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Effects of adult onset mild calorie restriction on weight of reproductive organs, plasma parameters and gene expression in male mice

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Abstract

Calorie restriction (CR) extends lifespan and delays onset of age-related diseases in various organisms, even when started later in life. Despite benefits for health and lifespan, CR's negative impact on reproduction is documented in some animals. Studies employing approximately 40% CR detected a delay in sexual maturation and impairment of fertility, which were combined with extension of the reproductive period. In contrast, mild CR (10-20%) is apparently not deleterious to reproduction. Hence, we hypothesized that mild CR started at 8 months of age would prolong reproductive capabilities and improve health parameters of male mice. To test this hypothesis, we assessed the effects of 10 and 20% CR on reproductive organ weights, selected plasma parameters and hepatic/testicular gene expression in normal male mice of heterogeneous genetic background. Starting at 8 months of age (adult), mice were assigned to 3 regimen groups: 10% CR (n = 8), 20% CR (n = 9) or *ad libitum* (AL; n = 8). Four months of CR were sufficient to reduce glycemia in a non-fasted protocol. Mild CR initiated in adulthood did not significantly impact final body weight, most of the analyzed plasma parameters or weight of androgen-dependent organs. Moreover, CR did not interfere with expression of the assessed testicular genes, or most of the hepatic genes, but it did cause an increase in the levels of peroxisome proliferator-activated receptor gamma (*Pparg*) and mouse sulfotransferase (*mSTa*); and a decrease in glucose-6-phosphatase-α (*G6pc*) mRNA, which might signify improvement of body condition. The important finding of our study was that a mild CR regimen, as low as 10 and 20%, was sufficient to impair glycemia in a non-fasted state, and also the levels of plasma IGF-1, corroborating the concept that mild CR has the potential for improving health and longevity, even when started later in life.

Keywords: calorie restriction, gene expression, glycemia, IGF-1 levels, male mice.

Introduction

There is robust evidence that calorie restriction (CR) extends median and maximal lifespan and delays the onset of age-related diseases in organisms ranging from yeast to mammals (Weindruch and Sohal, 1997; Masoro, 2000, 2005; Smith *et al*., 2004; see review in Colman and Anderson, 2011), even when started later in life (Weindruch and Walford, 1982; Weindruch *et al*., 1982; Takahashi and Goto, 2002; Sanz *et al*., 2005; Podkowka-Sieczka *et al*., 2008). Nevertheless, the specific mechanisms by which CR prolongs lifespan are not yet established or completely understood. One of the proposed pathways by which CR prolongs lifespan is the insulin-like growth factor 1 (IGF-1)/insulin signaling pathway (Bartke, 2005, 2006; Bonkowski *et al*., 2006; Yamaza *et al*., 2007; Bonkowski *et al*., 2009; Colman and Anderson, 2011; Speakman and Mitchell, 2011). Also, CR has been linked not only to a reduction in the circulating levels of IGF-1, but also of inflammatory cytokines (see review in Fontana and Klein, 2007). On the other hand, CR leads to increased levels of sirtuin 1 (SIRT1; Cohen *et al*., 2004; Wang *et al*., 2007, Corbi *et al*., 2012). Sirtuin 1 is the mammalian homologue of Sir2 (silencing information regulator 2), a longevity inducer in *Saccharomyces cerevisiae* and *Caenorhabditis elegans* (Kaeberlein *et al*., 1999; Tissenbaum and Guarente, 2001). In an elegant study, Rodgers *et al*. (2005) showed that SIRT1 protein is induced in the mouse liver after fasting. Moreover, these authors observed that under fasting conditions, SIRT1 is able to control hepatic gluconeogenic/glycolytic pathways by deacetylating peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC1α), which represses glycolytic genes. In view of these findings, we aimed to investigate the hepatic expression of *Igf1*, *Sirt1* and *Pgc1a* after mild CR in adult mice. Other genes were also selected on the basis of their putative connection to CR effects. These included the insulin receptor (*Insr*), which is crucial to insulin sensitivity and is largely expressed in hepatocytes and adipocytes (Desbuquois *et al*., 1993);

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peroxisome proliferator-activated receptor gamma (*Pparg*), which acts as an insulin sensitizer (Ferré, 2004), with its levels declining with age, and CR apparently preventing this decay (Sung *et al*., 2004); glucose-6-phosphatase-α (*G6pc*), which is involved in the process of gluconeogenesis and catalyses the hydrolysis of glucose-6-phosphate into glucose and phosphate (Chou and Mansfield, 2008); phosphoenolpyruvate carboxykinase (*Pck1*), which is a key effector in gluconeogenesis, catalyzing the reaction where oxaloacetate + guanosine triphosphate are converted to phosphoenolpyruvate + CO2 + guanosine diphosphate (Chakravarty and Hanson, 2007); and finally, mouse sulfotransferase (*mSTa*), which is responsible for the biotransformation of several compounds, including steroid hormones such as dehydroepiandrosterone (DHEA; Takahashi *et al*., 2009), a hormone produced by the adrenal cortex. This hormone reportedly declines with age in primates (Goncharova *et al*., 2010), an outcome prevented by CR (Mattison *et al*., 2003).

In contrast to the major benefits of CR on health and lifespan, a negative impact of CR on reproduction is also well documented. The nutritional state clearly affects sexual maturation, as indicated by a delay of puberty in birds and mammals subjected to CR (Merry and Holehan, 1979; Ottinger *et al*., 2005; Zeinoaldini *et al*., 2006; Brito *et al*., 2007). However, there are also reports on the extension of reproductive life by CR (Merry and Holehan, 1979; Holehan and Merry, 1985; McShane and Wise, 1996; Selesniemi *et al*., 2008). We previously reported that 20% CR did not appear to be deleterious to reproduction in different stocks of mice (Rocha *et al*., 2007a,b). Indeed, the overall impact of CR can vary depending on the degree of restriction and the species considered (Mockett *et al*., 2006; Shanley and Kirkwood, 2006), and moreover, within a species, CR effectiveness may vary among strains (Harrison and Archer, 1987; Harper *et al*., 2006; Ferguson *et al*., 2008). Thus, it could be expected that CR effects specifically related to reproduction might vary as well. The potential negative influence of CR on reproduction is of particular concern when contemplating the use of CR, or CR mimetics, to prolong human lifespan. Therefore, we also aimed to do a reproductive assessment of adult male mice subjected to CR, and the parameters utilized were the weight of reproductive organs, testosterone levels and gene expression in the testis. The genes were selected based on their role in testis function and on their potential as targets of CR. These genes included: androgen receptors (*Ar*), which are found in the three main types of testicular somatic cells, i.e., Sertoli, Leydig and peritubular myoid cells (Shan *et al*., 1997; Zhou *et al*., 2005); cytochrome P450 aromatase (Cyp19a1 - *Arom*), which is responsible for the conversion of testosterone to estrogens (Santen *et al*., 2009); luteinizing hormone receptor (*Lhr*) and follicle-stimulating hormone receptor (*Fshr*), which play a key role in the control of Sertoli and Leydig cell functions, respectively; 3-β-hydroxysteroid dehydrogenase/isomerase (*Hsd3*) and cytochrome P450c17 (*Cyp17*), which are steroidogenic enzymes; and *Igf1*, which reflects the local production of IGF-1 in the testis. Of note, the percentage of food restriction in the majority of studies is around 40% (Weindruch and Sohal, 1997), and we hypothesized that a milder CR would dissociate the effects cited above, i.e., the treatment would bring health benefits without inhibiting reproduction in male mice. To test this hypothesis, we assessed the effects of two levels of mild CR, i.e., 10 and 20%, on selected reproductive parameters in adult male mice with a heterogeneous genetic background. Selected plasma parameters and hepatic gene expression were also examined to detect possible health-related effects of CR. We expected that the results would provide an initial test of our hypothesis that mild CR started at 8 months of age can prolong reproductive capabilities and improve health parameters of male mice.

Materials and Methods

Animals

Male mice (*Mus musculus*) were produced in the Southern Illinois University animal facility in a closed and non-inbred colony. Animals were group-housed 4-5 per cage, under a 12 h light/12 h dark cycle, lights on at 0600 h, and a temperature of 22 ± 2ºC. All animal protocols utilized in this study were approved by the Southern Illinois University Laboratory Animal Care and Use Committee, and were conducted in accordance with the NIH Guidelines for the Care and Use of Experimental Animals. Our facility had sentinel animals which were tested for bacterial and viral infections every 3 months with negative results.

Adult-onset calorie restriction

Animals had free access to tap water and pelleted rodent food (Lab Diet 5001, not-autoclaved, 23.4% protein, 4.5% fat, 5.8% crude fiber; PMI Nutrition International, Richmond, IN, USA). Starting at 8 months of age (adult), mice were assigned to 3 regimen groups: 10% CR (n = 8), 20% CR (n = 9) or *ad libitum* (AL; n = 8). These levels of CR were achieved by providing treated animals 90 or 80% of the amount of food consumed by the AL group, respectively. The AL group was constantly monitored for food consumption, and its average daily consumption values were used to calculate the amount of food to be given daily to the CR groups between 1500 h and 1700 h. The treatment lasted for 12 months. During this period, weight of animals and of food consumed was assessed weekly.

Glycemia after overnight fasting and overnight regular food protocol

Four months after initiation of CR, plasma glucose levels were measured under two conditions: 1) after overnight (12 h) food removal - FAST; and 2) after overnight routine feeding protocol - NON-FAST (described in the preceding section). The FAST protocol was performed by removing all food from the AL cages and any remaining food from the CR cages. This was done at 2100 h, when there was usually very little or no food left in CR cages. Blood drops were collected the next morning, between 0900 and 1100 h, by cutting off the tip of the tail. A glucometer (Lifescan, Johnson & Johnson, New Brunswick, NJ, USA) was used to measure peripheral glucose levels. Blood collection in both protocols was performed one week apart in the same animals.

Tissue collection

At 20 months of age, the animals were fasted overnight, anesthetized with isoflurane (Aerrane, Baxter HealthCare Corporation, Deerfield, IL, USA), bled by cardiac puncture and sacrificed. Liver, testes and seminal vesicles + coagulating glands were quickly removed, weighed and frozen in dry ice. Liver and testes were stored at -80°C and further processed for RNA extraction. Blood treated with 0.5M EDTA was centrifuged, plasma collected and kept at -80°C for measurements of glucose, insulin, IGF-1 and testosterone.

Analytical methods

Plasma levels of glucose were measured using the colorimetric method by Sigma (Sigma Diagnostics, St. Louis, MO, USA). Insulin levels were assessed by an Enzyme-linked Immunosorbent Assay (ELISA) kit (Crystal Chem Inc., Downers Grove, IL, USA; intra-assay precision CV < 10.0%). Plasma IGF-1 was measured using another ELISA kit (Immunodiagnostic Systems Inc., Fountain Hills, AZ, USA; intra-assay precision CV < 6.4%). Testosterone levels were assessed by radioimmunoassay (RIA; Diagnostic Products Corporation, Los Angeles, CA, USA; intra-assay precision CV < 12.0%). All samples were run in duplicates, and kits were used following the manufacturer's instructions. All diet groups were assessed in the same run to avoid inter-assay variation.

Testis homogenates

A fraction of about half a testicle was weighed, fractioned and homogenized inside tubes containing 500 µl of ice cold phosphate buffer (50 mM, pH 7.4) with 0.25 M sucrose. The tubes were then centrifuged at 600 x g for 10 min at 4°C to precipitate cellular debris. The supernatant was collected, transferred to new tubes and centrifuged at 10,000 x g for 20 min at 4°C to separate the mitochondrial pellet. Finally, the supernatant was kept at -80°C until it was used for RIA measurement of testicular testosterone content.

Radioimmunoassay

Testosterone levels in testis homogenates were assessed by RIA (Diagnostic Products Corporation, Los Angeles, CA, USA; intra-assay CV < 12.0%). Samples were run in duplicates, the volume of homogenate was 50 µl, the amount recommended for plasma in the instructions from the manufacturer. In order to avoid inter-assay variations, all diet groups were assayed in the same run.

RNA extraction

Testis and liver total RNA was extracted using the guanidinium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (1987). All RNA samples were run in 1.5% agarose gel containing ethidium bromide to check RNA quantity and quality.

cDNA

In order to produce cDNA from 2 µg of RNA, iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) was utilized, according to the manufacturer's instructions. Samples were run in an Eppendorf Mastercycler Personal;: the cycle consisted of submitting the samples to 25°C for 5 min, 42°C for 30 min, then 85°C for 5 min, and keeping them at 4°C in the end.

RT-PCR

Real-time reverse-transcriptase polymerase chain reaction (real-time RT-PCR) amplification was carried out using an iScript one-step RT-PCR kit with SYBR Green (Bio-Rad Laboratories, Hercules, CA, USA) and SmartCycler (Cepheid, Sunnyvale, CA, USA). The primers used are listed in Table 1. Data were normalized by housekeeping gene β-2-microglobulin (*B2m*) expression. The thermal cycler was set up to perform 40 cycles of three temperature steps each, as follows: denaturing at 95ºC, annealing at 62ºC and extension at 72ºC. Negative controls were present in every run and consisted of PCR mix plus the primers, but lacking the cDNA sample. The melting curve information and 2% agarose gel electrophoresis were used to confirm the PCR products.

Table 1. Primers used for RT-PCR.

Gene	GeneBank accession no.	Forward (5' - 3')		Backward (5' - 3')	
		Start	Sequence	Start	Sequence
B2m (*B2m)*	NM_009735	137	aagtatactcacgccaccca	298	aagaccagtccttgctgaag
Igf1 (*Igf1*)	NM_010512	24	catcatgtcgtcttcacacc	152	ggtccacacacgaactgaag
Sirt1 (*Sirt1*)	NM_019812	508	gtgatgacgatgacagaacg	669	ccagctcaggtggaggaatt
Ppargc1a (*Pgc1a*)	NM_008904	498	cagaagagccgtctctactt	646	ctcggtcttaacaatggcag
Insr (*Insr*)	NM_010568	1335	cttggacaaccagaacctga	1477	ccttagttccggagacttct
Pparg (*Pparg*)	NM_011146	608	gtcagtactgtcggtttcag	756	cagatcagcagactctgggt
G6pc (*G6pc*)	NM_008061	263	gtggtcggagactggttcaa	399	gtctcacaggtgacagggaa
Pck1 (*Pck1*)	NM_011044	147	cctcagctgcataacggtct	283	ccatcgcagatgtggatata
mSTa1 (*mSTa1*)	MUSSMSTA1X	155	ccaagtcaggaacgaactgg	308	cgtggtccttccttattgat
Ar (*Ar*)	NM_013476	961	ccaaaggattggaaggtgag	1107	tgtagtagtcgcgattctgg
Cyp19a1 (*Arom*)	NM_007810	181	ttcaataccaggtcctggct	330	agtgtctcctctccactgat
Lhr (*Lhr*)	MUSLHRAA	849	cactgctgtgctttcaggaa	1000	ccactgagttcattctcctc
Fshr (*Fshr*)	NM_013523	1329	ccatactaagagccagtacc	1501	ccttgcattccagttgcatg
Hsd3b1 (*Hsd3*)	NM_008293	490	catcttctgcagctcagttg	630	agtactgccttctcagccat
Cyp17a1 (*Cyp17*)	NM_007809	997	agcatatccttgtcacggtg	1110	tcttcctcttcacctcagga

Statistical analyses

The software SPSS 10.0.1 (SPSS Inc. Headquarters, Chicago, IL, USA) and GraphPad Prism 4.02 (GraphPad Software Inc., San Diego, CA, USA) were used for statistical analyses and graph generation. Results are presented as means ± standard error of the mean (SEM). One-way ANOVA was used to evaluate the effects of the diet. Tukey and Games-Howell were used as *post-hoc* tests, whenever appropriate. Data considered too skewed after descriptive analysis were submitted to Kruskal-Wallis (non-parametric) test. Results of RT-PCR are shown as fold-change of CR groups compared to the AL group. These specific data were processed using SmartCycler (Cepheid, Sunnyvale, CA, USA) and Microsoft Excel (Redmond, WA, USA) software. The values for threshold cycle numbers (ΔCt) obtained from each gene investigated were normalized to the housekeeping gene β-2-microglobulin (B2M) by subtracting ΔCt B2M from ΔCt gene. For all statistical analyses, $P < 0.05$ was considered significant.

Results

To determine how animals that are under different diet regimens adjust their metabolism to 12 h of fasting, we assessed glycemia after 4 months of CR (when they were 12 months old) by collecting blood samples with and without overnight fast. We observed reduced ($P < 0.001$) levels of glucose in the plasma of treated animals in comparison to the AL group, but only in NON-FAST condition (Fig. 1). Under this protocol, 10% CR led to a reduction of 23% in glucose levels compared to AL, and 20% CR rendered glycemia 18% lower in comparison to AL. No differences were seen among treatments when glycemia was measured in the FAST protocol. Body weight was not significantly different among diet groups at this time point (data not shown).

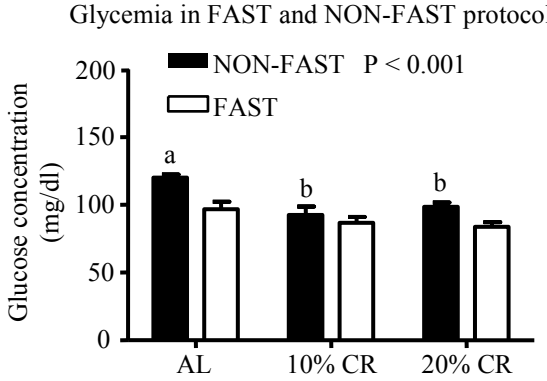

Figure 1. Glycemia under FAST and NON-FAST protocols, after 4 months of mild CR (10 and 20%) in male mice. Values are shown as mean ± SEM. Columns that do not share the same letter are different at the level of significance indicated in the insert. Columns without superscript letters indicate no statistical differences. CR: calorie restriction.

At the age of sacrifice (20 months), there were no significant differences in body weight among groups, 10% CR and 20% CR animals were only 0.3 and 9% lighter than AL animals, respectively (Table 2). Biometric parameters analyzed were overall not significantly affected by diet. Testis weight and gonadosomatic index (GSI; gonad weight/body weight x 100; Fig. 2A), sex accessory gland (seminal vesicles + coagulating glands) weight and index (sex accessory gland weight/body weight x 100; Fig. 2B), as well as emptied seminal vesicle weight and index (seminal vesicle weight/body weight x 100; Fig. 2C) were not significantly different among diet groups.

Table 2. Effects of mild CR on body weight, some plasma parameters and testosterone levels in 20-month-old male mice.

Parameter	AL	CR 10%	CR 20%
Body weight (g)	30.3 ± 1.7	30.2 ± 1.4	27.7 ± 0.9
Glucose (mg/dl)	89.0 ± 3.8	92.1 ± 5.8	84.9 ± 6.1
Insulin (ng/ml)	1.3 ± 0.7	0.7 ± 0.2	1.3 ± 0.4
IGF-1 (ng/ml)	397.4 ± 20.0[a]	322.5 ± 25.7[b]	290.6 ± 23.6[b]
Testosterone in plasma (ng/dl)	170.0 ± 156.2	33.2 ± 12.2	24.9 ± 7.2
Testosterone in testis homogenate (ng/dl)	9355.4 ± 6133.1	5510.7 ± 2365.6	3213.9 ± 1021.5

Note: Values are shown as mean ± SEM. Different superscripts denote a significant difference ($P < 0.05$ or less). The absence of superscript letters indicates no statistical differences. Number of animals were: AL = 8, CR 10% = 8 and CR 20% = 9. Glucose and IGF-1 levels were analyzed using ANOVA; Insulin and testosterone levels were analyzed using Kruskal-Wallis test.

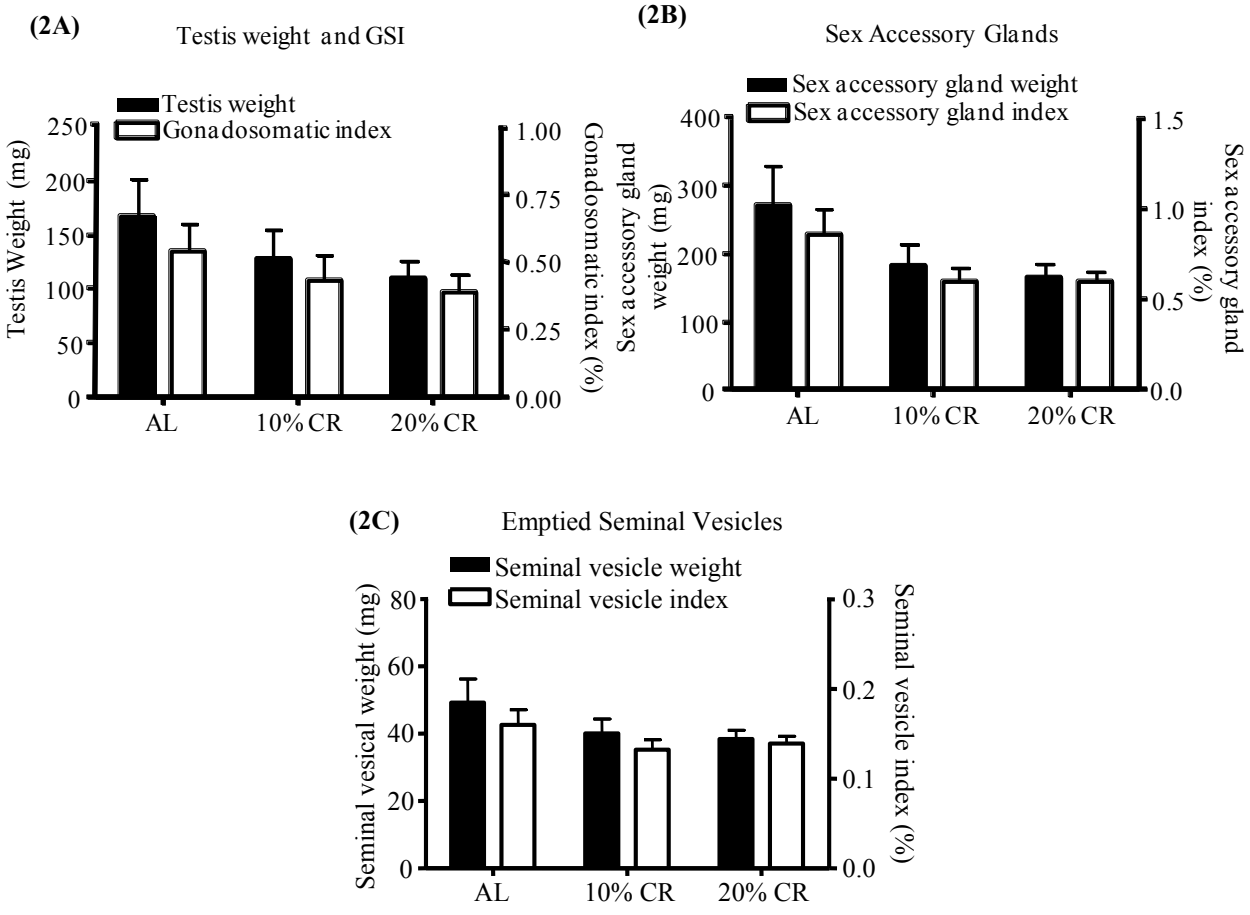

Figure 2. Effects of 10 and 20% CR on reproductive organ weights of male mice. A: Absolute and relative (GSI) testis weight. B: Weight of accessory sex glands (seminal vesicles + coagulating glands). C: Emptied seminal vesicle weight. Values are shown as mean ± SEM. The absence of superscript letters indicates no statistical differences. CR: calorie restriction.

Peripheral glucose levels at the end of experiment (age 20 months), were measured using the standard protocol of 12-h of fast before sacrifice. Endpoint glucose and insulin plasma levels were not significantly different across groups (Table 2). No significant impacts of mild CR on insulin levels were observed in the present study. There was a significant ($P < 0.01$) suppression of IGF-1 levels in the plasma of CR animals under 10% CR (19% reduction) and 20% CR (27% reduction) compared to the AL group (Table 2). When comparing individual group means, plasma IGF-1 levels in the AL group were higher than in the 10 or 20% CR groups, while the latter groups did not differ between each other.

To further investigate the effects of mild CR on reproduction, we also measured the levels of testosterone in peripheral circulation and in testis homogenates. The result was that testosterone levels either in plasma or testis homogenates were greatly variable (Table 2).

No significant differences were observed in the expression of mRNA for *Igf1*, *Sirt1*, *Pgc1a*, *Insr*, and *Pck1* in the liver of mice from different groups (Fig. 3A). Despite the changes in plasma IGF-1 levels, *Igf1* gene expression in the liver was not significantly affected by the CR levels imposed. The CR treatment increased mRNA levels of *Pparg* (39% in the 10% CR group; 57% in the 20% CR group; $P < 0.009$) and reduced the expression of *G6pc* mRNA (72% in the 10% CR group; 69% in the 20% CR; $P < 0.001$) compared to the AL group. We observed a striking increase ($P < 0.001$) in hepatic *mSTa1* mRNA levels after CR, which led to a rise of over 3800% in the relative levels of this message in the 10% CR group, and over 8100% in the 20% CR group, compared to AL (Fig. 3B). No significant diet-related differences were detected in testicular mRNA levels of *Ar*, *Arom*, *Lhr*, *Fshr*, *Hsd3*, *Cyp17* and *Igf1* across the diet groups studied (Fig. 3C).

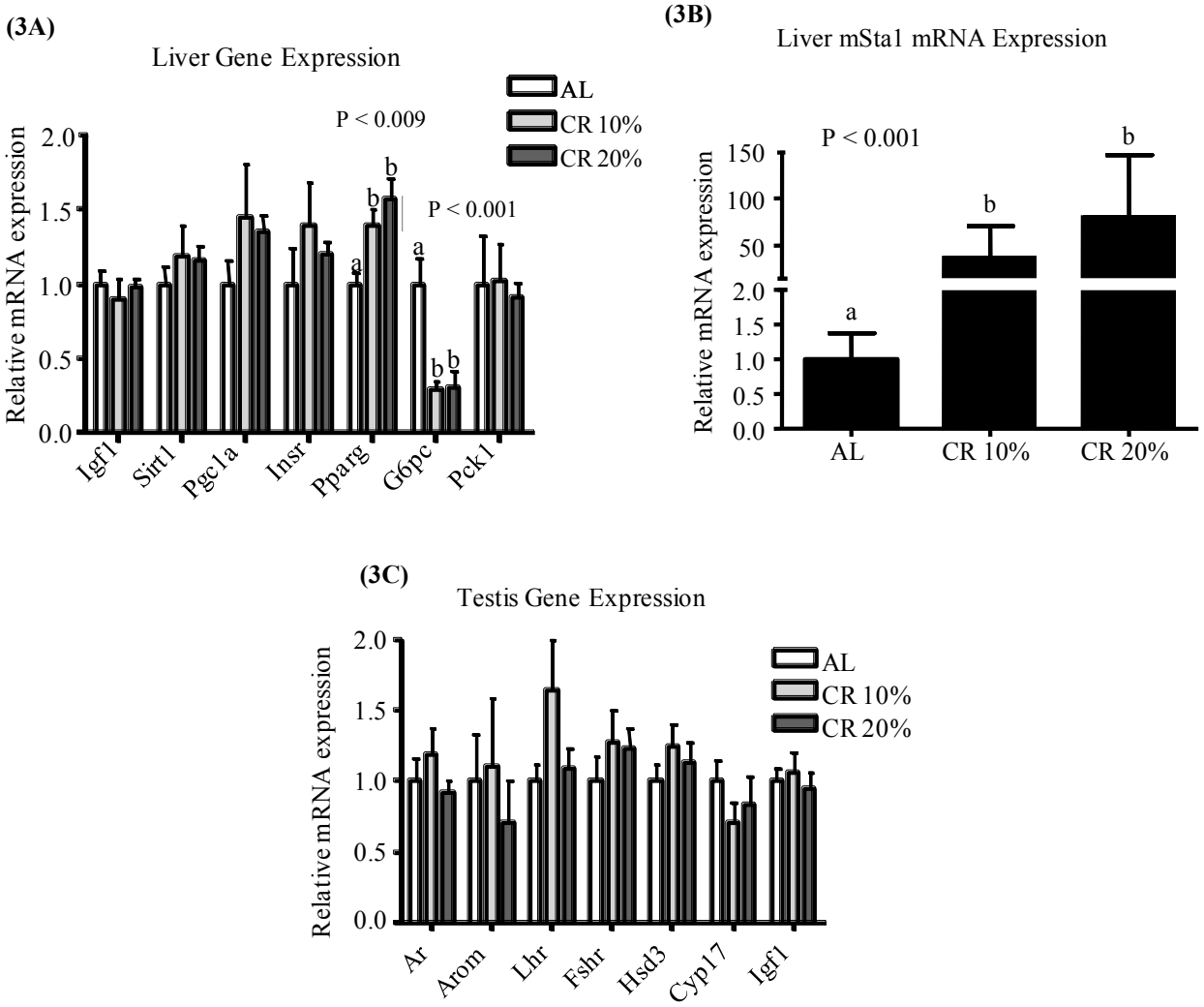

Figure 3. Effects of 10 and 20% CR on gene expression in male mice. A: hepatic gene expression; B: expression of *mSTa* mRNA in the liver; C: testicular gene expression. Values are shown as mean ± SEM. Columns that do not share the same letter are different at the level of significance indicated in the insert. Columns without superscript letters indicate no statistical differences. CR: calorie restriction; gene abbreviations are explained in the body text.

Discussion

Glycemia assessed after 4 months of CR was reduced in the CR groups, compared to the AL group, only in the NON-FAST condition. Under the FAST protocol, no differences were seen among treatments. Our group previously reported similar results, with no significant differences in glucose levels between AL and 20% CR animals (Rocha *et al*., 2007a). That was because mice in this study were also submitted to 12 hour-fasting, a common-sense protocol for sacrificing laboratory animals. Martin *et al*. (2007) reported comparable results from male and female Sprague-Dawley (SD) rats subjected to varied diet protocols and sacrificed after a 12 h fast. Thus, we suggest that the overnight fast might have a bigger impact on AL than CR animals; this explains the similar levels of glucose across the groups. Presumably, glycemia measured without a period of fasting reflects the prevailing levels of glucose in the animals.

The body weight of all animals presented no differences at the age of sacrifice. Reduction of body weight after CR is expected and well documented (Masoro, 2002, 2005). Weight of reproductive organs was also mostly not affected by diet. CR reportedly decreases the weight of reproductive organs (Chen *et al*., 2005), but under the conditions of our study, no significant differences were detected in the absolute or relative weight of reproductive organs. Lack of differences in biometric parameters in the present study is probably related to late onset of CR, after animals were fully grown (8 months of age), and to the mild levels of CR applied (10 and 20%).

Endpoint glucose and insulin plasma levels were not significantly different across groups. Martin *et al*. (2007) also reported no differences in insulin levels among diet groups in male and female SD rats, with the exception of one group of males which presented reduced insulin levels after 6 months of 20% CR. Insulin levels usually parallel glucose levels under fasting conditions (Larue-Achagiotis and Le Magnen, 1983; Shulman *et al*., 2002), which might explain the similarities observed for both parameters. One possible explanation for the lack of differences in glucose and insulin levels after CR in the present study may rely on the association of mild CR treatment with the assessment of these parameters after an overnight fast (discussed previously). Of note, a restriction of 30% in calories is sufficient to promote a significant reduction in peripheral glucose and insulin levels, as well as an increase in insulin receptor protein levels in the liver of normal mice (Bonkowski *et al*., 2009)

Interestingly, we observed a significant suppression of peripheral IGF-1 levels after CR. Plasma IGF-1 levels in the AL group were higher than in the 10 or 20% CR groups, while the latter groups did not differ between each other. The present study was not meant to assess the longevity of the animals; instead, we searched for markers of health condition which could be considered as predictors of lifespan extension. For instance, it is known that CR improves insulin sensitivity and lowers glycemia and IGF-1 levels (Masoro, 2005; Fontana, 2009). These are some of the proposed mechanisms by which CR prolongs lifespan (Bartke *et al*., 2001; Barger *et al*., 2003; Speakman and Mitchell, 2011). In mice, lower IGF-1 levels are associated with a longer lifespan (Bartke, 2005), a reduced mitogenic action of IGF-1, and the consequently delayed occurrence of neoplasias, and are likely among the mechanisms behind these effects (Blakesley *et al*., 1997; Jenkins and Bustin, 2004). Dunn *et al*. (1997) and Rogozina *et al*. (2009) reported that CR reduced the levels of IGF-1 in cancer-prone mouse lines and observed that this reduction was linked to a lower incidence of tumors in the CR-treated animals. Our findings confirm other reports of a reduction of IGF-1 by CR and suggest that even mild CR (20%) could lead to life extension.

The great variability observed in testosterone levels either in plasma or testis homogenates in the preset study lead to a lack of differences among treatments. A great variation in testosterone levels, especially in mice, is a well-known phenomenon and probably reflects the absence of testosterone binding globulin and the pulsatile fashion of LH release in this species (Bartke *et al*., 1973; Bartke and Dalterio, 1975). In order to validate the extremely variable values detected in the present study, we compared ranks of testosterone levels in plasma and testis homogenates. The ranks matched closely, and the two animals with the highest levels of testosterone in plasma also had the highest levels of testosterone in testis homogenate. The correlation factor for the values of testosterone in plasma and in testis homogenates was $r = 0.937$ ($P < 0.01$).

We observed no significant differences in the expression of mRNA for *Igf1*, *Sirt1*, *Pgc1a*, *Insr*, and *Pck1* in the liver of mice from different groups (Fig. 3A). Despite the changes in plasma IGF-1 levels, *Igf1* gene expression in the liver was not significantly affected by the CR levels imposed. A reduction of IGF-1 levels in response to CR was previously reported in rats and mice (Masoro, 2003, 2005), as well as other species (Katic and Kahn, 2005). Another gene involved in the hepatic response to CR is the *Pgc1a* gene, which, together with *Sirt1*, is involved in the putative mechanisms of lifespan extension (Rodgers *et al*., 2008). Corton and Brown-Borg (2005) reported increased levels of both *Pgc1a* and *Sirt1* after CR in their review on the role of PGC-1 in CR and longevity. Other authors reported a significant increase of *Sirt1* after CR (Cohen *et al*., 2004; Crujeiras *et al*., 2008), and of PGC-1 after fasting (Ding *et al*., 2006) or 30% CR (Chiba *et al*., 2009). We observed no differences in *Sirt1* and *Pgc1a* mRNA levels in the liver of mice among all diet groups, again, likely reflecting the mild degree of CR in the present study. We did not detect differences in the expression of

Insr mRNA among the groups studied, while Bonkowski *et al*. (2009) reported increased protein levels of INSR in the liver of normal mice subjected to 30% CR, and Zhu *et al*. (2005) observed that 40% CR caused a rise in hepatic mRNA levels of *Insr* in rats.

The CR treatment increased mRNA levels of *Pparg* (39% in the 10% CR group; 57% in the 20% CR group; $P < 0.009$) and reduced the expression of *G6pc* mRNA (72% in the 10% CR group; 69% in the 20% CR; $P < 0.001$) compared to AL group. Zhu *et al*. (2004) reported an increase in hepatic *Pparg* mRNA levels in rats after 40% CR, and these observations are similar to our results. Pparg is an insulin sensitizer (Ferré, 2004), which has its levels of mRNA and protein decreased by ageing, but CR seems to prevent this decay (Sung *et al*., 2004). Thus, the apparent increase by CR in *Pparg* mRNA levels observed in our 20-month-old animals may represent a prevention of the age-related decline in the expression of *Pparg*. Masternak *et al*. (2005) reported no differences in hepatic *Pparg* mRNA levels in mice under 30% CR, but noted a significant increase in mRNA and protein levels of Pparg in livers from long-lived growth hormone receptor/binding protein knockout (GHR-KO) mice compared to wild type counterparts. These knockouts are known for their greater longevity and outstanding insulin sensitivity, and their higher levels of hepatic Pparg probably contribute to this phenotype (Masternak and Bartke, 2007). The reduction in *G6pc* mRNA levels in our animals corroborates with the role of CR in improving body condition, since G6pc over expression results in increased glycemia (Barthel and Schmoll, 2003). However, Spindler (2001) and Chiba *et al*. (2009) found increased levels of *G6pc* mRNA in the liver of mice and rats subjected to CR. The protein PCK1 is known for its role in gluconeogenesis and for its response to nutritional state (Tilghman *et al*., 1974), but we did not see differences in the expression of this gene when comparing all diet groups. Velez and Donkin (2005) also reported no alterations in *Pck1* mRNA levels after submitting cows to 50% CR, but Chiba *et al*. (2009) observed increased levels of this message in the livers of rats subjected to 30% CR.

The levels of hepatic *mSTa1* mRNA were remarkably high in the CR groups compared to the AL group. The reason we assessed *mSTa* levels was because the sulfotransferases, together with sulfatases, are the main enzymes responsible for the processes involving sulfate transfer (Bojarová and Williams, 2008). By doing this, the sulfotransferases regulate the bioavailability of several endogenous molecules, such as steroid hormones (Borthwick *et al*., 1995). Dehydroepiandrosterone (DHEA) and its sulfated metabolite DHEA-S are examples of such hormones and are produced by the adrenal cortex (Kroboth *et al*., 1999). In humans and in some animal species, the levels of these hormones decrease with age, and for this reason constitute markers of senescence (Mazat *et al*., 2001; Roth *et al*., 2002). Since this decline is impaired by CR, Heilbronn *et al*. (2006) proposed DHEA-S as one of the biomarkers of CR and longevity in rodent and primate models. Our results are in general agreement with the literature since we observed an increase in *mSTa* mRNA levels after mild levels of CR, and this may have led to higher levels of DHEA-S.

No significant diet-related differences were detected in testicular mRNA levels of all genes assessed, that is, *Ar*, *Arom*, *Lhr*, *Fshr*, *Hsd3*, *Cyp17* and *Igf1* across all diet groups. Gonadotropin-releasing hormone (GnRH) is released by the hypothalamus, stimulating the pituitary to release follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Sertoli cells are the main target of FSH and, under its effect, exert crucial roles on the spermatogenic process. Leydig cells are the main targets of LH, which initiates in these cells a cascade of events that lead to the production of testosterone, and also estradiol, using cholesterol as the main substrate. Mitochondria and smooth endoplasmic reticulum of Leydig cells possess several steroidogenic enzymes that are crucial for this process, including 3-β-hydroxysteroid dehydrogenase/isomerase (HSD3), cytochrome P450c17 (Cyp17) and cytochrome P450 aromatase (Cyp19a1 - AROM) (Stocco and McPhaul, 2006). The testis not only produces testosterone but is also its main target, and androgen receptors (AR) are found in the three main types of testicular somatic cells, i.e., Sertoli, Leydig and peritubular myoid cells (Shan *et al*., 1997; Zhou *et al*., 2005). Of note, the nutritional state controls not only the onset of puberty but also continued adequate reproductive function throughout life (Schneider, 2004). In the present study, we assessed the expression of the aforementioned genes in the testis of mice subjected to three dietary regimens, i.e., AL, 10% CR and 20% CR. The levels of expression in the different groups were not significantly different for any of the genes analyzed, although there was a tendency for a slight increase of the message for those genes in the 10% CR group, perhaps representing some compensatory mechanisms or consequences of altered testosterone availability.

In conclusion, mild CR initiated during adulthood in male mice did not significantly impact final body weight, plasma insulin or testosterone levels and weight of androgen-dependent organs, although this regimen significantly impaired peripheral glucose levels in a non-fasted state. Additionally, CR did not interfere with the expression of the analyzed testicular genes or most of the hepatic genes assessed, but it did increase hepatic *Pparg* and *mSTa* and decreased *G6pc* mRNA levels, thus resembling some of the previously described effects of more severe CR protocols. One important finding of our study was that a mild CR regimen, as low as 20, and even 10%, was sufficient to impair glycemia in a non-fasted state, and also the levels of plasma IGF-1, corroborating the concept that mild CR has the potential for improving health and possibly longevity,

even when started later in life.

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