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Calorie Restriction in Rodents: Caveats to Consider

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Abstract

The calorie restriction paradigm has provided one of the most widely used and most useful tools for investigating mechanisms of aging and longevity. By far, rodent models have been employed most often in these endeavors. Over decades of investigation, claims have been made that the paradigm produces the most robust demonstration that aging is malleable. In the current review of the rodent literature, we present arguments that question the robustness of the paradigm to increase lifespan and healthspan. Specifically, there are several questions to consider as follows: (1) At what age does CR no longer produce benefits? (2) Does CR attenuate cognitive decline? (3) Are there negative effects of CR, including effects on bone health, wound healing, and response to infection? (4) How important is schedule of feeding? (5) How long does CR need to be imposed to be effective? (6) How do genotype and gender influence CR? (7) What role does dietary composition play? Consideration of these questions produce many caveats that should guide future investigations to move the field forward.

Keywords

nutrition; diet restriction; protein restriction; fasting; low calorie diets; longevity; mortality; lifespan; healthspan; chronic disease

Introduction

Eight decades have passed since the publication of the paper by McCay et al. (McCay et al., 1935) describing the impressive prolongevity effects of retarding the growth of rats by restricting food available to them. This paradigm of calorie restriction (CR), also known as diet restriction (DR), has emerged over that period to become one of the most widely used tools of biogerontologists for dissecting biological mechanisms of aging. The appeal of the paradigm is its robustness as evidenced by the wide number of invertebrate and vertebrate species exhibiting prolongevity effects in response to a wide variety of CR regimens.

Moreover, its appeal is strengthened because the beneficial effects on lifespan typically also encompass positive effects on healthspan. The latter includes delay in onset and reduction in incidence of many chronic diseases as well attenuation of many age-related functional declines, including mobility and cognition.

In response to the familiar refrain describing the robustness of the CR paradigm that has been the focus of many past reviews, we will couch the current review within a context of denting and tarnishing its reputation by presenting several major caveats that now need to be considered in moving the field forward. We believe that such an approach is timely and certainly necessary. Consistent with our charge in this endeavor, the review will be limited to rodent studies of CR, but the points we raise certainly apply across the wide range of approaches and animal models that use this paradigm. Moreover, the points raised in the review are certainly relevant to considerations of how to apply the CR paradigm to human health.

To this end, we will attempt to summarize what we know and what we do not know regarding CR in rodents, and we will focus primarily on effects of CR on lifespan and healthspan. Thus, a deep dive into mechanisms of CR is not the main objective of this effort. The product will best be viewed within the context of other reviews provided in this Special Issue as well as recent reviews appearing elsewhere that offered critiques of the CR paradigm (Roth and Polotsky, 2012; Sohal and Forster, 2014a).

At What Age Does CR No Longer Produce Benefits?

One of the first caveats to consider regarding the robustness of CR for retarding aging in rodents is the age at which it is imposed. This consideration raises important practical questions regarding the relevance of CR as an intervention in humans. Without going into details regarding this issue, there remains considerable controversy regarding the health benefits of dieting for elderly persons (Porter Starr et al., 2014; Waters et al., 2013). However, even within the context of CR research in rodents, the question remains: Is there an age at which CR loses its effectiveness in terms of significantly increasing lifespan and/or healthspan?

By far, the most applied paradigm in rodent research involves the initiation of CR shortly after weaning but typically post pubescent, ranging from 4–12 weeks of age. This paradigm remains the most robust regarding effects of lifespan and healthspan, although later sections of the review will raise mitigating issues such as genotype and type of diet and consider possible negative effects on health and healthspan. The question is at what age does CR begin to lose its anti-aging benefits?

Early in the development of the CR field using rodent models, a critical question was whether the prolongevity effects of CR required retarding development. McCay and colleagues (McCay et al., 1941) addressed this issue in an early study in which rats ranging in age from 200–450 days were subjected to their CR paradigm adjusted to maintain body weight. Because no statistical analysis was conducted in this early study, Ingram and Reynolds (1983) (Ingram and Reynolds, 1983) reanalyzed the data presented in the original

article to confirm that increased lifespan in the CR group begun at mature ages. Other early studies corroborated these findings by showing that mature rodents responded nearly as well to CR as young post-weaning animals. For example, Yu et al. reported significant increases in lifespan in Fischer-344 (F344) rats placed on 40% CR at 6 mo of age(Yu et al., 1985). Similarly, Weindruch and Walford reported significant increases in lifespan in two long-lived mouse strains (C57BL/6 and B10C310) when put on a 40% CR regimen at 12 mo of age(Weindruch and Walford, 1982). Pugh et al. confirmed this longevity effect of CR in male C57BL/6 (B6) mice treated at 12 mo of age and also reported significant reductions in cancer incidence in the CR group compared to controls (Pugh et al., 1999b).

Many subsequent rodent studies have confirmed that CR begun up to 12 mo of age promotes significant prolongevity effects. However, even this conclusion must be tempered with considerations of genotype and feeding schedule. For example, Goodrick et al. employed every-other-day (EOD) feeding to impose CR in A/J, C57BL/6J, and B6AF1/J mice at different ages (Goodrick et al., 1990). When initiated at 1–2 months of age, the regimen of intermittent feeding (IF) produced significant increases in lifespan in all three strains. However, when initiated at 6 mo, IF had significant prolongevity effects only in B6 and the hybrid strain. Moreover, when started at 10 mo of age, IF significantly reduced lifespan in A/J mice, and no had significant effects on lifespan in the other two strains. Forster et al. noted similar age x genotype interactions with a regimen of 60% CR (Forster et al., 2003a). When initiated at 4 mo of age, CR increased lifespan in C57BL/6Nnia and B6D2F1/Nnia mice; however, there was no significant lifespan effect in DBA/2Nnia mice. When initiated at 24 mo, CR reduced lifespan in all three strains, with the greatest reduction in DBA mice. Similar negative effects on lifespan were reported by Ross in which CR was initiated in 300 day old Sprague-Dawley (SD) rats (Ross, 1977). Lipman et al. noted no significant lifespan effects when 33% CR was initiated in Long-Evans (LE) rats at 18 mo of age (Lipman et al., 1995). In a more extensive study, Lipman et al. (1998) reported no significant lifespan effects or reductions in tumor burden when 32% CR was introduced to 18- and 26-mo old male F344xBN F1 rats (Lipman et al., 1998).

Thus, at question is whether there are ages in rodents at which CR is no longer effective or even detrimental to lifespan and healthspan? Dhahbi et al. initiated CR (~40%) in 19-mo old male C3B6F1 mice and reported significant increases in lifespan accompanied by reduced tumor rates as well as a global gene transcriptional profile that resembled life-long CR(Dhahbi et al., 2004). Lee et al. also confirmed that CR (40%) initiated in 14-mo old male B6C3F1 mice produced a global gene expression profile that indicated slower aging(Lee et al., 2002). As one caveat, it is possible that mice are more responsive than rats to late-life CR. Additionally, even among mice it is now clear that genotypes respond differently to the same level of CR. For example, in an extensive survey of age-related lesions in mice on 40% CR since early life, Harbison (Harbison et al., 2016) observed that male and female C57BL/6Nnia mice experienced much greater disease reduction in response to CR compared to DBA/6Nnia mice. In p53-deficient mice that have greater susceptibility to spontaneous and inducible tumors, 40% CR as well as a 1-day fast per week significantly reduced tumor burden when initiated at 10 mo of age (Berrigan et al., 2002).

In addition to the positive effects of late-life CR on cancer risk, there are other studies suggesting beneficial effects on many other indices of aging at a molecular level. For example, Goto and colleagues (summarized in Goto et al. have published several studies examining the effects of CR (30–40%) induced in late-life over short periods (2–3.5 mo) (Goto et al., 2007). A few illustrative findings are as follows: (1) Half-lives of numerous proteins were increased in mouse hepatocytes taken from 23-mo old animals subjected to 2 mo CR; (2) age-related reductions in proteasome activity were attenuated in liver and muscle of 26.5 mo old rats on CR for 3.5 mo; (3) levels of protein carbonylation were reduced in liver mitochondria and skeletal muscle cytoplasm in 30-mo old rats on CR for 3.5 mo; and (4) carbonyl modification of histones was increased to young levels in 28-mo old rats on CR for 2 mo, a finding consistent with restored transcription.

Other studies have documented that the glucose/insulin axis remains partially sensitive to CR manipulations at advanced ages. As an example, Park et al. subjected 18 mo male F344 rats to 30% CR for about 4–6 weeks, and they observed that CR significantly improved glucose tolerance and insulin sensitivity, but without correcting impairment in several parameters of insulin signaling in muscle (Park et al., 2006). Pires et al. initiated 40% CR in 1- and 2-yr old Wistar rats and reported improved glucose tolerance and insulin sensitivity in both groups after 21 days of treatment, with greater responses in the younger cohort; however, no significant diet differences were observed when they examined glucose-stimulated insulin secretion in isolated pancreatic islets (Pires et al., 2014).

The function and health of the heart and kidney also appear to respond favorably to late-life CR. For example, Yan et al. initiated a 2-mo CR regimen in 20-mo old mice, an age at which distinctive cardiomyopathy is observed (Yan et al., 2013). CR-treated mice showed cardiac function equivalent to young mice as well as reduced myocardial fibrosis and apoptosis. F344xBNF1 rats subjected to 40% CR at 17.5 mo of age had reduced nephropathology compared to controls with less damage to mitochondrial DNA in the tubular epithelial cells (McKiernan et al., 2007). As another example, a regimen of 40% CR in 24 mo old rats for only 10 days suppressed indices of oxidative stress and inflammation in the aged kidney, including lowered production of reactive oxygen species, lipid peroxides COX-2 activity, ##NF-ΞB##, and iNOS (Jung et al., 2009). A similar regimen initiated in 25-mo old male SD rats also improved kidney morphology, including decreased glomerular volume and fibrosis, and reduction in senescence-associated β-galactosidase staining and levels of 8-hydroxydeoxyguanosine, a well-known marker of mitochondrial DNA oxidative damage (Ning et al., 2013). In a recent paper, Dai et al. observed that just a brief 10 week intervention with 30% CR in 26 month old C57BL/6 female mice reversed the pre-existing age-dependent cardiac hypertrophy and diastolic dysfunction (Dai et al., 2014).

In considering how late in life can CR exert positive effects on healthspan, studies examining brain and behavior are essential to review. Sharma and Kaur (Sharma and Kaur, 2007) reported improvements to several brain parameters following 3 mo CR (40%) in 18-mo old rats, including increased levels of brain antioxidants, HSP-70, and neural cell adhesion molecule (NCAM) as well as reduction in glial fibrillary acidic protein (GFAP) levels. In a follow-up study, Kaur et al. confirmed several of these benefits when CR was initiated in 24-mo old Wistar rats (Kaur et al., 2008). Additional brain markers, such as

synapsin-1, showed notable improvements in another follow-up study examining several brain regions after 21-mo old rats were started on CR for 3 mo (Sharma et al., 2010). Quintas et al. reported that late-life CR increased SIRT1 in rat hippocampus (Quintas et al., 2012). Cardoso et al. found that 18-mo old rats subjected to CR for 6 mo exhibited some protection against age-related decline in neuropeptide Y (NPY)-and somatostatin (SS)-containing neurons in the hippocampus (Cardoso et al., 2014).

Behavioral benefits of CR have also been observed. For example, Means et al. reported that 40% CR begun at 14-mo in C57BL/6Nnia mice attenuated age-related decline in grip strength, motor coordination, and spontaneous alternation behavior, but did not have significant effects on performance in the Morris water maze (MWM) (Means et al., 1993). Geng et al. noted significant benefits to motor performance (open field) and cognition (MWM) as well as for survival in 18-mo old rats introduced to 40% CR for 6 months (Geng et al., 2007). Singh et al. introduced an IF regimen (EOD) to 21-mo old male Wistar rats for 3 months and noted significant improvements in motor performance (rotarod test) and cognition (MWM) (Singh et al., 2012). Salvatore et al. reported that 30% CR introduced to F344BNF1 rats at 12 mo of age significantly attenuated the longitudinal decline in locomotor behavior measured in the open field (Salvatore et al., 2016).

In summary, the question of what age does CR lose its prolongevity effects in rodents remains open. It is clear that increases in lifespan diminish when CR is initiated at advancing age. However, in order to provide the most definitive answer, several considerations need to be taken into account, such as species, genotype, and type of CR regimen. In contrast, there are many apparent benefits to CR on healthspan measures when initiated at advanced ages, even as old as 24 mo.

Does CR Attenuate Cognitive Decline?

One of the most critical questions regarding the robustness of CR on healthspan in rodents is whether the intervention can attenuate age-related decline in behavioral function. When considering motor function, recent reviews of the literature continue to substantiate the robustness of CR for attenuating age-related decline in a number of abilities (Mattson, 2012, 2014), although the literature remains less consistent in conclusions with regard to the effect of CR on cognitive function.

Even in studies conducted a few decades ago, a clear conclusion on behavioral function was elusive. For example, Goodrick (Goodrick, 1984) reported attenuation of age-related decline in performance of a complex T-maze in aged male Wistar rats maintained on CR by EOD feeding. In a subsequent study of the effects of lifelong CR on behavioral performance in female C3B10RF1 mice, Ingram et al. noted a similar attenuation of the age-related decline in learning ability in the same maze(Ingram et al., 1987). However, instead of the food motivation protocol that Goodrick had applied, the latter study used avoidance of footshock as the motivational factor. This was an important alteration in protocol because of the possible confounding of motivational factors in which CR rats are provided food rewards. Similarly, Idrobo et al. reported that 12 mo of CR initiated in young B6 mice improved performance in a radial arm maze with food as the reinforcement (Idrobo et al., 1987).

Beatty et al. noted that EOD feeding in rats from 3–21 mo of age improved adaptation of the maze in old rats but did not significantly improve memory(Beatty et al., 1987). Bond et al. also reported no significant improvements in radial arm maze performance of aged rats on a 40% CR regimen since 6 weeks of age (Bond et al., 1989). Therefore, it would appear that consideration of CR effects on cognitive aging is more meaningful in studies applying negative reinforcement in their paradigms.

The application of the MWM paradigm to investigating effects of CR on cognitive performance is suitable because performance is negatively reinforced by requiring the rodent to escape from water by swimming to locate a hidden platform. Successful navigation requires the formation of a spatial map based on extramaze visual cues to locate the platform. Performance is typically assessed in two phases: 1) learning as measured by time or distance to locate the platform across trials conducted over a few days; 2) memory as measured by swimming in proximity to where the platform was previously located, referred to as a memory probe trial. Pitsikas et al. revealed that a 35% CR regimen from 3 weeks of age in male CD-COBS rats protected against age-related decline in motor performance as well as in performance in the MWM both in the learning and memory phases(Pitsikas et al., 1990). Stewart et al. noted significantly better learning and memory performance of 24-mo old F344 rats in the MWM when maintained on 40% CR since weaning; however, the performance of 30-mo old CR rats appeared to decline(Stewart et al., 1989). In a longitudinal study of male SD rats, Gyger et al. found no significant effects of 30% CR started at 3 weeks of age on learning in the MWM through 19 mo of age but did find significant improvement in the memory test as well as in a reversal test in which the platform was moved to a different location (Gyger et al., 1992). In a study of male B6 mice at 19 and 24 mo of age on 40% CR since 14 weeks of age, Bellush et al. found no significant effects on learning or memory performance in the MWM (Bellush et al., 1996). Similarly, Markowska (Markowska, 1999) observed significant attenuation of performance in sensorimotor tasks in F344 rats on 40% CR since weaning, but no significant effects on learning and memory performance in the MWM. In a follow-up study, Markowska and Savonenko (Markowska and Savonenko, 2002) again failed to find a CR effect on MWM performance in Brown-Norway rats but did note significant attenuation of age-related decline in MWM performance of F344x Brown Norway F1 rats (F344xBN). Thus, in these early studies, it was clear that findings regarding the benefits of CR on performance in the MWM differed among genotypes and depended on age of testing. In a previously cited study by Means et al., 40% CR initiated in 14-mo old B6 mice improved motor performance but had no significant effect on MWM performance (Means et al., 1993).

More recent studies using the MWM have continued to yield mixed results regarding the effects of CR on performance. Short-term CR from a young age has shown consistent evidence of being beneficial. For example, Ma et al. investigated 30% CR from 7–30 weeks of age in male B6 mice and noted significantly better performance in both learning and memory performance(Ma et al., 2014). In a study from the same group, Dong et al. confirmed that 30% CR for 10 mo improved both learning and memory in the MWM compared to controls (Dong et al., 2016).

Beneficial effects of life-long CR on learning performance of rats in the MWM have also been observed, but effects on memory were not apparent. For example, Fitting et al. investigated effects on motor and cognitive performance of 40% CR since 14 weeks of age in 36-mo old F344XBN rats (Fitting et al., 2008). Noting robust beneficial effects of CR on motor performance, these investigators also found that CR produced significant benefits on learning in the MWM, but not in memory probes. In agreement, Carter et al. explored cognitive effects of 40% CR from weaning in male F344BN rats of several different ages (8, 12–15, 25–27, 35–38 mo) (Carter et al., 2009). The results showed superior performance of CR rats in learning the MWM task, but no significant effects on the memory component. In an object recognition task, which is a measure of short-term memory, CR rats actually performed worse than controls.

When CR is initiated later in life, significant improvements have been documented. Kuhla et al. reported improved learning and memory performance of 19-mo old mice begun on a regimen of 40% CR at weaning (Kuhla et al., 2013). Geng et al. initiated 40% CR in 18-mo old rats and noted improved learning and memory in MWM performance at 24 mo (Geng et al., 2007). Similarly, Singh et al. subjected 24-mo old male Wistar rats to EOD feeding for 3 mo and reported improved MWM in both the learning and memory phases of the task (Singh et al., 2012).

There have been studies in which no significant effects on MWM performance were observed after short-term CR regimens were applied. As an example, Alomari et al. and Khabour et al. initiated EOD feeding in young Wistar rats for 6 weeks and observed no significant enhancement of performance in a radial arm water maze (Alomari et al., 2016; Khabour et al., 2010). In a longitudinal study using the MWM and 25% CR from weaning, Hansalik et al. also found no significant effects on performance in SD rats at 5, 10 and 18 mo of age (Hansalik et al., 2006). In a study of late-life induction of CR, Sarker et al. reported that 15-mo old B6 mice maintained on 30% CR for 12 weeks had improved performance in an active avoidance task but not in the learning and memory components of the MWM (Sarker et al., 2015).

Although several reports have been published that did not document significant beneficial effects on cognitive performance, it is rare that a report is published in which CR has a negative impact on performance. One such example is Yanai et al. In this study, male Wistar rats had their body weight clamped at a low level, which was ultimately about 35% of that of AL fed rats (Yanai et al., 2004). Thus, this was clearly a very severe form of CR. When tested at 7–12 mo of age and at 17–18 mo of age, their performance in the MWM was worse than that of AL controls. It could be that this degree of CR was indeed detrimental to cognitive performance due to hypoglycemia because an injection of glucose prior to testing improved learning.

Regarding other factors involved in mediating the effects of CR on cognition, there are two interesting studies to be mentioned. First, Komatsu et al. examined effects of 30% CR initiated at weaning in male SAMP8 mice, which is often cited as a model of accelerated aging (Komatsu et al., 2008). At 28 weeks of age, CR attenuated age-related deficits in a passive avoidance task, and this effect was robust across groups that were fed diets with

different compositions. In short, CR was effective in producing benefits, and it did not matter whether calories originated from protein, fat, or carbohydrate content of the diets. A second interesting study was conducted by Dhurandhar et al. in a transgenic mouse model of Alzheimer's disease (Dhurandhar et al., 2013). When CR was imposed at 8 weeks and maintained for 16 weeks, the impaired performance of these mice in the MWM paradigm was attenuated. The unique angle of this study, however, was that one group of mice received injections of a ghrelin agonist, which normally produces hunger and increased food intake. Even though the body weight of the ghrelin-treated mice was matched to the controls at a much higher level than the CR group, the performance of drug-treated mice was found to match that of the CR group.

In this brief review of the literature on the effects of CR on cognitive performance, we have avoided inclusion of rodent models of disease that would greatly expand our discussion and, instead, focused on rodent models of normal aging. The general conclusion is that no clear conclusion can be made regarding whether CR can protect against age-related cognitive decline. There has been little systematic research to sort out some of the factors that could help clarify this mixed bag of results. Suspected factors include the age of onset of CR, the age at which testing was done, the type of paradigm, and the numbers of animals evaluated. On the latter point, many of the studies had relatively small sample sizes that could have reduced statistical power to document significant effects of CR.

Are There Detrimental Effects of CR?

While the previous sections of this review have considered the robustness of the CR paradigm for increasing lifespan and improving certain aspects of healthspan, this section will consider possible negative effects of CR in rodents. Specifically, we will review reports that CR has detrimental effects on bone health, wound healing, and certain immune responses. The papers that are reviewed will relate to conventional paradigms of CR; thus, we have not considered effects of protein restriction, or restriction of specific amino acids, such as methionine.

Bone Health

Since it is well established that bone density is highly correlated to body mass, it is not surprising that many early studies reported that bone density was reduced in rodents on CR. Although previous reviews have concluded that CR appears detrimental to bone health, later studies have performed a more comprehensive analysis of bone health and offered a more nuanced conclusion (Huang and Ables, 2016). Many past studies on CR, when initiated in early life (3–14 weeks), established the detrimental effects on bone growth in rats and mice as measured by size, density, mineralization, and biochemical markers of bone (Devlin et al., 2010; Ferguson et al., 1999; LaMothe et al., 2003; Nnakwe, 1998; Talbott et al., 1998; Talbott and Shapses, 1998). Even when initiated at mature and older ages (6–23 mo), CR has been reported to produce detrimental effects to bone architecture and strength (Baek et al., 2008; Banu et al., 2001; Colman et al., 2007).

Most of these studies have typically examined whole body bone mineral content (BMC) and bone mineral density (BMD) by dual-energy X-ray absorptiometry (DXA) or done finer

analyses on only one skeletal site, typically the femur or tibia. Upon examination at multiple sites, however, the effects of CR do not appear universal and are dose dependent. For example, Berrigan et al. (2005)(Berrigan et al., 2005) examined female B6 mice introduced to 20, 30, 40% CR for 26 days beginning at 5 wk of age. These investigators found a reduction in whole body BMC and BMD only in mice on 30 and 40% CR. Similar findings were observed by DXA scans of the tibia but not the vertebral bone (Berrigan et al., 2005) (Berrigan et al., 2005). Other dietary conditions can alter the effects of CR on parameters of bone health. For example, Hawkins et al. introduced female SD rats to 60% CR at 6 mo of age and noted lower BMD in femoral and travecular bone in rats on a normal diet. In the same study, CR had no significant effects on BMD if the rats had been on an obesogenic diet prior to CR (Hawkins et al., 2010). In contrast, when Shen et al. fed female rats a high-fat diet for 4 mo before imposing a 35% CR for 4 mo, these investigators noted decreased BMD and strength in trabecular bone (Hawkins et al., 2010; Shen et al., 2013).

Exercise is another condition that can possibly interact with CR. As an example, Bodnar et al. examined bone quality in male SD rats exercised in running wheels from 5 mo of age compared to a CR group fed to maintain the body weight of exercised rats (Bodnar et al., 2012). At 23 mo of age, the CR group had cortical thinning of femoral and tibial shafts compared to non-exercised controls. Hattori et al. investigated exercise initiated at 4 wk in male SD rats with and without 30% CR for 13 wks. They noted a reduction in femoral bone strength in rats in the exercised group on CR compared to control rats (Hattori et al., 2014).

Other studies have noted beneficial effects of CR on bone health. In an early study, Kalu et al. examined the effects of lifelong 40% CR on several bone parameters in male F344 rats (Kalu et al., 1984). They found that maturation of the femur was delayed and this bone was lighter, less dense, and had less calcium; however, when they measured femur strength through biomechanical manipulations, they found improved bone strength from CR rats, when normalized to body weight. Additionally, there was no senile bone loss at 24 mo of age in CR rats.

This finding of improved bone strength has been replicated in later studies also applying normalization for body mass. For example, Lambert et al. imposed 35% CR in male Wistar rats at 2 mo of age and recorded increased strength in tibia and vertebrate measured by multiple mechanical test adjusted for body mass (Lambert et al., 2005). Similarly, Westerbeek et al. evaluated bone health in male F344xBNF1 rats begun on 40% CR beginning at 14 week of age. Using microcomputed tomography, they reported improved structural properties of bone at 8, 30–35 mo, and 35–40 mo of age when normalized to body mass (Westerbeek et al., 2008). Thus, these latter studies suggest that the difference in body weight induced by CR must be taken into account when considering the structural properties of bone in deciding whether the dietary regimen is detrimental to bone health. While certain properties of the bone are diminished in CR rats compared to controls, the underlying structure and the biomechanical properties might be improved.

Other aspects of skeletal health of rodents on CR have also been examined. As an example, Athanasiou et al. investigated the biomechanical properties of articular cartilage obtained from F344 rats at 6, 12, 18, and 24 mo (Athanasiou et al., 2000). Comparing control groups

to groups on lifelong 40% CR, these investigators found no significant differences in the biomechanical properties of mouse cartilage. McNeill et al. assessed the symptoms of osteoarthritis in 24-mo old male B6 mice introduced to 40% CR at 14 weeks of age (McNeill et al., 2014). At 24 mo of age, they found no significant differences compared to the control group; however, it is also important to consider that they found no significant decline in structural properties of the joints as compared to controls.

In summary, while it is clear that even short-term 30–40% CR can reduce indices of BMC and BMD in rodents of all ages, these findings do not necessarily mean that bone health has been compromised by this nutritional intervention. When taking into account differences in body mass, it appears that bone strength can actually be enhanced in CR animals and that senile bone loss can be attenuated.

Wound Healing—Another controversy in the literature regarding possible detrimental effects of CR is whether there is impairment in wound healing. Early studies reported that mice (Harrison and Archer, 1987) and rats (Reiser et al., 1995) on CR had slower rate of wound healing measured by *in vivo* assays. These findings appear consistent with *in vitro* studies demonstrating reduced cell proliferation in general in samples obtained from CR rodents. For example, Hsieh et al. reported that a variety of cells obtained from mice maintained on continuous or IF 33% CR for a few weeks showed reduced proliferation *in vitro* (Hsieh et al., 2005).

Studies focusing on collagen production as a main driver of wound healing have confirmed that even a few days of CR reduces collagen production (Spanheimer et al., 1991). Protein restriction greatly affects the rate of wound healing, as showed by the study of Otranto et al., who noted that 12 weeks of moderate (23%) to severe protein (100%) restriction initiated at 12 weeks in male and female rats disturbed wound healing in a dose-dependent manner with deposition of extracellular matrix and neovascularization greatly affected (Otranto et al., 2009).

In contrast to these reports, a few other studies have reported little to no adverse effects of CR on wound healing. For example, Emery et al. placed rats on 62% CR for 7 days prior to an abdominal incision made to simulate surgery (Emery and Sanderson, 1995). They observed no significant effects of CR on the rate of muscle protein synthesis at the site of the wound either 2 or 7 days after surgery, and the tensile strength and the collagen content of the wound were also unaffected by food restriction. Imposing a fasting regimen of 4 days every 2 weeks, Havati et al. observed no detrimental outcome to wound healing in young mice (Hayati et al., 2011). Investigating cutaneous wound healing in male Wistar rats at various ages, Roth et al. found no significant effects of a lifelong 30% CR regimen when measured at 3, 7, 12, or 18 mo of age (Roth et al., 1997).

A highly valuable discovery within this limited literature on wound healing in rodents was that refeeding of CR animals is greatly beneficial to recovery in situations where negative effects of the regimen are noted. In an innovative study, Reed et al. (1998) examined cutaneous wound healing at different ages (4–6, 15–17, and 30–33 mo) in two hybrid mouse strains (B6D2F1 and B6C3F1) fed AL (Reed et al., 1996). In addition, another group of the

aged mice (30–33 mo) had also undergone long-term CR (40%) compared to AL feeding. Moreover, a subgroup of these aged mice that had been on life-long CR were refed to AL levels for 4 weeks prior to wounding. Negative effects on healing were observed in mice on CR at all ages compared to AL controls; however, in the old CR group that had been refed, the rate of wound healing was similar to that of younger AL-fed mice. Hunt et al. replicated these findings in young rats (Hunt et al., 2012). Specifically, male F344 rats were placed on 40% CR at weaning and maintained until 7 mo of age when a subgroup of CR rats was then placed on AL feeding 48 hours before cutaneous wounding was imposed and maintained on AL throughout healing. Rats maintained on CR had significantly slower healing compared to AL controls; however, the refed rats were equivalent in healing to the controls. Thus, it would appear that detrimental wound healing observed in CR rats can be easily remedied by short periods of refeeding. In the report previously cited by Hsieh et al., whereby a variety of cell populations taken from CR mice exhibited impaired proliferation *in vitro*, a short period of refeeding quickly reestablished their proliferative capacity (Hsieh et al., 2005).

One last major point that must be emphasized regarding this topic is that reports of wound healing deficiencies typically involve CR imposed in young animals and generally run for a short period of time. In their review of the literature, Wolf and Pendergrass (1999) noted that short-term CR does retard cell proliferation when measured *in vitro*, but cell replication in samples taken from older CR-fed animals does not seem to be impaired (Wolf and Pendergrass, 1999). Moreover, in a careful histological examination of skin quality in 24-mo old F344 rats, Bhattacharyya et al. (2005) noted an attenuation of many age-related changes, e.g. depth of epidermis, dermis and fat layers, by CR (Bhattacharyya et al., 2005).

Infection—Another robust hallmark of CR in rodents is the increased protection afforded against a wide range of chronic diseases (Kristan, 2008). In contrast, there are numerous reports describing reduced susceptibility of CR animals to various infectious agents, which are reviewed in this section (Kristan, 2008). Effros et al. is an oft-cited paper that first reported the increased protection of aged mice to infection (Effros et al., 1991). Specifically, (24–26 mo) C3B10RF1 mice maintained on a 60% CR regimen since weaning had a greater response to an injection of influenza virus, as evidenced by increased antigen presentation and T-cell proliferation compared to AL fed controls. Thus, this finding supported the robust effects of CR on immune responses.

In a later of series for papers, Gardner and colleagues (Gardner, 2005; Gardner et al., 2011) noted that CR impaired responses to the influenza virus. CR mice suffered a much higher risk of mortality. The major difference between the Effros and later studies was the route of administration of the virus. Effros et al. imposed ip injections of influenza virus, while the Gardner studies used an intranasal route of administration which was more consistent with the typical route of infection (Gardner et al., 2011). Gardner et al. noted decreased survival of aged (22 mo) B6 mice on lifelong 40% CR following infection (Gardner, 2005), and Ritz et al. (2008) noted the same even in young (6 mo) B6 mice on CR(Ritz et al., 2008). Both studies observed decreased natural killer (NK) cell activity in lungs of CR mice. From the same series of studies, Clinthorne et al. (2013) confirmed reduced NK cell maturation in young (6 mo) B6 mice on 40% CR(Clinthorne et al., 2013), and Duriancik and Gardner (2016) observed reduced dentritic cells in mice treated similarly(Duriancik and Gardner,

2016). Goldberg et al. (2015) confirmed and extended these findings when they observed increased mortality among 18-mo old B6 mice on 40% CR since 14 weeks of age before being infected with West Nile virus (sc injection)(Goldberg et al., 2015). Appropriate T-cell response to the viral infection in CR had been compromised. These investigations have concluded that an appropriate immune response to this particular viral infection presents too much of a metabolic demand in CR mice, as they lose additional weight following treatments. As evidence, Clinthorne et al. (2010) noted that two weeks of AL feeding to 6 mo old B6 mice that had been on 40% CR since a young age significantly improved survival and NK cell production and function following influenza viral infection(Clinthorne et al., 2010).

Impaired response of CR mice to other types of infection has also been reported. For example, Shi et al. (1998) examined the effects of a nematode worm infection *(Heligmosomoides polygyrus)* in female BALB/c mice and noted increased number of worms in CR (~30%) mice accompanied by reduced T-cell function(Shi et al., 1998). Kristan (2007) also investigated effects of a nematode infection *(Heligmosomoides bakeri)* in mice on 6 mo CR (40%) and infected for one month(Kristan, 2007). While CR mice mounted an equivalent immune response (eosinophils and immunoglobin G1) and had similar body weight changes compared to AL controls, they were infected with more worms and more eggs compared to AL controls.

Cecal ligation and puncture (CLP) has been a model used to investigate response to CR mice to bacterial infections and resulting sepsis. Sun et al. (2001) was the first study to report that CR mice (6-mo old) subjected to CLP exhibited greater inflammation and mortality as evidenced by many immune parameters, e.g. higher serum levels of inflammatory cytokines, TNFa and IL-6(Sun et al., 2001). Similarly Kang et al. (2002) reported reduced survival following CLP in mice that were on a severe CR regimen (75%) 7 days prior to the infection(Kang et al., 2002). Direct infection with bacterial strains, such as *Helicobacter hepaticus*, to induce colitis have also been reported to increase mortality in SMAD3−/−mice on 30% CR for 20 wk compared to control mice (McCaskey et al., 2012). Ikeda et al. (2001) used ip glycogen injections to induce peritonitis in male B6 mice fed AL or 50 and 75% CR for 7 days prior to infection(Ikeda et al., 2001). They observed decreased CD11b and CD18 expression in peripheral mononuclear cells in the CR mice; however, only one day of refeeding could restore immune responses to control levels.

It should be noted that not all studies have reported negative effects of CR in response to infection. Outcomes appear to depend on the CR regimen as well as the infectious agent. For example, Hasegawa et al. (2012) subjected male B6 mice to 8 days of EOD before CLG cecal ligation(Hasegawa et al., 2012). They noted decreased mortality in CR mice, less organ injury, and reduced inflammatory response. As another example, Peck et al. (1992) induced a Salmonella infection via ip injection in female mice A/J subjected to 50% CR for 3 weeks prior to injection and observed reduced mortality in the CR group compared to controls(Peck et al., 1992). In contrast, protein restriction increased mortality compared to controls. Hunt et al. (1993) inoculated CBA/T6 mice with cerebral malaria (plasmodium berghei) after imposing CR sufficient to produce 10% body weight loss across 7 days (Hunt et al., 2012). Mice on CR had reduced mortality compared to controls. Ahmed et al. (1991)

initiated CR (30 and 50%) in weanling mice for 11 weeks followed by rotavirus infection (Ahmed et al., 1991). While they noted reduced microvilli in the 50% CR group compared to controls, there were no detectable histological differences between CR and control groups in villi following rotavirus infection. As a general model of inflammation, MacDonald et al. (2011) injected young mice with lipopolysaccharide (LPS) and reported that a 50% CR regimen for 4 weeks attenuated proinflammatory gene expression (COX-2, leptin) in the hypothalamus and increased anti-inflammatory gene expression (SOCS-3, IL-10) (MacDonald et al., 2011).

In summary, several studies have noted increased susceptibility to infections of several types in mice on CR regimens. It appears that this susceptibility may be due to great metabolic demands required to mount a proper immune response to infection. As observed in the section on wound healing, refeeding only for a few days restores the ability to mount such a response. In addition, there are several studies indicating no negative effects or even beneficial effects of CR on response to several types of infection. Finally, an interesting aspect of this literature is the dearth of studies using rat models. Thus, the generalization of the findings of impaired response to infection in CR rodents is limited to mouse models.

How Important is the Schedule of Feeding?

Over the decades there has been considerable debate about whether the actual reduction in calories was necessary for producing the robust anti-aging effects of CR or whether the paradigm induced changes in the schedule of feeding that also produced beneficial effects not directly related to the calories consumed (Masoro, 2005). This debate has generated many new rodent studies manipulating meal frequency (Mattson, 2014). In general, the following two types of paradigms can be considered: (1) intermittent energy restriction; (2) time-restricted feeding.

Intermittent Energy Restriction (IER)

In general, the IER paradigm refers to reductions or eliminations in energy intake periodically. Some examples include every-other-day (EOD) fasting, fasting 2–3 days a week, or restricting energy to a fraction (1/4) of the AL level a few days a week. Interest in evaluating such paradigms in rodent models has increased because of further consideration of translation to humans. Specifically, the consideration is that application of CR to humans might be more tolerable if imposed only a few days a week rather than every day.

Many studies have been conducted utilizing the EOD regimen of IF with 2–3 days of fasting. The results are impressive, indicating beneficial effects on a wide range of function and conditions(Anson et al., 2005). For example, systematic studies from the National Institute on Aging have focused on effects on cardiovascular function. Wan et al. (2003) were the first to demonstrate that EOD feeding initiated in young rats (SD) reduced blood pressure and heart rate after only 6 months (Wan et al., 2003). Mager et al. (2006) showed that EOD feeding in SD rats produced better autonomic control of cardiac function after only 1 month (Mager et al., 2006). Follow-up studies revealed the marked protection of EOD feeding on protection against cardiac ischemia in young rats compared to controls (Ahmet et al., 2005; Wan et al., 2010). Other studies from this group (Ahmet et al., 2010), however, did note

some abnormalities in cardiac function following an EOD regimen. Specifically, after 6 months on the regimen SD rats exhibited diminished cardiac reserve.

Tikoo et al. (2007) reported that EOD feeding for 8 weeks in SD rats protected against nephropathy and hypertension following induction of diabetes via treatments with streptozotocin (STZ)(Tikoo et al., 2007). Gehrig et al. (1988) noted that 4 mo EOD feeding in SD rats protected against pathology and inflammation following renal ischemia at 86 week of age (Gehrig et al., 1988).

Given the previous discussion of possible detrimental effects of CR on protection against infection, one study has reported positive results for IF. Specifically, Campos-Rodriguez et al. (2016) observed that IF imposed for 40 weeks in BALB/c mice offered greater protection to Salmonella infection compared to controls (Campos-Rodriguez et al., 2016).

Although both mutations increase longevity, glucose control is impaired in Ames dwarf mice and growth hormone (GH) deficient mice. Arum et al. (2014) noted that EOD feeding induced in older mice of these genotypes improved their glucose control.

Many studies have reported beneficial effects of IF on brain and behavioral function. For example, Li et al. (2013) studied effects of EOD feeding beginning at 7-wk old in CD-1 mice and reported better performance at 11 mo in maze learning and fear conditioning as well as lower oxidative stress markers in brain compared to AL controls (Li et al., 2013). Halagappa et al. (2007) studied effects of EOD feeding initiated at 3 mo of age in 3xTgAD triple transgenic mice and tested at 17 mo (Halagappa et al., 2007). They observed the regimen attenuated amyloid pathology and decline in cognitive abilities compared to controls. Tajes et al. (2010) imposed IF in young SAMP8 mice for 8 wks and observed increased SIRT1 expression and BDNF compared to controls(Tajes et al., 2010). Even IF interventions begun at middle age (Singh et al., 2015) or late age (Singh et al., 2012) in Wistar rats can improve cognitive performance and motor coordination compared to controls.

Despite the interest in IF paradigms, relatively few studies have conducted direct comparisons between IF and CR imposed every day. Examining metabolic differences in mice begun at 8–12 weeks of age on either 30% CR or EOD feeding, Westbrook et al. (2014) observed at 29–33 mo that mice on EOD feeding had a significant reduction in oxygen consumption (VO2) in both fed and fasted states, while CR increased both the range of VO2 and the difference in minimum VO2 pattern under fed and fasted states (Westbrook et al., 2014). Examining diet responses in p53 deficient mice started at 10 mo of age, Berrigan et al. (2002) reported that 40% CR was more effective that IF (1 day fast a week) in preventing cancer (Berrigan et al., 2002). Using 16-wk old B6 mice, Anson et al. (2003) conducted a direct comparison in between EOD feeding and 40% CR implemented for 20 weeks (Anson et al., 2003). While effects on glucose/insulin responses were similar between the regimens, there were stark differences in terms of other responses. Specifically, mice on the EOD regimen had increased levels of serum IGF-1 and β-hydroxybutyrate, while CR mice had reduced levels. In addition, IF provided greater neuroprotection in hippocampal neurons following infusion of the excitotoxin, kanic acid, compared to CR mice. The most

relevant observation to this comparison was that the EOD mice were consuming about the same level of calories as control mice eating AL.

Other laboratories have begun to explore novel regimens of IER. For example, Brandhorst et al. (2015) have proposed a “fasting mimicking diet” (FMD) in which B6 female mice are fed a low protein, low sugar and high fat diet providing 10–50% of normal caloric input for 4 days twice a month (Brandhorst et al., 2015). When 16-wk old mice were placed on FMD, they experienced body weight and fat loss while consuming the same level of caloric intake as control mice over time. Cancer incidence was significantly reduced and onset delayed, bone loss was retarded, and survival was improved. Additionally, several measures of healthspan, including motor (rotarod) and cognitive (spontaneous alternation, novel object recognition, and Barnes maze) performance was improved in the mice on FMD. Thus, there is emerging evidence that fasting regimens can be shorter in duration than EOD feeding, which has been studied extensively, and still offer beneficial effects on many parameters of aging.

Time-Restricted Feeding (TRF)

In general, this type of diet regimen refers to limiting daily caloric intake to a window, e.g. 4–6 hours. This paradigm has received considerable attention within the obesity field, with less investigation within the biogerontology literature. Nelson and Halberg (1986) first reported that a 25% CR regimen started at 6 weeks of age in female CD2F1 mice was equally effective in increasing lifespan compared to AL feeding whether food was delivered as a single meal early during the dark period, early during the light period, or as 6 smaller meals distributed across the dark period (Nelson and Halberg, 1986). Similarly, Masoro et al. (1995) found that the increased survival of F344 rats undergoing 40% CR was unaffected by whether meals were provided at one time during the dark period or in two meals, one during the light and the other during the dark (Masoro et al., 1995).

In contrast to these findings in the aging literature, there are numerous studies in the obesity literature that could be cited in support of the health benefits of TRF. Most of these involve limited food access to a few hours in young mice on high fat diets (HFD). For example, Duncan et al. (2016) investigated B6 mice on a HFD (60% calories from fat) versus low fat (10% of calories from fat) and HFD with feeding restricted to 8 hours during the dark period (Duncan et al., 2016). This TRF regimen reduced body and liver weight gains (but not fat) and improved glucose tolerance. Similarly Hatori et al. (2012) also limited feeding of a HFD (61%) to 8 hr during the dark period in B6 mice and noted reduced evidence of obesity, hyperinsulinemia, hepatic steatosis, and improved motor coordination with no differences in caloric intake (Hattori et al., 2014). The period of restricted feeding appears to be a critical element in the benefits of these regimens. For example, Sundaram and Yan (2016) found that 8 hr of restricted feeding to be more effective than 12 hours of feeding on a number of obesity-related parameters in mice on a HFD (Sundaram and Yan, 2016). Haraguchi et al. examined a variety of TRF regimens in mice on a HFD. When feeding was restricted to 4 hours during either the light or dark periods, body weight and fat were reduced even though caloric intake was not affected (Haraguchi et al., 2014). These beneficial effects were reduced when feeding was restricted to 8 hours and even 2 hours.

As mentioned above, these promising findings related to beneficial effects of TRF in mice on high fat diets have not been fully investigated within aging studies. The early negative findings from studies by Nelson and Halberg (Nelson and Halberg, 1986) and Masoro (Masoro et al., 1995) likely have dissuaded follow-up; however, these positive findings from obesity literature will undoubtedly spur many new investigations. Additional studies, insights, evolutionary origins, and rationale for this renewed interest in TRF can be further explored in two excellent reviews recently published (Longo and Panda, 2016; Mattson, 2014).

How Long Does CR need to be Imposed to be Effective?

Most research on CR has assumed that long-term treatment (> few months) is required to invoke mechanisms that produce protection against physiological insults, diseases, and age-related conditions. In their excellent review of the literature on the benefits of short-term dietary restriction, Robertson and Mitchell (Robertson and Mitchell, 2013) define such interventions as ranging from one day to several months. We have already cited several studies in the preceding sections that show beneficial health responses to various CR regimens that could be fall under this definition. For example, Park et al. reported that 30% CR for 4–6 weeks improved glucose tolerance and insulin sensitivity (Park et al., 2006); better cognitive performance was reported in mice after 12 weeks (Sarker et al., 2015) and 16 weeks of CR (Dhurandhar et al., 2013); improved inflammatory responses in the brain was found following 4 weeks of CR in mice (MacDonald et al., 2011); and increased protection against streptozotocin-induced diabetic complications was observed in rats on EOD feeding for 8 weeks (Tikoo et al., 2007).

The literature on benefits of short-term restriction first emerged in studies of protection against ischemia and other models of physiological insult. For example, 2–4 weeks of 30% CR in mice could protect against ischemia reperfusion injury to the kidney and liver (Mitchell et al., 2010). Three months of EOD feeding provided neuroprotection against cerebral ischemia in rats (Yu and Mattson, 1999) and MPTP toxicity in mice (Duan and Mattson, 1999). Even 48 hours of fasting prior to focal brain ischemia can attenuate neuronal injury (Marie et al., 1990). Examining effects in the stroke-prone hypertensive rats model, Chiba and Ezaki (2010) reported increased protection against ischemic stroke in rats provided 50% CR for 2 weeks prior to insult (Chiba and Ezaki, 2010).

Longo and colleagues have discovered the beneficial effects of short-term CR and fasting to enhance benefits of chemotherapy in in vitro and in vivo mouse models (Brandhorst and Longo, 2016; Lee et al., 2012; Lee et al., 2010; Raffaghello et al., 2008). As an example, when mice were submitted to a 48-hour fast resulting in a 20% weight loss, the chemotherapeutic effects of cyclophosphamide and etoposide were enhanced (Raffaghello et al., 2008). Lee et al. noted that two fasting cycles of 48 hours each proved equally effective as chemotherapy in reducing several types of tumors (Lee et al., 2012). Fasting also proved effective in enhancing the anti-tumor effects of radiation in mice (Safdie et al., 2012).

In summary, the long-held conviction that the health benefits of CR required long-term exposure has turned to a more balanced view that short-term exposure of a few weeks or

even a few days can also be beneficial. This research area is rapidly expanding given its translation significance. However, whether these short-term interventions should be also cover under the term CR is questionable, as CR has traditionally been linked to long term interventions.

Genotype and Gender Influences on CR

Most of the data published on the effect of CR on lifespan and mortality in mice suggests that there is a close-to-linear relationship between the percentage of restriction and the percentage of increase in lifespan achieved (Merry, 2002, 2005; Speakman and Hambly, 2007; Weindruch, 1996). In recent years, this well-established tenet of CR has been called into question, particularly, the key concept of the universality of lifespan extension with CR across species or even strains of the same species (for a recent review (Sohal and Forster, 2014b)). Recently, a meta-analysis using data available from studies conducted between 1934 and 2012 illustrated that the lifespan-extending effects of CR in mice ranged from a modest 4 to 27%, and those results were particularly weak in the recombinant inbred strains of mice (Swindell, 2012). It is important to acknowledge that most CR studies in the meta-analysis were done under 40% CR condition. We know now that this level of CR may be excessive for some strains of mice. The interaction between the degree of CR and different aspects of physiology has been recently examined (Derous et al., 2016a; Derous et al., 2016b; Lusseau et al., 2015; Mitchell et al., 2015a; Mitchell et al., 2016a; Mitchell et al., 2015b; Mitchell et al., 2015c) and survival (Mitchell et al., 2016b).

Some of the strongest evidence supporting the disparity of responses to CR in terms of survival were the two experiments that performed lifespan analysis in the ILSXISS recombinant inbred strains of mice (Liao et al., 2010; Rikke et al., 2010). The studies were run in two independent laboratories with similar overall conclusion: The effect of CR on survival is strain- and sex-dependent. There are some other important observations that we would like to highlight about these studies. First, none of the inbred strains had a strong (30 to 40%) increase in mean or maximum lifespan and in the majority of the strains tested, CR had either no effect or caused a shortening of lifespan (Liao et al., 2010; Rikke et al., 2010), although no cause of death or pathology was reported. In fact, only 5% of the ILSXISS males and 21% of the females showed a beneficial response to CR. Second, site differences in the response to CR were observed; for example, females from mouse strains 117 and 115 had opposite results depending on the particular site where the experiment was carried out, which could point to an environmental factor. Third, and most importantly, the authors reported an inverse correlation between changes in adiposity and lifespan (Liao et al., 2011). This latter observation could have profound implications in our understanding of the role of adiposity during CR; however, it also could just mean that the 40% CR regimen was just too stringent for the non-responsive strains. Surprisingly, since the publication of these manuscripts, no one has tried to replicate or use the potential advantage of having well characterized recombinant inbred strains to dig deeper in the origins of this differential response to CR and the interaction with sex and genetic makeup.

Further evidence of the disconnect between the universality of lifespan extension with CR comes from the inbred DBA/2J, a strain of mice that is generally classified as ‘short-lived’

and unresponsive to 30–50% CR even though large differences of mean lifespan have been reported. Indeed, results from different studies and laboratories found a slight reduction to a greater than 60 % increase in mean lifespan in response to CR (Bronson and Lipman, 1991; Fernandes et al., 1976; Forster et al., 2003b, a; Lipman, 2002; Turturro et al., 1999). A lower level of CR rather than the standard 30–50% CR may perhaps benefit DBA/2J mice. To address this question systematically, Mitchell et al. recently performed a longevity study using both sexes of DBA/2J mice and two levels of CR, 20 and 40%, and found that when maintained on 40% CR, DBA females exhibited a 37.3% increase in maximum lifespan and males a 29.8% increase (Mitchell et al., 2016b). Interestingly, DBA males on 40% CR had an 18.8% reduction in the first portion (Q1) of their lifespan, which was consistent with the earlier report of Forster (Forster et al., 2003a). This negative effect in the Q1 in DBA males was mitigated when using a 20% CR regimen. In contrast, the response of DBA females to either 20 or 40 % CR was almost identical and positive. In the same study, B6 mice on 20% CR had a significant improvement on survival, with a mean lifespan extension of 40.6% in females and 24.4% in males. However, 40% CR had a detrimental effect on the survival of B6 females, with a small 13.3% increase in maximum lifespan. These results support the concept that there must be a strong interaction between genotype, gender, and grade of CR. Although B6 is considered a CR-responsive strain, the differential response to 40% CR has been previously reported in these mice (Harrison and Archer, 1987; Harrison et al., 1984).

The controversy about the CR responses of different strains of mice challenges the dogma that CR always leads to improvements in health and survival. One possible explanation for the different outcomes in the data is that all strains do respond positively to CR in terms of disease burden, but that the ideal level of restriction may be strain-specific when assessing lifespan extension. Efforts are now invested to further investigated this interplay between mouse strains, the level of restriction, and their impact on survival. The DBA results of Mitchell et al. (2016) and those from John Speakman’s group in which the effects of different grades of CR in B6 mice elicit differential responses (Derous et al., 2016a; Derous et al., 2016b; Lusseau et al., 2015; Mitchell et al., 2015a; Mitchell et al., 2016a; Mitchell et al., 2015b; Mitchell et al., 2015c) open the door for new studies aimed at identify the underlying biological responses. Thus, one could predict that some of the recombinant inbred strains will have a beneficial response to CR if they were tested at a lower level of dietary restriction. This approach could set the stage for improving our understanding of the complex interaction between degrees of CR and health and survival outcomes.

These complexities have been also observed in the responses to CR in rhesus monkeys, were two independent non-human primate studies have reported similar improvements in health but different survival outcomes in response to 30% CR (Colman et al., 2009; Mattison et al., 2012a). Recently, the two sites responsible for the non-human primate studies published a paper aimed at clarifying the similarities and caveats reported between the two studies (Mattison et al., 2017).

Interestingly, and perhaps not surprisingly, emerging results from interventions aimed to mimic CR by means of genetic or diet manipulation/supplementation also show clear sex differences. In most cases, there has been high variability in the individual sex response (Austad and Bartke, 2015; Austad and Fischer, 2016). Perhaps, these sex differences could

be easily explained because of the intrinsic differences in metabolism and clearance of some of these compounds; however, when we look at genetic manipulations, it becomes harder to understand these dimorphic responses. Clearly, there is much to learn by deepening our understanding of these differences.

The emerging variability in the responses of the different strains and sexes to CR challenge the current dogma that the response to CR is universal and beneficial. Clearly, since most studies are done using a single strain and sex, we are limiting our ability to fully understand if each of the strains and sexes do respond to their own optimal restriction. This could be illustrated by the results in Mitchell et al in which each strain and sex responded differently to two different levels of restriction (Mitchell et al., 2016b). Perhaps, taking advantage of this differential response we could gain better insights on the underlying mechanisms of CR, identify specific biomarkers that could predict the responsiveness, or lack thereof, of each individual strain and sex. Without a doubt, if we could understand where the optimal level of restriction lies for an individual, it will substantially improve our understanding of CR. In the future, the field needs to center efforts on trying to understand the complex interactions that are emerging from these studies. Particularly, puzzling are the emerging separations between health and survival benefits in the response to CR, both present in the mice (Mitchell et al., 2016b) and non-human primate literature (Mattison et al., 2017; Mattison et al., 2012b)

What Role Does Dietary Composition Play?

Restricting the caloric intake of a rodent is proven more complex than expected, and the consistent implementation of a CR diet has become an important caveat in rodent studies (Anson et al., 2005; Cerqueira and Kowaltowski, 2010; Speakman and Mitchell, 2011; Speakman et al., 2016). The complexities and logistics of such feeding protocols are not trivial and have resulted in a variety of diets and feeding regimens (Anson et al., 2005; Masoro, 2005, 2006, 2009; Speakman and Mitchell, 2011). In this section, we will cover diet composition with a focus on macro nutrients, particularly proteins. We have already covered in the previous sections some of the differential physiological responses that distinguish CR from protein restriction (PR) and other aspects of diet composition. The impact of various feeding regimens were recently extensively reviewed (Le Couteur et al., 2016a; Le Couteur et al., 2016b; Masoro, 2005, 2006; Solon-Biet et al., 2015; Speakman and Mitchell, 2011; Speakman et al., 2016). The role of optimal dietary composition for promoting lifespan extension in ad libitum or CR condition remains unknown. A number of diets, with a range of ingredients, have been used for CR and aging studies (Pugh et al., 1999a), but so far only a handful of studies have attempted to compare diets to ascertain if dietary composition influences the response to CR. For example, there is little know about the specific role of fat on longevity in the context of CR. Only a handful of longevity studies have been completed where the diets were manipulated only in their lipid content and composition. To date most of the studies published were performed in short-lived rodent models and have produced conflicting results. For example, in the context of short-live strains, it has been reported that lifespan is either increased in mice fed a diet containing fish oil versus corn oil (Halade et al., 2010; Jolly et al., 2001), or is decreased (Berdanier et al., 1992; Tsuduki et al., 2011) and

in a recent longevity study in B6C3F1 male mice fed they observe also a shortening of lifespan relative to their controls (Spindler et al., 2014).

Another issue comes from the standard method used to perform a CR study. Typically, is done providing food *ad libitum* to a group of control animals and to restrict a given amount of food for the CR group. In such protocol, if there is no supplementation of the diet with the necessary micro- and macro-nutrients, the CR animals consume less total calories based on the level of restriction, along with a reduction in all the levels of nutrients, which in some case could lead to malnutrition. Studies that involved a specific reduction of protein in the diet have found that rodents displayed responses that were similar to those of CR-fed animals (Cerqueira and Kowaltowski, 2010). However, most of the early reports in this field have suggested that manipulations of the macro and micro-nutrient composition do not have a major impact on survival (Iwasaki et al., 1988; Yu et al., 1985; Yu et al., 1982). To counter this earlier argument, a few recent studies have provided highly systematic manipulation of protein content in mouse studies (Le Couteur et al., 2016a; Le Couteur et al., 2016b; Simpson et al., 2015). For example, a 40% PR diet, while maintaining a very similar calorie intake, recapitulated the CR effects on mitochondrial ROS production and oxidative damage in rats (Ayala et al., 2007; Sanz et al., 2004). Such effects were not replicated when decreasing other macronutrients (Sanz et al., 2006a; Sanz et al., 2006b; Sanz et al., 2006c), pointing perhaps to a particular role for protein or amino acid restriction. Furthermore these authors also show a beneficial effect of PR on survival without changing the caloric intake, but this effect on survival was distinct to that of the 40% CR group (Ayala et al., 2007; Sanz et al., 2004). Severe restriction of dietary protein or specific amino acids also can extend the lifespan of rodents, independently of caloric intake (Pamplona and Barja, 2006).

Recently, the concept that PR is responsible for the effects of CR has re-emerged through the adoption of an approach called the ‘Geometric framework’ (Raubenheimer and Simpson, 1997; Raubenheimer et al., 2012; Simpson and Raubenheimer, 2007). Originally developed in insects, this approach can dissect out the contrasting impacts of nutrition on multiple output variables, including lifespan (Piper et al., 2011; Raubenheimer et al., 2012). This framework was successfully implemented in the re-analysis of invertebrate survival data and clearly supported the notion that the effects of CR involved more than calorie manipulation, but instead involved a complex interaction between the macronutrients that lead to optimization of different aspects of invertebrate biology. In a true translation effort, Solon-Biet et al. tested whether this geometric framework approach could be implemented in rodents and would the predictions made in insects be true in rodents (Solon-Biet et al., 2014). These authors designed an incredibly complex experiment in which protein:carbohydrate:fat ratios were modified, resulting in a design that included 25 different types of diet and following all mice until death (Solon-Biet et al., 2014). The data convincingly showed that the key factor driving the survival effect was the protein to carbohydrate ratio, not the calories. The same group recently reported that short-term exposure to three of the previously reported diets under AL and CR conditions led to a metabolic impact of CR on glucose homeostasis that was very similar to the AL group fed a low protein diet, and that CR plus the low protein diet did not add further benefit than CR alone (Solon-Biet et al., 2015).

Protein is made up of individual amino acids. When the reports began to emerge on the effects of PR, the question arose as whether it was protein itself or a particular amino acid or group of amino acids. Research on this topic led to the discovery that restriction of methionine does replicate some of the biological effects of CR, including an increase in lifespan in rats (Orentreich et al., 1993; Richie et al., 1994) and mice (Malloy et al., 2006; Miller et al., 2005; Sun et al., 2009). Another effective amino acid in extending lifespan in rats is Tryptophan. Low-tryptophan diets, is an easy and relatively simple form of dietary restriction that leads not only to increases in maximum lifespan (Ooka et al., 1988; Segall and Timiras, 1976; Segall et al., 1983), but also postpones the onset of tumors (De Marte and Enesco, 1986), retards the aging-related loss of temperature homeostasis (Segall and Timiras, 1975) and slow the senescent deterioration of the coat in female rats (Segall and Timiras, 1975). However, because animals fed low levels of tryptophan in the diet promptly enter a “self-imposed” restriction of food intake (Segall and Timiras, 1975), some of the observations reported to date may be due, at least in part, to just this voluntary CR effect. More recently a couple of groups have reported beneficial effects of either restriction or supplementation of diets with branch chain amino acids (BCAAs). D’Antona et al showed that a BCAA-enriched mixture increased the average life span of mice as well as some markers of mitochondrial health markers in cardiac and skeletal muscle, reporting as well an enhancement in physical endurance (D’Antona et al., 2010). Recently, Fontana et al reported that feeding mice a diet specifically reduced in BCAAs improved glucose tolerance and body composition similar to a standard PR diet (Fontana et al., 2016).

This argument about how each of the macro nutrients plays into the effects of CR became a matter of scientific debate soon after McCay published his seminal paper in 1935 (McCay et al., 1935), and has undergone several cycles of popularity over the years, although the persistent and standing affirmation in the field is that, in rodents, the effects of CR and PR, while overlapping in some aspects, they are mechanistically different. Speakman et al, acknowledges that there is an independent impact of PR on lifespan, but it works over a very different range of restriction than that of CR (50 to 85%: relative to a reference diet versus CR which is 10–65% relative to AL), and has a smaller impact on survival (Speakman et al., 2016). More recently, Speakman et al wrote an extensive report covering both, the history and controversies in PR versus CR and performed an elegant and thorough analysis of the published data since the early 30’s (Speakman et al., 2016). The authors concluded that the restriction effect on lifespan in rodents is due to a reduction in total calories and not protein or any other macro nutrient, at least in rodent studies, in agreement with others (Pamplona and Barja, 2006).

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