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Does caloric restriction extend life in wild mice?

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Summary

To investigate whether mice genetically unaltered by many generations of laboratory selection exhibit similar hormonal and demographic responses to caloric restriction (CR) as laboratory rodents, we performed CR on cohorts of genetically heterogeneous male mice which were grandoffspring of wild-caught ancestors. Although hormonal changes, specifically an increase in corticosterone and decrease in testosterone, mimicked those seen in laboratory-adapted rodents, we found no difference in mean longevity between *ad libitum* (AL) and CR dietary groups, although a maximum likelihood fitted Gompertz mortality model indicated a significantly shallower slope and higher intercept for the CR group. This result was due to higher mortality in CR animals early in life, but lower mortality late in life. A subset of animals may have exhibited the standard demographic response to CR in that the longest-lived 8.1% of our animals were all from the CR group. Despite the lack of a robust mean longevity difference between groups, we did note a strong anticancer effect of CR as seen in laboratory rodents. Three plausible interpretations of our results are the following: (1) animals not selected under laboratory conditions do not show the typical CR effect; (2) because wild-derived animals eat less when fed AL, our restriction regime was too severe to see the CR effect; or (3) there is genetic variation for the CR effect in wild populations; variants that respond to CR with extended life are inadvertently selected for under conditions of laboratory domestication.

Key words: aging; caloric restriction; cancer; domestication; wild mice.

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Introduction

Reducing caloric intake substantially below the *ad libitum* (AL)-feeding level extends life in multiple species including yeast (*Saccharomyces cerevesiae*) (Jiang *et al*., 2000), fruitflies (*Drosophila melanogaster*) (Mair *et al*., 2003), nematodes (*Caenorhabditis elegans*) (Lakowski & Hekimi, 1998), the crustacean *Daphnia longispina* (Ingle *et al*., 1937), a spider, *Frontinella pyramitela* (Austad, 1989), and a range of laboratory mouse and rat genotypes (Weindruch & Walford, 1988). We will call this extension of life by caloric restriction (CR) the 'CR effect'. In laboratory rodents, considerable evidence suggests that CR not only extends life but also broadly slows aging in many physiological functions (Weindruch & Walford, 1988). Whether or not primates exhibit the CR effect is not yet known (Lane *et al*., 2004), although studies in several species, including humans, are in progress (Mattison *et al*., 2003; Fontana *et al*., 2004; Heilbronn *et al*., 2006).

However, CR does not ubiquitously extend life. For instance, in medflies (*Ceratitis capitata*), reduced caloric intake had either no effect on longevity (at feeding levels ranging between 60% and 95% AL) or actually reduced longevity (at 50% AL and below) in both sexes (Carey *et al*., 2002). In addition, although five of ten rotifer species studied exhibited strong life extension in response to CR and two other species showed a marginal longevity increase, three species showed a substantial decrease in longevity (Kirk, 2001). Even among laboratory rodents, occasionally studies fail to observe the CR effect (Harrison & Archer, 1987; Forster *et al*., 2003) in certain genotypes under certain conditions.

All mammalian (and most nonmammalian) studies on CR have utilized animals adapted over many generations to laboratory life. That is, they have been subjected to dozens to hundreds of generations of selection for traits favored under laboratory husbandry such as rapid growth, early maturity, larger adult body size, and a high reproductive rate. These are all conducive to high colony productivity under benign, food-rich environments. However, this selective regime can also lead to shortened life (Promislow & Tatar, 1998; Sgrò & Partridge, 2000; Linnen *et al*., 2001; Miller *et al*., 2002a) and other less obvious traits such as the loss of melatonin production by the pineal gland (Goto *et al*., 1989) and greater telomere length (Hemann & Greider, 2000). The more rapid growth and larger body size of laboratory domesticated animals compared with their wild progenitors could be due to inadvertent selection for higher food consumption in laboratory populations. Indeed, this is true for laboratory vs. wild house mice. Laboratory mice eat roughly 20% more than wild mice under AL laboratory conditions on a weight-adjusted basis (Austad & Kristan, 2003). One possible consequence of this observation is that CR in comparatively gluttonous laboratory mice with idiosyncratic phenotypes that are the result of inadvertent laboratory selection might have different effects than in mice more recently derived from the wild.

Mechanisms by which the CR effect is produced are still not clear. Among the candidate mechanisms are neuroendocrinological alterations (Nelson *et al*., 1995). Although most hormone concentrations, including reproductive steroids, are reduced in rodents by CR early in life, they then decline less rapidly with aging than AL animals, such that by late life they may actually cross over and be higher in CR relative to AL animals (Merry & Holehan, 1985; Chen *et al*., 2005). By contrast, the stress hormone corticosterone is moderately elevated by CR in laboratory rodents and has even been hypothesized to play a role in the CR effect (Klebanov *et al*., 1995; Nelson *et al*., 1995).

In order to examine whether CR might similarly affect longevity and hormone concentrations in mice not subjected to many generations of laboratory selection, we captured wild house mice, allowed them to reproduce in the laboratory, and subjected their male grandoffspring to 40% CR. We used grandoffspring to avoid confounding our study with maternal effects from life under field conditions. These 'wild-derived' mice have previously been shown to be smaller, reach reproductive maturity later, and live about 20% longer than a stock created by the intercrossing of four laboratory strains (Miller *et al*., 2002a). In both CR- and AL-fed mice we measured body mass throughout life, fecal steroid concentration (testosterone and corticosterone) from the initiation of CR until more than 2 years of age, and longevity. We used fecal rather than serum hormone measurements because it is a completely noninvasive technique (feces are collected during normal weekly cage changes) which should not affect the stress levels or longevity of our study animals in any way. Also, it mitigates the considerable difficulties of assessing basal stress measurements without the confound of iatragenic stress (Sabatino *et al*., 1991; Harper & Austad, 2000b). Finally, because hormones are excreted relatively slowly compared to their secretion rate, fecal hormone measures integrated variation in circulating hormone levels over a longer period of time than serum measurements and thus give a clearer picture of chronic hormone exposure.

We also performed complete gross and histologic evaluation of tissue pathology on all animals recovered soon enough after death such that their tissues and/or organs had not been too autolyzed for assessment. In some cases, when tissues were too autolyzed for histopathologic assessment, we could still determine the cause of death from gross necropsy. All animals for which we could establish a cause of death and/or assess histopathologic lesions were used in our analyses.

Results

With imposition of the CR regime at 3 months of age, mice quickly lost weight from a prerestriction level of about 20 g to about 15 g over a 3-month period, at which point their weight stabilized until about 140 weeks of age when it increased slightly and remained so for the rest of life (Fig. 1A). This late-life increase in weight was due both to the fact that the longest surviving CR animals were heavier throughout restriction than the shorter survivors and that the longest-lived CR mice actually gained

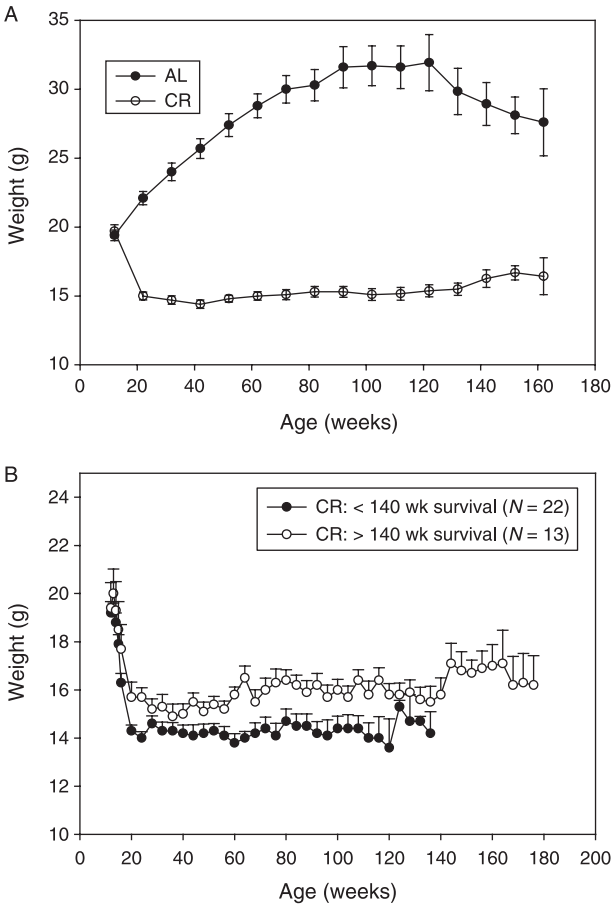

Fig. 1 (A) Longitudinal trends in body weights of *ad libitum* (AL) and caloric restriction (CR) wild-derived mice. (B) Longitudinal trends in CR animals that are long- [> 143 weeks (1000 days)] or short-lived (< 143 weeks).

weight faster from 150 weeks of age compared to the previous period (Fig. 1B). The relative constancy of the mean CR weight throughout most of life (Fig. 1A) is a bit misleading as individual animal weight fluctuated substantially despite their constant feeding regime. In fact, peak individual weight among CR animals was 17.35 ± 0.29 g, more than 2 g higher than the population mean over most of the study.

By contrast, the AL mice continued to gain weight from 12 weeks until they reached a peak body mass of about 31 g at 96 weeks of age. They maintained that weight until about 122 weeks, after which their weight declined for the remainder of life (Fig. 1A). The late-life decline in weight was primarily due to individual weight loss rather than differential survival of lighter individuals. At the maximum difference in mass between the groups (roughly 2 years of age), CR animals weighed 47.6% as much as controls.

As in previous reports in rodents, CR induced an increase in corticosterone relative to controls at virtually all ages (Fig. 2A). Peak corticosterone concentration in CR animals was observed at 36 weeks of age, and declined thereafter. By 84 weeks of age, corticosterone concentration had stabilized as there were no statistically significant changes after that point (paired *t*-

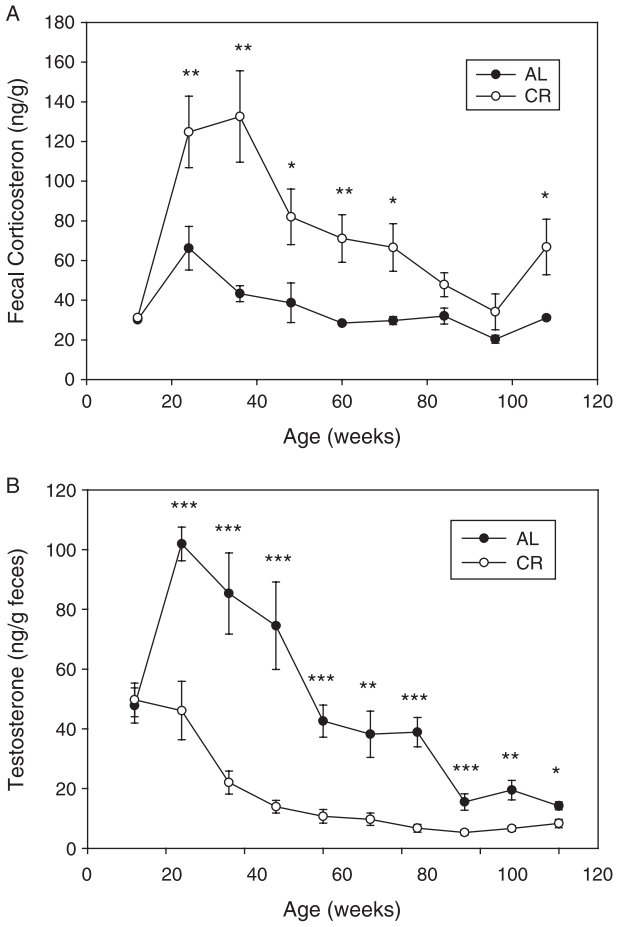

Fig. 2 Fecal (A) corticosterone and (B) testosterone concentrations in caloric restriction (CR) vs. *ad libitum* (AL) mice. ***$P \leq 0.001$; **$0.001 < P \leq 0.01$; *$0.01 < P < 0.05$ for differences between CR and AL at equivalent times.

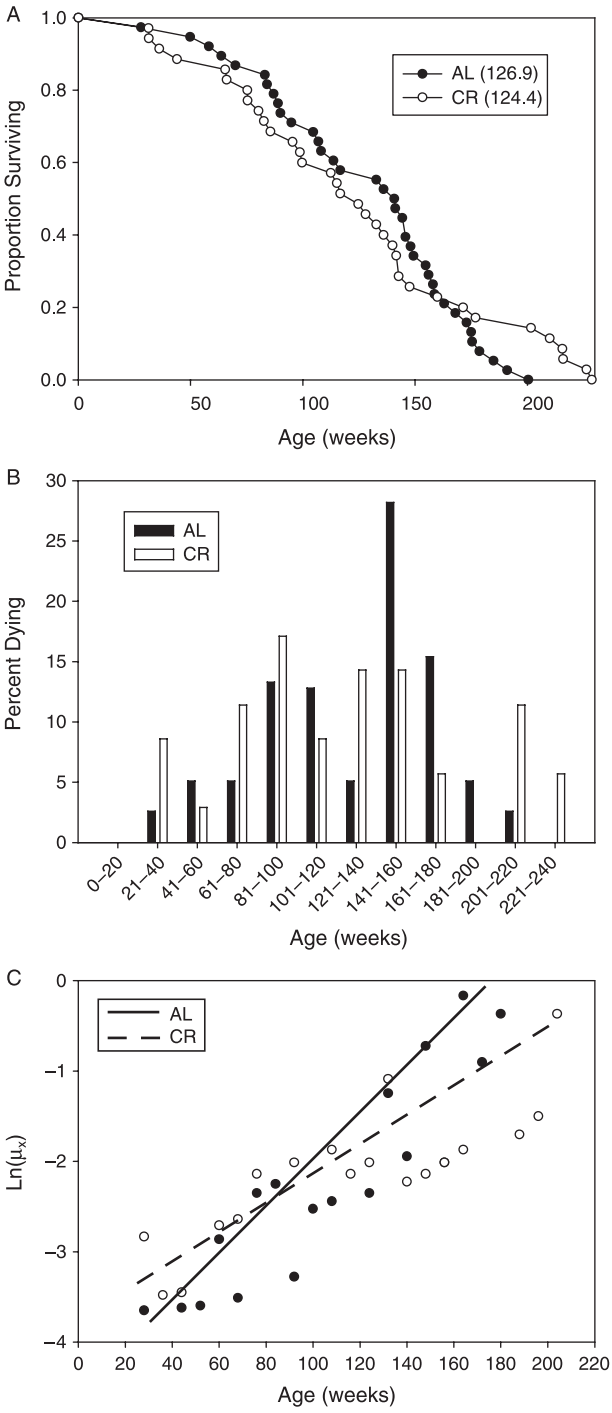

Fig. 3 Longevity of *ad libitum* (AL) and caloric restriction (CR) animals. (A) Comparative survival shows no statistical difference. Numbers in parenthesis are mean values for each group. (B) Distribution of deaths in the two groups. CR animals show more early deaths but fewer later deaths than the AL group. (C) Age-specific hazard rates and Gompertz plots for the two groups. Despite the lack of a difference in survival between the groups, there is a statistical difference in Gompertz parameters ($P = 0.014$).

tests, $P = 0.09$ and $P = 0.17$, for 108 and 96 weeks, respectively). For AL animals, peak corticosterone occurred somewhat earlier (at 24 weeks of age) and was only about half as high as in CR animals. The subsequent hormone decline with age was not nearly as dramatic, with no significant continuing pattern of decline after 60 weeks of age.

Also as in previous studies, CR substantially reduced testosterone which was significantly lower compared with AL animals (Fig. 2B) in early adult life. However, unlike previous studies, lower testosterone persisted in CR compared with AL animals until at least 120 weeks of age. In AL animals, testosterone peaked at 24 weeks of age and fell to less than one-fifth peak level by 96 weeks of age. By contrast, testosterone in CR animals was highest at 3 months of age just prior to the initiation of CR. Thereafter, it declined gradually until leveling out by 84 weeks.

Survival did not significantly differ between AL and CR groups (Kaplan–Meier, log rank test, $P = 0.523$). AL animals lived an average of 888.5 ± 48.7 days compared with 870.6 ± 67.4 days for CR animals. Mean longevity was also statistically indistinguishable between groups ($t_{72} = 0.218$, $P = 0.828$). However, visual inspection of the distribution of deaths in the two dietary groups (Fig. 3B) suggested that while more CR animals died early in the experiment (age < 600 days), more CR animals survived until relatively late (age > 1350 days). In fact, the six longest-lived individuals in the experiment were all on restricted

diets. The longest-lived AL mouse died at 1403 days compared with 1601 days for the longest-lived CR mouse.

Analysis of age-specific mortality patterns can reveal subtle differences that might be overlooked by survival or longevity analyses (Finch, 1990; Pletcher *et al*., 2000). An age-specific mortality comparison of the two groups using maximum-likelihood methods indicated that a Gompertz model fit the data better than Gompertz–Makeham, logistic, or logistic-Makeham models. Futhermore, this analysis revealed significantly different Gompertz parameters ($P = 0.014$) (Fig. 3C). The initial mortality rate (intercept of the Gompertz curve) is higher (–4.22 vs. –5.29) in the CR relative to AL group, whereas mortality rate doubling time (inversely related to the Gompertz slope), which is often considered to be a measure of aging rate, was longer for CR compared with AL animals (10.9 months for CR vs. 6.4 months for AL).

Twenty-four AL and 25 CR animals underwent necropsy. Mice in both feeding groups died from various causes, chief among these a range of cancers, as well as several cardiac problems and renal failure due to extensive mineralization. One death in each group appears to be infectious in origin (AL: staphylococcal dermatitis; CR: bacterial nephritis). As has been reported numerous times, CR appeared to have a protective effect against tumors (Table 1). Almost 60% of the AL animals had at least one tumor at necropsy compared with only 12% of the CR group ($P = 0.001$, Fisher's exact test). The only three CR animals with tumors were among the oldest individuals in the study (mean age at death = 1449 days). A significant number of animals in both groups had no histopathologic lesions.

Discussion

Hormonal changes with age in our wild-derived animals were similar in many, although not all, details to those reported from serum assays in laboratory rodents. For instance, CR elevates serum corticosterone levels compared to AL controls in young laboratory rats and mice (Klebanov *et al*., 1995; Han *et al*., 2001) as it did in the feces of our mice (Fig. 2A). Free corticosterone is elevated in CR rats throughout life relative to age-matched AL controls (Han *et al*., 2001) and similarly fecal corticosterone concentration [which is the product of the excretion of free hormone only (Palme *et al*., 2005)] was elevated in CR relative to AL animals throughout life in our wild-derived mice. Unlike serum measures of basal glucocorticoids, which tend to remain reasonably stable or slightly increase throughout adult life in laboratory rodents and primates (Sapolsky, 1992; Hauger *et al*., 1994), fecal corticosterone measures in this study declined dramatically from 9 months of age in the AL group and declined more subtly from 6 months in the CR group. Whether this pattern represents a clear difference from laboratory rodents or primates is not clear. Because fecal steroids must be metabolized and/or excreted before appearing in feces, any alteration in their rate of metabolism or excretion relative to the rate of feces formation could also explain age-related changes. As we did not measure serum corticosterone concentration throughout life, we can not distinguish between an actual decrease in circulating level and a decline in the rate of corticosterone metabolism and/or excretion.

Table 1 Necropsy results

	Ad libitum	Caloric restriction
Total necropsied	24	25
Number with tumors	14 (58.3%)	3 (12%)
No histological lesions	5 (20.8%)	4 (16%)

Neoplastic lesions reported here were not necessarily the cause of death. We simply report the number of animals in which any tumors were observed.

Chen *et al*. (2005) found that serum testosterone in AL Brown–Norway rats fell by 79% between 5 and 24 months of age. Similarly, we saw fecal testosterone reduced by 81% between 6 and 25 months in our AL mice. In CR rats, serum testosterone was reduced to less than half that in AL animals after 2 months of restriction (Chen *et al*., 2005). Our wild-derived mice showed a similar drop in fecal testosterone after 3 months restriction. However, whereas CR rat testosterone remained stable between 5 and 25 months of age and did not decline farther until 28 months, our CR mice showed a continuing gradual reduction in fecal testosterone between 6 and 21 months of age. Unlike in laboratory rats, testosterone levels in our AL and CR animals never converged, much less crossed over even as late as 30 months of age (Fig. 2B).

Our most striking finding was that although hormonal responses to CR were similar to previously published work, wild-derived mice on CR did not live longer on average than AL controls. As has been previously emphasized by several investigators, there is something to be gained by comparing not just mean longevities and survival curves but also age-specific mortality trajectories. In this instance, Gompertz modeling of AL vs. CR mortality patterns showed a statistically significant difference between the respective lines, in that initial mortality rate was higher for CR animals but the Gompertz slope was lower. The two parameters (initial mortality rate and Gompertz slope) offset one another with respect to mean longevity, so that even though the lines were significantly different, neither mean longevity nor Kaplan–Meier survival curves differed statistically. A note is warranted about the statistical power inherent in our sample sizes of 39 AL vs. 35 CR animals. If the CR effect were as robust as typically found for a standard laboratory strain, say, C57BL/6 males, then a sample of 14 animals in each group is sufficient to detect a true difference of 175 days mean longevity with 80% probability at the $P = 0.05$ level (data from NIA/NCTR Biomarkers study, as shown in Sprott & Austad, 1996; Fig. 1). Given the larger variation in age-at-death in our wild-derived mice, a sample of 51 per group would be necessary to detect a similar difference in longevity. However, the difference seen in our study is only 18 days in mean longevity, with the AL groups having the greater absolute value.

There are several possible explanations for this result. First, it is possible that mice not adapted to laboratory conditions fail

to exhibit the CR effect, and that the effect in mammals is an artefact of the laboratory domestication process. AL food consumption level, after all, is about 20% reduced in wild-derived mice on a weight-adjusted basis compared with laboratory-adapted animals (Austad & Kristan, 2003), indicating that a certain degree of gluttony has evolved over several hundreds of generations of inadvertent laboratory selection. Also, it could be argued that because wild-derived animals are not laboratory adapted, they have considerably higher basal stress levels than laboratory mice. Therefore, the extra stress imposed by CR was deleterious. However previous work in our laboratory suggests this is not true, as fecal corticosterone concentration was slightly higher in C57BL/6 mice compared with first-generation offspring of wild-caught mice (Harper & Austad, 2000a).

A related interpretation is that the above hypothesis is valid, but only for this particular long-lived wild population. It is clear that not all wild mouse populations are the same (Berry & Bronson, 1992). They will be adapted to local conditions and, if introduced to a new habitat in recent times, be subject to complex founder effects as well as local selective regimes. For instance, the Idaho mice used in this study have been shown to be endocrinologically distinct from a mouse population on the tropical island of Majuro as well as from laboratory-adapted mice. In particular Idaho mice had significantly lower serum insulin-like growth factor I (IGF-I) and leptin levels, and Majuro mice had higher glycated hemoglobin level compared with one another or with an outbred laboratory stock (Miller *et al*., 2002a). The low IGF-I in Idaho mice is of particular interest given the recent report that growth hormone receptor knockout mice (which also have dramatically reduced IGF-I) also do not exhibit increased lifespans in response to CR (Bonkowski *et al*., 2006).

Another conceivable interpretation is that because they are not adapted to the laboratory, wild-derived mice would have lived longer in the natural environment had they been restricted to the same extent. We think it is important to note that although many authors have speculated that the CR effect is an adaptive response to naturally occurring food shortages (Harrison & Archer, 1989; Masoro & Austad, 1996), there is no direct empirical evidence for or against that hypothesis. Although food restriction is somewhere between difficult and impossible to perform experimentally in a controlled fashion under natural conditions, a common paradigm among population ecologists is to supplement the food of free-living animals by enhancing natural food supplies. Generally animals respond to food supplementation with increased body weights, earlier reproduction, and enhanced population growth, showing that animals do generally eat less than they would prefer to in nature. One possible prediction then would be that food-supplemented animals should be shorter-lived than unsupplemented controls. Boutin (1990) reviewed seven such studies in birds and in every case the food-supplemented birds exhibited increased, rather than decreased, survival. Similarly for mammals, the same author found that in 14 studies food supplementation increased adult survival rather than decreased it. In addition, 25 studies found no effect of food supplementation on survival, and only two (of 41) studies found decreased survival with increased food. Juvenile survival increased with food supplementation in 18 studies, there was no effect in nine studies, and decreased in only one study. The only one of these studies to be performed in house mice found that supplementation increased body weight and population productivity but had no effect on survival (DeLong, 1967). Thus, there is little direct evidence that food restriction extends life in nature.

An additional possible interpretation is that because wild-derived animals eat less, the standard CR protocol of 40% restriction is too severe, and that the CR effect would have been observed if we had restricted the animals to a lesser extent. Higher CR mortality early in life might be viewed as supporting this interpretation, given that in a genetically heterogeneous population, some animals would be more sensitive to undernutrition than others. While this interpretation is certainly potentially valid, several observations make us question it. For instance, higher mortality among CR animals early in life occurs commonly in studies using standard protocols, in which a robust CR effect can be seen (Yu *et al*., 1982; Turturro *et al*., 1999). Also, restriction much more severe than ours (i.e. up to 65% reduction from AL intake) has been reported to extend life dramatically in mice (Weindruch *et al*., 1986). Although the body weights of our restricted mice were low (~15 g) throughout life, and substantially lower than the weights of restricted laboratory strains (Turturro *et al*., 1999), our AL animals also weighed less than most laboratory strains. Only small DBA/2 laboratory strain males approach the adult weight of our males. Moreover weights of our restricted animals overlap the weights of mice trapped in nature (Austad & Kristan, 2003). The difference between AL and CR weights in our study (CR body mass was 47.6% body mass of AL animals at maximum difference) is greater than the difference in feeding level. However, due to differences in activity level and thermoregulatory costs in CR and AL animals, such a difference is not unexpected. A recent study of C57BL/6 laboratory mice using, as we did, singly housed AL and 40% CR animals found that body mass in CR animals averaged across the lifespan was 50% that of AL controls (Ikeno *et al*., 2005).

Although there was no difference in mean longevity between AL and CR animals, the six longest-lived animals in our study all came from the CR group (Fig. 3A,B). A similar pattern of late-life reduced mortality rate has been seen in another genetically heterogeneous, but fully fed, wild-derived mouse population (Miller *et al*., 2002a). A plausible explanation for both these trends might be genetic variability in survival capacity and the ability to exhibit the CR effect. It has been previously reported that even in laboratory mice, there is genetic variability for physiologic correlates of the CR effect (Rikke *et al*., 2003, 2004).

Although our study was not designed to assess genetic effects, some hint that genetic effects are implicated might be evident if some particularly large fraction of the longest-lived CR animals were clustered in particular parentages. Our study animals were derived from nine unique parentages and 18 individual litters. Four of these parentages produced the ten

longest-lived CR animals (> 1000 days). These parentages also produced 18 (46%) of all AL animals and 60% of all CR animals in our study. However the distribution of animals from these parentages that lived greater than, vs. less than, 1000 days does not differ from chance ($\chi^2 = 0.39$, $P = 0.843$). Of course, despite this analysis genetic variation could still be playing a role in the results. However, available data do not support that conclusion. A study more appropriately designed to detect genetic effects will be required to clarify this point.

It might be wondered whether our results are due to the fact that our colony was a clean conventional colony rather than an specific pathogen free (SPF) barrier colony. That is unlikely for several reasons. First, our necropsy results suggest that death due to infection is rare (one case in each experimental study group). Second, the CR effect was discovered and for most of its history reproduced time after time in conventional colonies (Weindruch & Walford, 1988). In fact among the most striking life-extension effects of CR reported to date, in which animal diets were restricted as much as 65%, were from mice housed in clean conventional facilities (Weindruch *et al*., 1986).

Another point worth noting is that our survival curves do not have the semirectangular shape that one often sees in healthy colonies of laboratory rodents. There are, again, several possible explanations for this observation. First, not all laboratory colonies show such a phenomenon. Specifically, some laboratory genotypes, in particular DBA/2, rarely exhibit anything close to 'rectangular' survival curves even in SPF colonies (see, for example, Fig. 2 in Sprott & Austad, 1996). Second, such a pattern could be due to genotypic or phenotypic heterogeneity with respect to lifespan. As our animals are genetically heterogeneous, this would not be surprising.

Our necropsy results show a dramatic antitumor effect of CR even in the absence of robust life extension. Almost 60% of AL animals had tumors at the time of death compared with only 12% of CR animals. The only three CR animals with tumors died at very old ages (averaging about 4 years). This result is consistent with the hypothesis that the antitumor effect of CR may be due to elevated circulating corticosterone (Birt *et al*., 2004) which has been seen in laboratory mouse and rat CR studies as well as the current study.

Tumor incidence is often, but not always, correlated with longevity in mouse studies. For instance, a hyperactive p53 mutant was found to reduce cancer incidence but shorten life (Tyner *et al*., 2002) and reduced activity of the mitochondrial antioxidant MnSOD increases cancer incidence but does not affect longevity (Van Remmen *et al*., 2003).

Our data bear on several previous findings concerning the impact of body weight on longevity in the aging and CR literature. Both Bertrand *et al*. (1980) and Weindruch *et al*. (1986) noted a positive correlation between rodent (rats and mice, respectively) body mass and longevity under CR at particular ages, but found no similar correlation among AL controls, suggesting that thrifty, energy-efficient (or reduced activity) phenotypes are particularly long-lived under CR. Given that CR animals living longer than 140 weeks were significantly heavier on average after weight had stabilized (from 24 weeks of age) than those living less than 140 weeks (Fig. 1B) (15.79 g vs. 14.28 g, $t_{33} = 4.182$, $P < 0.0005$), this appears to also be the case in our study. Although we found no significant correlations between body mass and longevity at 12 (at the beginning of restriction), 24, 36, or 48 weeks in CR animals, we did find a significant positive relationship between peak body mass and mean restricted body mass (after weight stabilization at 24 weeks) and longevity (Pearson $r = 0.567$, $P < 0.001$ and $r = 0.345$, $P = 0.043$, respectively). In the AL group, we found no significant correlations between body mass and longevity at any age unlike previous reports in other outbred populations (Miller *et al*., 2002b).

Our data also bear on the generality of the CR effect. If mice not subjected to genetic selection for rapid growth, early maturity, and high fecundity do not show the CR longevity effect, then it seems unlikely that such an effect will be found in other mammal species such as humans that have not been genetically selected for rapid growth and high fecundity (Demetrius, 2004; Phelan & Rose, 2005). On the other hand, if either hypothesis – that our restriction regime was too stringent or genetic variation for the CR effect is favored by laboratory selection – is correct, then our study may have little relevance to the likelihood that CR will extend life in other mammal species. The fact that we did observe an anticancer effect of CR even in mice failing to live longer, does suggest that laboratory selection plays no role in that effect. Ultimately the question of the generality of the CR effect in mammals, and in particular whether there will be such an effect in humans, is an empirical issue about which much more will be learned from ongoing studies of CR with primates. It should be noted that although one frequently sees in the literature that CR 'probably works in primates', the data are not yet available to support such a claim (Lane *et al*., 2004). One thing our study does not question is the tremendous utility that investigation of the CR effect has had, and will continue to have, for understanding fundamental mechanisms of aging.

We intend additional studies of CR in wild-derived mice to distinguish among the possible interpretations of the work presented here. The existence of mouse genotypes that clearly did not exhibit the CR effect would be helpful in a comparative sense in distinguishing between those molecular, cellular, and physiological responses to CR that are causally involved in its effects and those that are merely by-products.

Experimental procedures

Colony establishment

Wild house mice (*Mus musculus*) were live trapped in and around agricultural buildings located on the University of Idaho campus in Moscow, Idaho, USA. Wild mice are small compared to laboratory-reared animals. Wild-trapped males averaged 19.0 g, females 15.0 g. Once captured, both parental stock and their offspring were maintained in identical rooms under a 14 : 10 lighting regime to ensure individuals remained

reproductively active. These breeder animals were fed Purina rat chow and given tap water *ad libitum* (AL). To ensure avoidance of inbreeding depression, in all but one case (an uncle–niece mating), males were placed with unrelated females. Our colony could best be described as clean conventional. It was not a barrier facility, although animals were individually housed in micro-isolator cages. Of the 49 animals undergoing necropsy, two apparently died of infectious diseases (bacterial nephritis, staphylococcal dermatitis).

Experimental animals

Male mice to be used in our experiments were weaned at 21 days, fed AL, and group housed until 12 weeks of age. At this time, they were separated into either the AL or CR groups, both of which were housed individually. When more than one male was available from the same birth litter, they were split between the two experimental groups. Thus, litters with even numbers of males were equally divided between CR and AL groups. When litters had odd numbers of males, the odd male was assigned to the experimental group having fewer animals. If both groups had equal numbers of males, then the odd male was randomly assigned to either CR or AL group. Because of the small litters and generally poor breeding success of early generation wild-derived mice (Miller *et al.*, 2000), animals entered the study in eight cohorts as they reached 3 months of age. At the beginning of the experiment we had 40 animals in each group. However, due to the accidents, inadvertent injuries, and escapes, we ended up with 39 animals in the AL group and 35 in the CR group.

Animals were observed daily and weighed weekly during their cage change. Animals found dead were immediately transported to the Washington Animal Disease Diagnostic Laboratory at Washington State University 8 miles from Pullman, Washington, USA.

Feeding regimens

The feeding regimen in our experiment was designed to approximate that used in the NIA/NCTR biomarker study (Turturro *et al.*, 1999). Mice were raised from weaning on Harlan 92051 mouse diet (see Appendix for diet composition). This diet was modified from the diet used in Weindruch *et al.* (1986). For 1 week prior to initiating the feeding protocol, daily food consumption was calculated for each cohort to determine AL-feeding rate. Animals in the CR group were gradually restricted in 10% weekly increments from the previously calculated AL rate beginning at 3 months of age. Thus, at 13 weeks they were consuming 90% AL, at 14 weeks 80% AL, and so on, such that by 4 months of age, each CR animal was consuming 60% the AL amount. Restricted animals were switched to Harlan mouse 92173 diet, which contains approximately equal micronutrients and protein compared to the control diet when fed at 60% AL. During the 7-day feeding trial, mice averaged 2.86 ± 0.22 g (= 11.66 ± 0.88 kcal) consumption per day. Thus CR mice received 1.72 ± 0.14 g (= 6.41 ± 0.48 kcal) per day. This represents a 40% reduction in amount eaten and a 45% reduction in calories.

Hormone measurement

Fecal hormone measurements were obtained from the same subset (n = 25 and 13 for testosterone and corticosterone per dietary regime initially, with reductions in numbers as animals died) of animals from each group every 12 weeks from 1 week prior to the imposition of the final feeding regime until 108 and 120 weeks of age for corticosterone and testosterone, respectively. Fecal samples were collected approximately 24 h after weekly cage changing and stored in 95% ethanol at –20 °C until hormones were extracted and analyzed. Details of extraction and analysis may be found in Harper & Austad (2000). These assays have been validated extensively by assaying serial dilutions of samples, as well as assaying samples spiked with known amounts of hormone, as well as purposely inducing stress, and by comparing fecal samples with simultaneously collected serum (Harper & Austad, 2000).

Statistical analysis

Results are presented as means ± SEM. Most statistical analyses were performed with SPSS 13.0 for Windows. The survival analysis was performed with the SPSS survival procedure. Mortality analysis was performed with WinModest 1.02, a program developed by Scott Pletcher which among other things uses maximum likelihood techniques for fitting and testing common mortality models.

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Appendix

Composition of the mouse diets

Control diet TD.92051		Restricted diet TD.92173	
	g kg^{-1}		g kg^{-1}
Casein	207.0	Casein	345.5
DL-methionine	2.0	DL-methionine	3.34
Sucrose	272.0	Sucrose	199.2
Corn starch	272.0	Corn starch	199.2
Corn oil	135.0	Corn oil	100.0
Cellulose	60.0	Cellulose	65.99
Brewers yeast	4.0	Brewers yeast	6.67
Vitamin mix, Teklad (40060)	10.0	Vitamin mix, Teklad (40060)	16.7
Mineral mix, AIN-76 (170915)	35.0	Mineral mix, AIN-76 (170915)	58.4
Calcium carbonate $CaCO_3$	3.0	Calcium carbonate $CaCO_3$	