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Metabolizable energy intake during long-term calorie restriction in rhesus monkeys^{1,2}

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Abstract

Calorie restriction (CR) is a dietary intervention shown to increase maximum life-span. The aim of this study was to compare the metabolizable energy of the pelleted semi-purified diet with estimated energy intake from food weight. Energy density of diet, urine and feces were measured by bomb calorimetry in rhesus monkeys (23–29 years old) on CR (CR, n=11) and control (C, n=9). Food moisture was measured to be 2-fold higher ($9 \pm 1\%$) than indicated on the label ($\sim 5\%$). The measured gross energy of diet was 4.4kcal/g dry weight of CR and 4.5kcal/g dry weight of C diets. In a two-day trial, food intake (mean $\pm$ SD) was 112 ± 20 g and 136 ± 26 g of dry mass/d in the CR and C monkeys, respectively ($p = 0.003$). The fraction of the diet absorbed (CR=0.91; C=0.95) was different ($p < 0.001$) between CR and C monkeys. Using these coefficients, the metabolizable energy intake averaged over 6 months was 450 ± 53 kcal/d and 534 ± 97 kcal/d in CR and C monkeys, respectively (Diff=16%; $p=0.03$). These values were compared with energy expenditure (EE), as measured annually by indirect calorimetry (490 ± 61 kcal/d in CR and 532 ± 62 kcal/d in C monkeys). Adjusted for changes in body composition (2 ± 10 kcal/d in CR and 7 ± 12 kcal/d in C), energy balance was not different from zero in CR (-42 ± 42 kcal/d) and C (9 ± 61 kcal/d) monkeys. Use of diet weight is a reasonable estimate of the level of CR when food waste is assessed.

Keywords

energy balance; absorption efficiency; food waste; monkey chow composition; energy expenditure

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Introduction

Calorie restriction (CR) without malnutrition has been shown to delay aging and attenuate age-related diseases in various organisms (Wiendrich & Walford, 1988; Hunt et al, 2006). Reduced energy intake rather than a reduction in any specific macronutrient has been shown to be the primary cause of the life span extension (Iwasaki et al, 1988; Masoro, 1988). CR's ability to retard aging has been attributed to many reasons including a decrease in the metabolic rate (Harman, 1981) and associated reduction in oxidative damage (Bevilacqua et al, 2004; Masoro, Shimokawa & Yu, 1991; Sohal & Weindruch, 1996). Although it is well established that an acute decrease in energy intake decreases lean mass-adjusted metabolic rate (McCarter, 1991; McCarter, Masoro & Yu, 1985; McCarter & McGee, 1989), it is still debated whether this reduction is maintained during long-term CR or if the long-term reduction is explained by a decrease in body and organ mass (Gallagher et al, 1998).

While the effects of reduced energy intake have been studied extensively, many studies have estimated the degree of restriction based on the degree of reduction in the mass of diet that disappears from the food holder. This, however, may not accurately reflect the true degree of restriction. For example, it is known that the energy released when food is completely combusted in a bomb calorimeter does not exactly equal the metabolic energy provided to the body due to digestibility and incomplete utilization – ie energy losses in urine and feces. This reduction in energy available to the body has given rise to the term 'metabolizable energy' (ME), which is defined as the energy available to the body after correcting for losses in urine and feces (Moe, 1994). The systematic approach to measuring metabolizable energy was refined by Atwater and coworkers as extensively discussed in USDA Handbook #74 (Merrill & Watt, 1973). This body of work resulted in the determination of the general Atwater factors, which are the well-known values of 4, 9, and 4 kcal/g of metabolizable energy for average dietary carbohydrate, fat and protein, respectively. In addition, specific Atwater factors for the macronutrients in specific foods have been developed for an increasingly wide range of food items.

Calorie restriction in itself may also influence the metabolizable energy of a diet. Long-term CR has been shown to cause a variable decrease in the organ masses (Weindruch & Sohil, 1997) including intestinal mass which decreases to a greater extent than the degree of restriction (Weindruch & Sohil, 1997; Greenberg, 1999; Greenberg & Boozer, 2000). This decrease in intestinal mass could lead to a decrease in absorption of nutrients (Karasov et al, 2004). Notwithstanding, the feeding behavior in CR monkeys is such that all the food given to them is eaten in a relatively short amount of time followed by long post-absorptive periods. This may lengthen the transit time in the gut leading to better absorption of nutrients. Published data, however, is lacking with regard to either of these hypothesized effects of long-term CR on the absorption of nutrients.

The main aim of this study was to accurately determine the difference in metabolizable energy intake between CR and C monkeys. We assessed the effects of reduced food intake on the digestibility and metabolizability of food by measuring the waste energy in two consecutive 24-hr urinary and fecal collections in 23-29 year old male monkeys. We also tested the accuracy of the estimates metabolizable energy by comparing the results against measured energy expenditure and changes in body composition.

Methods

Animals

Twenty (n=11, CR; n=9, C) late middle aged (23-29 years old) rhesus monkeys (*Macaca mulata*) that are part of an ongoing, long-term (14 y) caloric restriction study (Ramsey et al,

1997) were studied. When the study was initiated, the CR group was restricted by 30% based on individually measured food intake prior to the imposition of CR. During the subsequent 14 years, the control group has reduced its voluntary energy intake and the weight of diet provided to the CR group was reduced accordingly on two occasions. There was an 18% restriction based on weight of diet of CR compared to C at the time of this study. Animals are fed a semipurified diet (Teklad, Madison, WI) containing 15% lactalbumin, 10% corn oil, and ~65% carbohydrate. Additional details of this study were previously published (Ramsey et al, 1997; Kemnitz et al, 1993). The protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the Graduate School at the University of Wisconsin.

Food intake measurement and gross energy intake

Throughout the duration of the main study, food intake was measured daily and these values were averaged by week and then by year. Because the food intakes were stable and composition was constant, we utilized the measured intake on the same two consecutive days for which we performed urinary and fecal collections without the aid of fecal markers. As part of the standard protocol, a measured amount of food was put into individual diet hoppers in the morning. At the end of the day any remaining food in the hoppers was collected and measured. Standard procedure for the main CR study and hence for the 12 month intakes reported herein were that any whole or large partial pellets that fall through the cage floor were counted and converted to the nearest gram. Food intake was calculated for each individual monkey as the difference between the weights of food given to the monkeys minus the food left in the hoppers including the pellets and partial pellets from the pans under the cages. During this two-day period of metabolizable energy study, we altered this procedure by collecting and weighing all of the small pieces of uneaten food (pellets plus partial pellets or crumbs) that fell to the pan. These crumbs of food were collected, dried in an oven at ~50°C to remove the urinary moisture contamination and weighed.

In the current study, the moisture content of the food pellets was measured as the weight lost when feed was dried in an oven at 50°C. Food pellets were sampled from various food-barrels within a lot. Each barrel of CR and C food was sampled three times before the barrel was empty and from three portions of the surface, per sampling. We sampled various barrels from three different lots to get a good estimate of the moisture content of food. These collected food pellets were dried in an oven at ~50°C until two consecutive weights were the same (indicating complete drying). These dried food samples were combusted in an adiabatic bomb calorimeter to obtain the gross energy (GE) of CR and C food pellets.

Fecal and urinary collections

While the monkeys remained in their usual cages (home cage), two consecutive twenty-four hour urine and fecal samples were collected from each animal on consecutive days. The pans beneath the cages were tilted in a manner such that the voided urine flowed into the anterior portion of the pans where they drained into narrow-neck containers, to minimize evaporation. The containers were emptied twice daily and the volume of the collections recorded. At the end of each day, homogenous samples from each 24-hr urine collection were aliquoted into polypropylene tubes (n=4 tubes; 40ml/tube) and stored at -20°C until further analysis. The fecal samples from the pans were also collected twice daily, measured and stored in separate polypropylene tubes at -20°C until further analysis.

Frozen urine and fecal samples were freeze dried under vacuum at -50°C (Virtis, Gardiner, NY) until the change in mass between hourly assessments of drying was less than 0.2% (approximately 24-36 hours). These dried samples were combusted in an adiabatic bomb calorimeter to obtain an estimate of excretory energy losses.

Bomb calorimetry

Gross energy content of food (GE), urine and feces was measured using a Parr bomb calorimeter (Model # 1241, Moline, IL). Briefly, energy of the dried material was determined by placing a weighed sample in the ignition cup of the bomb. Oxygen was added at ~35 psi and the bomb immersed in 2 L of water. The sample was ignited and the temperature increase of the water recorded. Gross energy was calculated using a boric acid calibration standard and expressed as kcal/g of dry matter. Replicate measures were made on pooled urine and fecal samples from each monkey.

Coefficients of apparent energy digestibility and metabolizability

The gross energy and excretory energy losses obtained from bomb calorimetry were used to calculate the coefficients (coeff) of absorption, digestion and metabolizability (Metab).

$$\text{Dry Matter Absorption coeff} = \frac{D_f - D_{fe}}{D_f}$$

$$\text{Energy Digestibility coeff} = \frac{(D_f \cdot GE_f) - (D_{fe} \cdot E_{fe})}{(D_f \cdot GE_f)}$$

$$\text{Metab. Energy} = \frac{(D_f \cdot GE_f) - (D_{fe} \cdot E_{fe}) - (D_U \cdot E_U)}{(D_f \cdot GE_f)}$$

D_f , g/d – dry food matter flux;

GE_f , kcal/g – gross energy of food;

D_{fe} , g/d – dry fecal matter flux;

E_{fe} , kcal/g – fecal energy;

D_U , g/d – urinary flux;

E_U , kcal/g – urinary energy.

The comparison of nutrient utilization efficiency using these coefficients has been criticized on statistical and other grounds, and an alternative approach based on analysis of covariance of excretory losses as a function of food intake has been proposed (Simpson & Raubenheimer, 1997; Simpson & Raubenheimer, 1999). Accordingly, we also evaluated excretory losses by that method as well.

Body composition

Whole body composition was measured semiannually in these animals from the inception of the study using dual energy X-ray absorptiometry (DXA; Model DPX-L, GE/Lunar Corp., Madison, WI). Briefly, animals were sedated with a mixture of ketamine HCl (10mg/kg body weight, IM) and xylazine (0.6mg/kg body weight xylazine, IM) for additional muscular relaxation in supine position (Coleman et al, 1999). For this study, the body composition measurement taken closest to the date of the study was used to calculate the rate of change in body energy stores of these monkeys. The caloric equivalents assigned to changes in body composition were 1.1 kcal/g lean mass and 9.5 kcal/g fat mass (Weir, 1949).

Energy balance was calculated as metabolizable energy (ME intake) – [energy expenditure (TEE) + caloric value of change in body composition (BC changes)].

Respiratory gas exchange

Energy expenditure (EE) was measured annually as part of the standard protocol of the main study (Ramsey et al, 1997). Animals were placed in one standard sized cage that is sealed within a transparent metabolic chamber with dimensions of 75cm wide × 75cm deep × 80cm high. The animals were housed in these chambers a day before the start of measurements for acclimatization. Respiratory exchange measurements were taken for 48 hours during 2 consecutive days. The chamber was placed in the room where other animals were housed to decrease the potential for stress induced by the change in cage environment (Ramsey et al, 1997). Room temperature was maintained at ~21°C to achieve thermoneutrality. The animals were fed a peanut-butter sandwich when in the metabolic cages in an amount to match the animals' habitual energy intake. We used the EE measurements closest to our 2-day study period for assessment of the energy balance in these monkeys.

Statistical analysis

Analysis of Variance (ANOVA) was used to identify the effect of treatment (CR vs. C) on various energy values. The loss of excretory energy was compared between CR and C monkeys using analysis of covariance (ANCOVA) with food intake as a covariate in the model. A $p \leq 0.05$ was required for statistical significance. All values are presented as mean $\pm$ standard deviation. All statistical analyses were performed using STATVIEW version 5.0.1 (SAS institute Inc, NC).

Results

Table 1 summarizes the characteristics of the animals at the time of this study (year 14 of CR). The two groups were of similar age and the CR monkeys had significantly lower mean body weight than the C monkeys at the time of this study ($p < 0.001$).

Food and gross energy intake

The measured moisture content of the feed was $9 \pm 1\%$ by weight, which is greater than the manufacturer's defined moisture of $\sim 5\%$ ($p < 0.05$). The gross energy values of dry weight of the two feeds were 4.4 ± 0.01 and 4.5 ± 0.01 kcal/g dry weight, for CR and C, respectively. The C animals lost 6 ± 10 g of dry wt/d of food as crumbs dropped to the bottom of the cage compared to negligible loss in the CR animals (0.4 ± 1 g of dry weight/d). The 2-day average food intake, after accounting for crumb differences, was measured to be 112 ± 20 grams of dry weight per day in the CR monkeys and 136 ± 26 grams of dry weight per day in the C monkeys. The gross energy intake significantly differed by 20% (CR = 447 ± 80 kcal/d and C = 559 ± 106 kcal/d) between CR and C ($F_{1,18} = 8$; $p = 0.01$) monkeys.

Coefficients of digestible and metabolizable energy

The fecal losses averaged for two-24hr periods were 12 ± 4 g of dry mass/d in the CR and 9 ± 5 g of dry mass/d in C monkeys ($p = 0.04$) (Table 2). The difference in fecal energy losses (CR = 42 ± 17 and C = 30 ± 11 kcal/d) approached significance ($p = 0.06$). Using the 2-day average energy loss in feces for individual monkeys, the digestible energy coefficients were calculated to be 0.91 ± 0.03 in the CR and 0.95 ± 0.03 in C monkeys and were significantly different between CR and C monkeys ($F_{1,16} = 21$; $p < 0.001$).

Two-24hr average urinary losses were measured to be 9 ± 3 g of dry mass/d in the CR and 8 ± 3 g of dry mass/d in C monkeys ($p = 0.2$) respectively (Table 2). The amount of energy lost in the urine (CR = 18 ± 8 kcal/d and C = 18 ± 7 kcal/d) and feces (CR = 42 ± 17 kcal/d and C = 30 ± 11 kcal/d) was not significantly different between CR and C monkeys (urine, $p = 0.94$). Using the 2-day

average losses in urine, the metabolizable energy coefficients were 0.87 ± 0.03 in the CR and 0.91 ± 0.03 in C monkeys ($F_{1,16} = 27$; $p < 0.001$).

Fecal plus urinary energy losses together increased with increasing gross energy intake for each animal, with no difference between C and CR in the slope of the relationship ($F_{1,16} = 1$; $p = 0.3$), but the CR animals excreted more energy per unit energy intake (4% greater excretory loss in CR monkeys; $F_{1,17} = 16$; $p < 0.001$) than the C monkeys (Fig. 1). Based on these excretory losses, the metabolizable energy values for the food pellets were 3.5 and 3.8 kcal/g for CR and C, respectively.

Six-month average food intakes (112 ± 15 g in CR and 127 ± 26 g of dry wt in C), obtained using the standard procedure for food disappearance in the main CR study, were adjusted by the respective energy coefficients (crumb loss, moisture and metabolizable energy) as reported above to calculate the metabolizable energy intakes (450 ± 53 kcal/d in CR; 534 ± 97 kcal/d in C) which differed by 16% between CR and C monkeys ($F_{1,18} = 5$; $p = 0.03$).

Total Energy expenditure (TEE) and changes in body composition

The TEE, as measured by indirect calorimetry, was 532 ± 62 kcal/d in C and 490 ± 61 kcal/d in the CR monkeys (Table 1). The C animals lost 0.3 ± 1 kg weight (0.1 ± 1 kg as fat mass; 0.2 ± 0.4 kg as FFM) and the CR animals lost 0.1 ± 1 kg weight (-0.0 ± 0.8 kg as fat mass; 0.1 ± 0.7 kg as FFM) as assessed between 2 consecutive DXA measurements, six months apart.

Energy Balance

When the energy equivalents of body composition changes ($CR = 2 \pm 10$ kcal/d; $C = -7 \pm 12$ kcal/d) were accounted for, the energy balance were numerically negative (-42 ± 42 kcal/d) in the CR and positive (9 ± 61 kcal/d) in C monkeys, but in neither case were the differences significantly different from zero ($p > 0.05$) (Figure 2).

Discussion

We have demonstrated that although use of weight of food disappearance provides a good measure of the level of CR, the estimate of metabolizable energy intake was in error in that it slightly overestimated the degree of energy restriction. The correction factors are individually small, but together do influence the true metabolizable energy intake values. These factors include feed moisture content, unmeasured food spillage, and energy lost in body waste.

Had we estimated the energy intakes using the Atwater general factors of 4, 9, and 4 kcal/g of carbohydrate, fat and protein to calculate metabolizable energy based on the manufacture's label, the average metabolizable energy intake would have been 3.8 kcal/g and 3.9 kcal/g dry weight of CR and C food, compared to our measured values of 3.5 and 3.8 kcal/g dry weight, respectively. These measured metabolizable energy values differed for two primary reasons. First, the metabolizable energy of 4, 9, 4 kcal/g for protein, fat and carbohydrate are average values obtained by Atwater by feeding subjects' a mixed diet. The diet fed to the monkeys in our study was comprised of a small number of purified food products, including simple sugar rather than starch and hence it is not surprising that one would obtain slightly different values between metabolizable energy calculated using Atwater factors and the measured metabolizable energy. Second, the moisture content of the diets were higher compared to factory defined percentages due to the hygroscopic nature of the diets, also leading to differences in the actual food intake values and hence the daily metabolizable energy intake.

Additionally, the food in the hoppers of these animals are weighed before and after feeding, but the food spillage within the cage pans and outside of the cage are usually only visually assessed with the exception of the current metabolizable energy study. Though not significant

due to high variability in the CR group, we found that the difference between the visually assessed and measured crumbs was $\sim 7\text{g/d}$ in C and 0.4 g/d in the CR monkeys. This can introduce measurement error in metabolizable energy intake of 1 kcal/d in the CR and 26 kcal/d in the C monkeys when food disappearance alone is used to estimate food intake.

Finally, using this long-term CR model, we have shown that the coefficient of metabolizable energy intake per unit gross energy intake was 4% lower under CR. Although there were no significant differences in the absolute excretory losses between CR and C animals, the energy lost per kcal of food ingested was significantly higher in CR compared to C monkeys. The differences in the coefficients of digestion in CR and C monkeys could be due to the decreases in intestinal mass induced by long-term CR. It has been shown that liver, kidney and the gastrointestinal tract are the first organs to be affected (Weindruch & Sohol, 1997, Hagopian et al, 2005). Nonetheless, the surface area of absorption is many times larger than the surface area needed for the flux of food through the gut and hence the decrease in intestinal mass may not explain the differences in absorption of nutrients in the CR monkeys. In fact, the similarity in slopes in Fig. 1 can be interpreted to mean that there was no significant difference in “true” digestibility or metabolizability between C and CR monkeys (Simpson & Raubenheimer, 1997; Simpson & Raubenheimer, 1999). Another possible explanation could depend on the proportion of fiber content in the two diets. In this study, however, care was taken in formulation of the diets such that the cellulose content (source of fiber) of both the diets was kept constant. Hence the amount of fiber the animals received was proportional to the amount of food ingested, and thus indigestible fiber should be proportional to intake. Therefore this would not be expected to explain the greater fecal energy losses in the CR monkeys. In addition, because the fiber contents were similar, the possibility of decrease in transit time (Hillemeier, 1995) inducing a decrease in absorption in the CR monkeys is unlikely. A third possibility could be that the endogenous sources contributing to the increase in energy loss (bile secretion, digestive juices secreted and mucosal sloughing etc) differ between the CR and C monkeys, which is an interpretation of the significantly different intercepts in Fig. 1 (Simpson & Raubenheimer, 1997; Simpson & Raubenheimer, 1999). Our study design did not include data collection that would provide evidence to differentiate between any of these possible mechanisms.

To further test the accuracy of the metabolizable energy intakes that we derived, we calculated the difference between metabolizable energy intake and energy expenditure for comparison with the caloric equivalent of body composition changes. Overall, the energy budgets balanced, which gives us confidence in our measurements.

The results of this study did have some limitations. The coefficient of variation (CV) of measured food intake was 2% in the CR and 7% in the C monkeys in the six-month period during which this study was conducted. These CV's would correspond to ~ 9 and 38 kcal/d , in CR and C monkeys respectively whereas our energy waste data was collected only for two days. The CV for the 2-day fecal and urinary losses was 24% and 34% in CR and C monkeys respectively which correspond to 14 and 16 kcal/d respectively. Similarly, energy expenditure was measured only for two, two-day periods. The within animal coefficient of variation of measured energy expenditure was 12% in the C and 13% in the CR monkeys calculated over two consecutive-year measurements which could explain nearly 64 kcal/d of the variability in flux in CR and C monkeys. There was, therefore, a possibility of day-to-day variability in all these measures and that our values were not fully representative of the six-month interval used in calculating energy balance.

Systematic effects associated with the conditions for measurement of energy expenditure can also potentially influence our results (Vishnevskii et al, 2004; Miyaoka et al, 2005). The measurement required that the animals be removed from their home cages and placed in the metabolic chambers. Even though the animals have been placed in these chambers for two days

every year for 14 years, the increase in urinary cortisol excretion rate in CR female monkeys was significantly higher in the metabolic cages compared to their home cages (data not shown). These results may indicate that the CR animals were subject to more stress when in the metabolic chamber and this might translate into higher energy expenditure in the chamber than in their home cages. It is not possible, however, to speculate on how this difference may translate to a difference in energy expenditure in kcal/d. Moreover, urinary cortisol values, showed high between-animal variability (37% CV) and were measured in female monkeys making translation of these values into energy equivalents difficult and could be one of the caveats of this analysis.

To conclude, we found that the measured metabolizable energy intakes of the CR and C animals to be 450 ± 53 (CR) and 534 ± 97 (C) kcal/d, which differed from the estimated energy intakes by 31 ± 4 and 17 ± 3 kcal/d, as obtained using the traditional technique of weighing the monkey diet consumption. This difference, however, is small or modest at worst and thus the careful use of weighed food disappearance provides a good estimate of metabolizable food intake combined with the classic use of Atwater factors to compare metabolizable energy in these animals. Nonetheless, to obtain this level of accuracy, one needs to assess factors such as moisture content and food wastage, which can be highly variable. Finally, we also found a significant 4% decrease in the absorption efficiencies under long-term CR, which is counter-intuitive and could not be explained by any specific mechanism thus far.

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Figure 1. Comparison of excretory loss and energy intake in CR and C monkeys

Analysis of covariance (ANCOVA) showed that the CR monkeys had a greater excretory energy loss per unit gross energy intake ($F_{1,17} = 16$; $p < 0.001$) compared to C monkeys. Treatment was used as a factor with excretory losses as the dependent variable and gross energy intake as the covariate in the model. There was no significant difference in the slopes ($F_{1,16} = 1$; $p = 0.3$).

Figure 2. Energy Balance

Each bar represents the mean $\pm$ S.D. of the energy equivalents. The food intake was calculated using 6-month average values of food intake and the coefficients of metabolizable energy for CR and C monkeys. Total energy expenditure (TEE) was measured using indirect calorimetry once during the 6-month period. Body composition (BC) changes were calculated as the difference in the fat and lean mass over 2 consecutive DXA measurements made 6 months apart. Energy balance calculated as- Energy balance= Metabolizable energy (ME intake) – [energy expenditure (TEE) + change in body composition (BC changes)].

Table 1Characteristics, energy intake and expenditure (Mean $\pm$ s.d.) in the older male rhesus monkeys

	Control	CR
n (no. of animals)	9	11
Age, years	24 $\pm$ 1	24 $\pm$ 2
Body weight, kg	13.3 $\pm$ 2 *	9.6 $\pm$ 1
Fat mass, kg	4.3 $\pm$ 0.6	1.6 $\pm$ 0.2
Lean body mass, kg	9.0 $\pm$ 0.3	8.1 $\pm$ 0.2
Energy expenditure, kcal/d	532 $\pm$ 66 *	490 $\pm$ 64

* represents a significant difference, compared with CR

Table 2

Dry matter and energy of the diet and excreta (mean ± s.d.)

trt	dry matter flux, g/d		gross energy content, kcal/g dry mass		Dry matter digestibility	Efficiencies Energy digestibility	Metabolizability
	Food	Feces	Urine	Feces			
C	136 ± 26	9 ± 5	8 ± 3	3.6 ± 0.7	0.94 ± 0.03	0.95 ± 0.02	0.91 ± 0.03
CR	112 ± 20	12 ± 4	9 ± 3	3.6 ± 0.5	0.90 ± 0.03	0.91 ± 0.03	0.87 ± 0.03
p-value	0.003	0.044	0.19	0.71	<0.001	<0.001	<0.001