 mo, respectively. One mouse (WW, age 27 d) died during the respirometry measurements, and total sample size is 87 here. For food intake, sample size is 86 because data for two mice were missing. $RMR_{22°C}$ compares RMR measured at 22°C between groups. RMR_{EXP} compares values measured under experimental conditions.

of substrates and the amount of mitochondrial uncoupling (Brand 2000). Uncoupling oxidative phosphorylation from ATP production by enabling protons to leave the intracellular space via uncoupling proteins reduces the production of ROS (Brand 2000). This process might in principle enable mice to combine high metabolic rates with long life spans, as has indeed been shown previously (Speakman et al. 2004). Specifically, in cold-exposed animals, it would be beneficial to uncouple oxidative phosphorylation, because when uncoupling occurs, energy is dissipated as heat available for thermoregulatory purposes (Brand 2000). Several studies have shown increases in uncoupling protein expression, specifically in BAT of cold-exposed rodents (Freeman et al. 1989; Florez-Duquet et al. 1998; Yamashita et al. 1999; Argyropoulos and Harper 2002; Jakus et al. 2002). This is one of several possible ways in which cold exposure might evoke responses counteracting the effect of energy turnover on life span. Other possibilities include changes in antioxidant defense. Some studies (e.g., Holloszy and Smith 1986) have shown a reduction in the incidence of tumors in mice exposed to cold, which may extend their life span. This low incidence of tumors may be linked to the low fat content of cold-exposed animals (Lu et al. 2006). Cold exposure may thus have positive effects on other aspects that influence survival and thereby counteract the negative effects of ROS on survival. If that is true, metabolic rate itself is apparently not a good predictor of life span.

Table 6: Lifetime energy potential

	WW	CC	CV (%)	*P*	CW
Body mass (g)	33.8	30.7			32.0
Total dry lean mass (g)	6.7	6.6			6.7
Organ mass (dry lean; g)	.61	.65			.63
DEE (kJ d^{-1})	47.8	70.6		.001	59.9
DEE_{BM} (kJ g^{-1} d^{-1})	1.4	2.3		.001	1.9
DEE_{DL} (kJ g^{-1} d^{-1})	7.1	10.7		.001	8.9
DEE_{OM}(kJ $g^{-1}d^{-1}$)	78	109		.001	95
Maximum life span (90%; d)	1,002	939			1,035
LEP (kJ)	47,941	66,257	23	.001	61,955
LEP_{BM} (kJ g^{-1})	1,416	2,155	29	.001	1,936
LEP_{DL} (kJ g^{-1}	7,156	10,046	24	.001	9,248
LEP_{OM} (kJ g^{-1})	78,467	10,2310	19	.001	98,617

Note. WW = mice housed at 22°C; CC = mice housed at 10°C (CC); CW = mice housed at 10°C until the age of 15 mo and 22°C thereafter. Lifetime energy potential (LEP; kJ $life^{-1}$) is the product of energy expenditure and life span and was calculated using average daily energy expenditure (DEE, kJ d^{-1}) measured at four ages (3, 11, 19, and 27 mo) throughout life in the test group, and median (data not shown) or maximum (90%) life span measured in the life-span group. In addition, LEP (kJ g^{-1}) was corrected for various measures of body composition measured at the same ages: BM = body mass; DL = dry lean mass; OM = "metabolic" organ mass (sum of dry lean heart, liver, kidney, and brain mass). Results for two-way ANOVA with factors group (WW and CC), age, and the group × age interaction are shown. First, we tested effects of these factors on whole-body and mass-specific DEE. Afterward, we estimated LEP for each individual by multiplying the mass-specific value of DEE with the maximum life span (90%) of its group and ran the same statistical test. CV represents the coefficient of variation calculated over the two groups (SD divided by mean × 100%). *P* values are shown for the two-way ANOVA. For completeness, values for CW mice are also shown in the last column.

In the course of evolution, animals have developed various systems to protect them from damage accrued by aerobic metabolism. Differences between taxa provide evidence for this evolutionary flexibility. Birds expend energy at a rate on average 1.5 times faster than a mammal of similar mass (Daan et al. 1990) but have greater longevity. Has the specific way of living of birds, possibly associated with reduced risks, resulted in increased investment in maintenance? There is evidence that birds have evolved mitochondria that produce less ROS per mL oxygen consumed than mammals, are better protected against ROS, and have lower oxidative damage (Barja et al. 1994; Herrero and Barja 1998, 1999). Comparing long- and short-lived mammalian species, the long-lived species also generally have lower levels of ROS production and higher protection against ROS (Barja 1998, 2002; Perez-Campo et al. 1998). Thus, during evolution, differences in ROS production have emerged relative to metabolic rate as well as in the capacity to defend against oxidative stress. Also within species, life-span increasing manipulations such as CR enhance maintenance processes (e.g., protein turnover, antioxidant enzymes, DNA repair; Lewis et al. 1985; Rao et al. 1990; Gredilla and Barja 2005). Aging could therefore be defined as the failure of maintenance and repair. Different maintenance mechanisms exist, and most of them have been shown to decrease with age. They depend on many genes, and a considerable investment of metabolic resources is necessary to keep up their activity. Individual mechanistic theories of aging revolve around the failure of specific maintenance systems and highlight different aspects of a complex process rather than being mutually exclusive explanations. Aging should be viewed as a multifactorial process. It does not result in a given cause of death. The system component that fails first may be largely a matter of chance.

No manipulation will affect one single aspect of the aging process in isolation, and this causes complications when testing a single theory such as the rate-of-living theory. Most manipulations of energy expenditure usually lead to changes in body mass (which by itself can affect life span) and body composition. The expression of energy expenditure thus requires a correction for body size. The best way to do this is still under debate (Ramsey et al. 2000; Speakman 2005*b*). Changes that may affect life span occur in numerous physiological parameters (e.g., hormone levels, fat content, antioxidants, protein turnover). This makes attributing differences in life span to a single process such as energy expenditure nearly futile. Our study rejects the rate-of-living theory in its simplest quantitative form, which states that the lifetime energy turnover per gram tissue (whether body, lean, or organ mass) remains constant when the instantaneous rate of turnover changes. These results do not prove the absence of a negative effect of energy turnover on life span because many studies have demonstrated such an effect in one form or another (Lyman et al. 1981; Wolf and Schmid-Hempel 1989; Daan et al. 1996). Taking different consequences of energy turnover into consideration implies that the relationship between energy turnover and life span cannot be a simple unidirectional process. In Figure 4, we conclude with a more realistic, if hypothetical, proposition. Energy turnover increases deleterious effects (e.g., ROS production) as proposed in the rate-of-living theory, which will cause a decline in expected life span. Simultaneously, a decrease in energy turnover rate may cause other negative effects mediated by conditional problems, such as excess body fat in captive rodents. Together, these two processes will generate an intermediate optimum where maximization of life span is concerned. This need not be the same energy expenditure that maximizes individual fitness, because fitness (i.e., the expected rate of gene propagation to the next generation) includes the additional component of reproductive output. On the basis of these opposing processes, we would not even necessarily expect to find evidence for the rate-of-living theory across the whole range of metabolic rates.

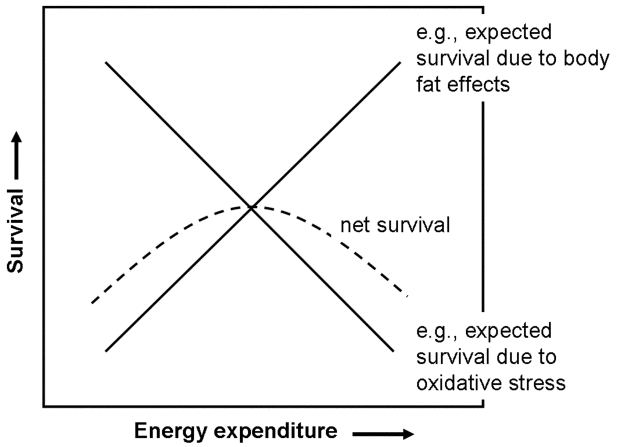

Figure 4. Schematic representation of the relationship between energy expenditure and survival. The graph shows the effect of two separate processes that occur with changing energy expenditure and that can affect life span. An increase in energy expenditure can have deleterious effects on survival, for example, via the increased production of reactive oxygen species, shown as a negative relationship. On the other hand, a decrease in energy expenditure may also have deleterious effects on survival, for instance, because it causes excess body fat, which is a health risk and will negatively affect life span (positive relationship). These two opposing processes then result in an optimal survival at a certain energy expenditure (*dashed line*). See text for further explanation.

In summary, among species in several taxa of homeotherms, there is generally an inverse relationship between energy expenditure and life span. Here, we show that within a species (laboratory mice), experimentally manipulating energy expenditure by decreasing ambient temperature did not affect life span in the direction predicted, and the quantitative expectation of a constant LEP was not upheld. These results refute the strict rate formulation of the rate-of-living theory. However, they do not undermine the fundamental relevance of energetics to the aging process, and they highlight the importance of understanding how different systems work together in aging animals.

Acknowledgments

We thank Saskia Helder for taking excellent care of the animals, Gerard Overkamp for technical assistance, and Berthe Verstap-

pen for performing isotope analyses. We also thank Peter Meerlo, Berber De Jong, and Jan-Albert Manenschijn for their practical help at various stages of the project. We would like to commemorate Professor G. Henk Visser, who initiated the work leading to this article. Professor Visser passed away on June 3, 2007. He was a great friend and colleague and is deeply missed.

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