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Effects of mild calorie restriction on reproductive parameters of pubertal and sexually mature male Wistar rats

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Abstract

Calorie restriction (CR) has been reported to have pro-longevity effects in a myriad of species. However, a deleterious effect of CR on reproduction is also observed, though seemingly negligible if CR is very mild. Full or partial suppression of reproduction by CR is worthy of concern when envisioning the use of this methodology to prolong lifespan in humans. Our aim was to investigate the effects of mild CR on several reproductive parameters, such as the onset of puberty, fertility, reproductive organ biometry, testis morphometry and testosterone and cholesterol plasma levels. Therefore, male Wistar rats were subjected to 30%CR from age 24 days until the ages of 50 and 150 days. Age-matched animals received food *ad libitum* (AL) and served as controls. The day of onset of balano-preputial separation, indicative of puberty, showed no difference between diet groups. In relation to the fertility parameters evaluated, considering mainly the number of pups per female, there was a trend for better performance in CR rats. Body weight, as well as the absolute and relative weights of the epididymal fat pad, was reduced by CR at both ages investigated, whereas testosterone and cholesterol plasma levels were not changed. The weight of ventral prostate, also the tubular diameter and luminal size were smaller in CR rats at age 50 days, but seemed to recover later since no differences were observed at age 150 days. In conclusion, our results suggested that 30%CR does not compromise reproduction in male Wistar rats.

Keywords: calorie restriction, reproduction, reproductive organ biometry, testis morphometry, Wistar rats.

Introduction

There is a growing interest in the study of the effects of calorie restriction (CR), especially due to its positive influence on longevity. The pro-longevity effects of CR were demonstrated in yeast (*Saccharomyces cerevisiae*), flatworms (*Caenorhabditis elegans*) and flies (*Drosophila melanogaster;* see review in Smith *et al*., 2004). Similarly to the reports on invertebrates, laboratory rats and mice, and also dogs, present life extension after CR (Weindruch and Sohal, 1997; Lane *et al*., 1999; Masoro, 2000, 2001; Kealy *et al*., 2002). Studies using monkeys suggest lifespan benefits attributed to CR (Barger *et al*., 2003; Ingram *et al*., 2004; Roth *et al*., 2004; Colman *et al*., 2009). Additionally, investigations in humans point to an improvement of some aging markers, like a reduction of peripheral levels of glucose and cholesterol, as well as blood pressure (Walford *et al*., 1992; Heilbronn and Havussin, 2003). In a paper published in 2005, Bordone and Guarente stated that "physiological changes that are elicited by CR contribute towards a condition of robust health, and that these same changes trigger greater longevity". On the other hand, despite lifespan benefits, CR reportedly delays puberty in birds and mammals (Merry and Holehan, 1979; Ottinger *et al*., 2005; Zeinoaldini *et al*., 2006), and reduces the frequency and size of litters in rats (Holehan and Merry, 1985; Masoro, 2001), even though it promotes an extension of reproductive lifespan (Holehan and Merry, 1985; McShane and Wise, 1996). Thus, nutritional status is clearly related to sexual maturation, and the effects of CR vary according to the degree of restriction and the species evaluated. For example, in rats, lifelong 40%CR resulted in a significant delay in puberty, with pronounced effects on reproductive functions of adult animals (McShane and Wise, 1996). Nonetheless, rats and quails growing under CR of 20-30% (mild) had their puberty delayed, but presented no long-term effects on reproductive function, and benefited from the lifespan extension effect (Nelson *et al.*, 1995; Merry and Holehan, 1979; Lane *et al*., 2001; Ottinger *et al*., 2005). The gonadal inhibition resultant of CR interventions represents a concern for researchers in this field, since CR or CR mimetics are intended to be used in the future to decelerate aging in humans (Masoro, 2001; Roberts *et al*., 2001). It is noteworthy that there are some recent indicatives that mild CR (10 and 20%) is non-deleterious to reproduction (Rocha *et al*., 2007a, b, 2012).

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The aim of the present study was to investigate the effects of 30%CR on several reproductive parameters in male Wistar rats subjected to CR from weaning to 50 and 150 days of age. We expected that this level of CR would confer improvement of some health parameters without impacting reproductive capability in these animals.

Materials and Methods

Animals and experimental design

Male Wistar rats aged 21 days were maintained in the vivarium of the Department of Morphology, Institute of Biological Sciences, Federal University of Minas Gerais, Brazil. All experiments followed approved guidelines for the ethical treatment of laboratory animals. Two experimental groups were set up according to the duration of CR and the age at sacrifice, as follows:

Experiment I - 50 days: Sixteen rats were placed in groups of four animals in plastic cages with free access to tap water, 12 h light (7 h)/12 h dark (19 h) and a temperature of 22ºC. Animals initially had free access to pelleted rodent food (Nuvilab CR1, not autoclaved, 22% protein, 4.5% fat, 8% crude fiber, Nuvital Nutrientes S/A, Colombo, PR, Brazil). Body weight (BW) and amount of food ingested were measured daily from age 21 days through the end of experiment. At 24 days, half of animals (n = 8) were randomly assigned to the *ad libitum* (AL) group and the other half (n = 8) to the calorie restriction (CR) group. Values for the amount of food to be given to CR animals were updated daily based on AL food consumption (Shaw, 1965; Rocha *et al*., 2007b). Therefore, 30%CR was attained by providing CR rats with 70% of the food consumed by AL animals. Food was provided to CR animals once a day, around 17 h. Rats were sacrificed at age 50 days.

Experiment II - 150 days*:* Sixteen males were subjected to the same proceedings described for Experiment I, the only difference was the age of sacrifice at 150 days. Prior to sacrifice, these animals were submitted to fertility testing (described elsewhere) at the age of 110 days.

Balano-preputial separation

Male Wistar rats from both experimental groups were examined daily for balano-preputial separation (BPS; Korenbrot *et al*., 1977) since the age of 21 days. BPS is considered an external (anatomical) sign of puberty onset in rodents and is characterized by a separation of the foreskin of the penis from the glans. Examination consisted of carefully pulling the foreskin of the penis. The BPS data were grouped for the ages of 50 and 150 days and the number of animals for each diet group was 16.

Fertility testing

Primiparous female Wistar rats aging approximately 150 days were used in a proportion of 4 females per each male in the experiment. Females were examined for estrous cycle on a daily basis, around 9 h. Briefly, female rats present estrous cycles of 5 days and the phases are: diestrus, proestrus, estrus, and metestrus. To determine phase of the estrous cycle in this study, vaginal smears were freshly prepared and the pattern of epithelial cells detached (Marcondes *et al*., 2002) was observed under a low-power light microscope. Only females in proestrus were mated, overnight, with males, at a ratio 1:1. Food was available *ad libitum* in mating cages containing AL males, while in mating cages with CR males, the amount of food provided to the couple was the amount updated for 2 CR animals. On the morning after, couples were separated and females checked for vaginal plug and for the presence of semen in vaginal tract. Fertility testing lasted 10 days, after that, males were returned to their original cage settings and females were monitored daily for pregnancy. Female sacrifice was performed on the 20th day of pregnancy, and the number of corpora lutea, resorptions and viable and non-viable fetuses were recorded.

Tissue collection and preparation

Twenty minutes prior to sacrifice, rats received an intraperitoneal injection of heparin (125 UI/kg BW, Liquemine® Produtos Roche Químicos e Farmacêuticos S.A., Rio de Janeiro, RJ, Brazil) and were then anesthetized with 5% sodium thiopental (50 mg/kg BW; Thiopentax, Cristália Produtos Químicos Farmacêuticos LTDA, Itapira, SP, Brazil). After that, perfusion with glutaraldehyde 4% phosphate buffer (0.05 M; pH 7.2-7.4) was performed through the left ventricle. Perfused animals had various organs collected and weighed as follows: testes, epididymides, seminal vesicles, ventral prostate + coagulating glands and epididymal fat pads. Testes were cut in 2-3 mm thick fragments and immersed overnight in the same fixative solution used in perfusion. Testis fragments were then routinely processed for histological and histometrical evaluations. Briefly, fragments were dehydrated in a graded series of ethanol baths and embedded in glycol methacrylate (Leica Historesin Embedding Kit, Leica Microsystems, Wetzlar, Germany). Four-micrometer testis sections were stained with 0.5% toluidine blue in 1% sodium borate, covered with Entellan® (Merck S.A., Rio de Janeiro, RJ, Brazil) and analyzed under a light microscope (Olympus CH30RF100, Olympus Optical CO. LTD., Japan). Gonadosomatic index (GSI), which

conveys the relation between testis weight and body weight (GSI = total gonad weight x 100/total body weight), was calculated for every animal. Likewise, indices (relative weights) for other reproductive organs, i.e., epididymis, seminal vesicles and ventral prostate, and for epididymal fat pads were calculated.

Morphometrical analyses

The average tubular diameter per animal was obtained from fifteen round or nearly round seminiferous tubule cross sections. The mean seminiferous epithelium height was determined by measuring two diametrically opposed epithelium walls in each tubule section. By subtracting epithelium height from tubular diameter, one can obtain the luminal diameter.

Hormonal measurements

Blood was collected by cardiac puncture prior to perfusion, added with 0.5 M ethylenediamine tetraacetic acid (EDTA) and centrifuged. The resultant plasma was kept at -80°C for measurements of cholesterol and testosterone, which were performed by TECSA Laboratories (http://tecsa.com.br). The method used for cholesterol was the endpoint colorimetric method for assaying total cholesterol, and total testosterone was assessed by the testosterone chemiluminescence immunoassay.

Statistical analyses

Statistical analyses were performed using Excel for Windows, SPSS 10.0.1 (SPSS Inc. Headquarters, Chicago, IL, USA) and GraphPad Prism 4.02 (GraphPad Software Inc., San Diego, CA, USA). All results are shown as mean ± standard error of the mean (SEM). Unpaired Student's *t* test was used to assess diet effect. The level of significance was set at $P < 0.05$.

Results

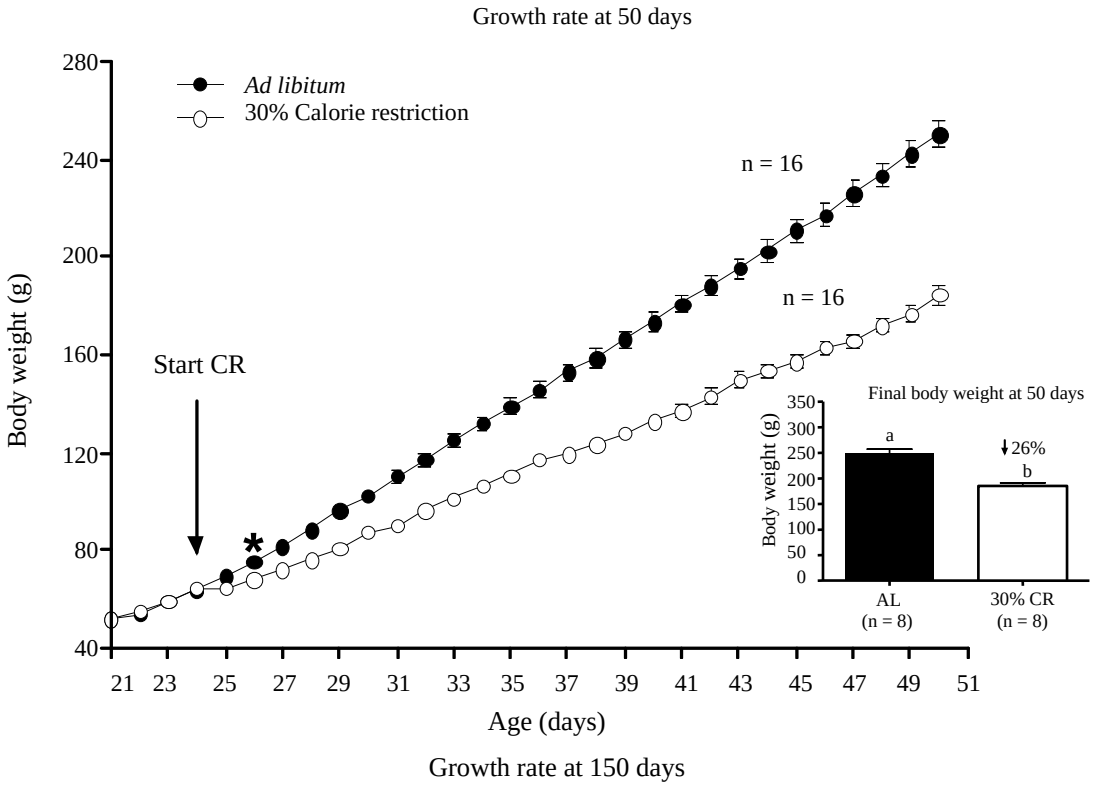
Calorie restriction had a significant effect on body weight (BW). The growth rates covering the whole experimental time period showed that, since the first few days of CR, BW became significantly different between treated and non-treated groups (Fig. 1). Final BW of CR animals was lower than AL animals at ages 50 days (26%; 250 ± 8 g in AL *vs.* 185 ± 6 g in CR; $P < 0.001$; insert in Fig. 1A) and 150 days (20%; 464 ± 13 g in AL *vs*. 370 ± 11 g in CR; $P < 0.001$; insert in Fig. 1B).

The onset of puberty, as measured by the mean day of BPS, was not impacted by CR ($P = 0.1$), and was 38.4 ± 0.3 days for CR animals, and 39.6 ± 0.5 days for AL animals. Also, based on the parameters evaluated, CR did not impact fertility performance in this study (Table 1). However, a trend ($P < 0.06$) for a higher mean number (12%) of viable fetuses was observed in females mated with CR males (12.2 ± 0.4), compared with the ones mated with AL males (10.8 ± 0.7). The average number of corpora lutea in ovaries was very similar in females mated with males from both diet groups (14.4 ± 0.6 in AL *vs*. 14.3 ± 0.7 in CR). Also, the number of losses, obtained by the subtraction of the number of viable fetuses from the number of corpora lutea, was not significant in females mated with males from both groups evaluated. Although no significance was reached, the average number of pups per male was numerically increased by 11% in the CR group (10.9 ± 0.7 in AL *vs*. 12.2 ± 0.4 in CR; $P = 0.1$).

Testes (Fig. 2A and 3A) and epididymides (Fig. 2B and 3B) did not have absolute weights impacted by CR at any age. Nevertheless, gonadosomatic indices were rather increased by CR at the ages of 50 and 150 days (Fig. 2A and 3A), whereas the epididymides indices were augmented only in 150-day-old animals (Fig. 3B). The absolute weight of epididymal fat pads was decreased by CR at both ages assessed (Fig. 2E and 3E). Seminal vesicle and ventral prostate absolute weights were also reduced by CR, but only in 50-day-old animals (Fig. 2C and 2D). Calorie restriction also affected relative organ weights (index) in some instances. Thus, a reduction in epididymal fat pad indices at both ages investigated was observed (Fig. 2E and 3E). Except for seminal vesicles at 150 days (Fig. 3C), in the other ages evaluated, the indices of seminal vesicles (Fig. 2C) and ventral prostate (Fig. 2D and 3D) were not impacted by CR. The results related to these selected organ weights and indices are summarized in Table 2.

At both ages studied (Table 3), the peripheral levels of cholesterol and testosterone were not significantly different between CR and AL groups. However, the testis morphometric data obtained revealed differences when the two groups were compared. Thus, the 50-day-old rats under CR had smaller tubular and luminal diameters (Fig. 4A, 4B, 5A), whereas no differences were noted for epithelial height at this age (Fig. 5A).. At the age of 150 days, no differences were noted in any of the morphometric parameters analyzed (Fig. 4C, 4D, 5B).

A



B

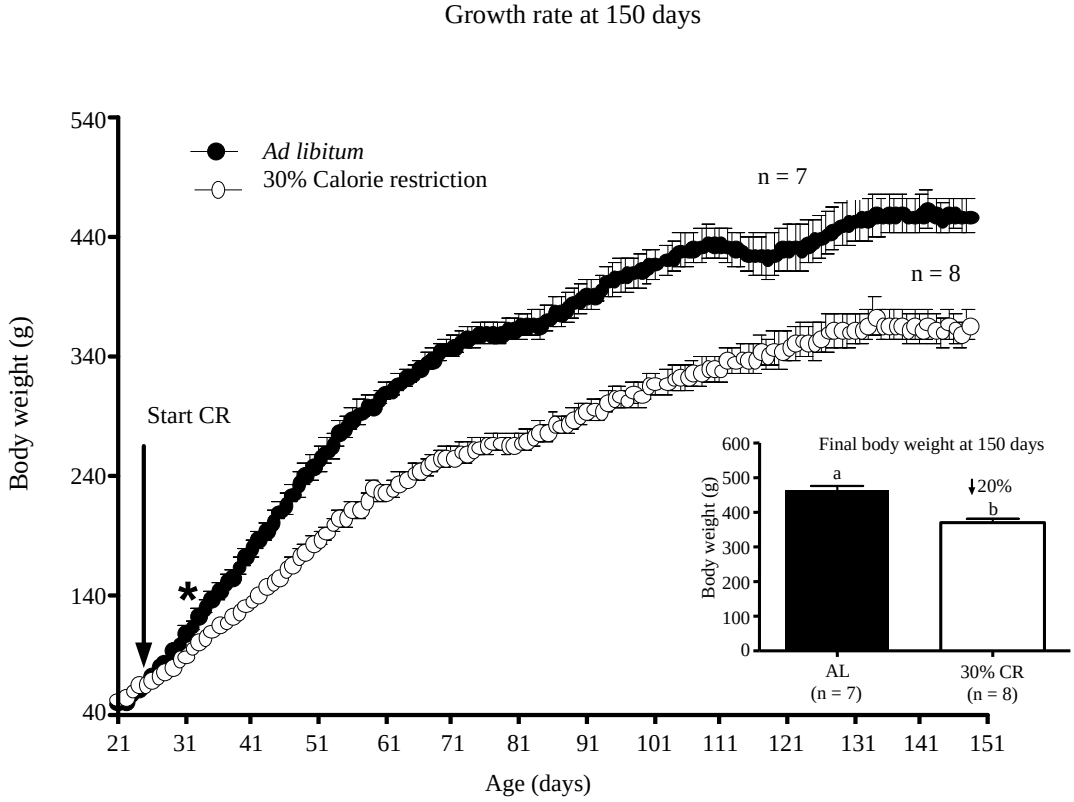


Figure 1. Growth rates of male Wistar rats sacrificed at the ages of 50 (A) and 150 days (B). Each time point represents the average BW per group. The figure reporting results of 50-day-old rats (A) combine measurements performed on the animals sacrificed at 50 and 150 days. From the day marked with asterisk on, differences in BW were significant. Rats received food *ad libitum* (AL) or were restricted (30%CR). Values correspond to means ± SEM. Final body weights of male Wistar rats at 50 (A) and 150 days (B) are shown in the inserts at the bottom right of the figures, and bars that do not share the same letter are statistically different ($P < 0.001$).

Table 1. Effects of 30%CR on reproductive performance of male Wistar rats.

Parameter	AL	30%CR
Live pups per female*	10.8 ± 0.7 (22)	12.2 ± 0.4 (25)
Corpora lutea*	14.4 ± 0.6 (22)	14.3 ± 0. 7 (25)
Resorptions*	1.5 ± 0.5 (22)	0.6 ± 0.2 (25)
Losses*	3.6 ± 0.9 (22)	2.1 ± 0. 6 (25)
Live pups per male	10.9 ± 0.7 (8)	12.2 ± 0.4 (8)

*Refers to females mated with males from the study; ( ) number of animals tested; Number of losses = number of corpora lutea minus total number of fetuses; Numbers represent mean ± SEM; Animals received food *ad libitum* (AL) or were restricted (30%CR).

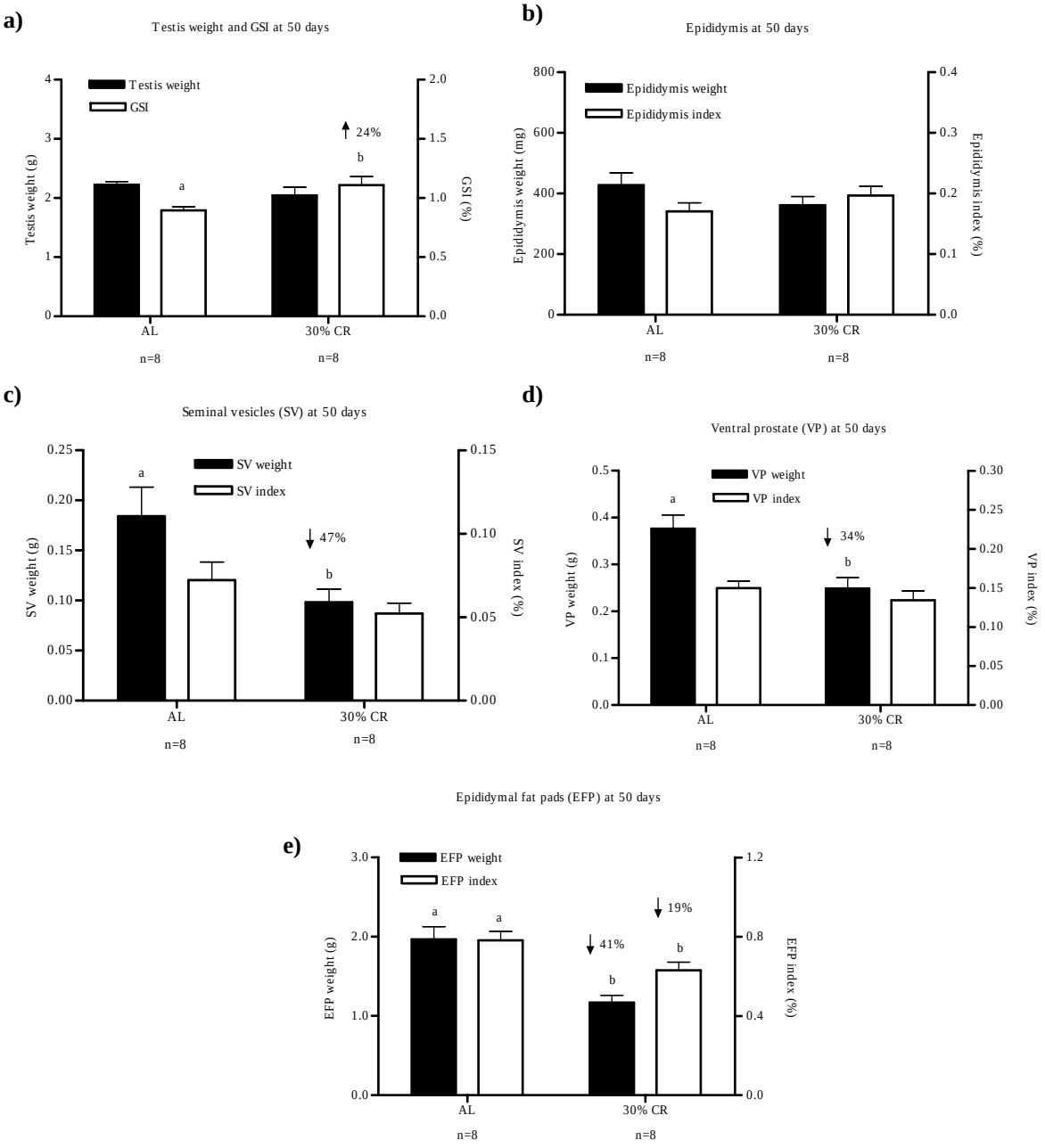


Figure 2. Effects of 30%CR on absolute and relative weights (indices) of several reproductive organs in male Wistar rats at the age of 50 days. Testis weight and GSI (A); epididymis (B); seminal vesicle (C); prostate (D); and epididymal fat pad (E). Bars represent means ± SEM, and those that do not share the same letter are statistically different at $P < 0.05$. Numbers preceded by arrows above the CR bars represent the percentage of reduction or increase in relation to the AL group.

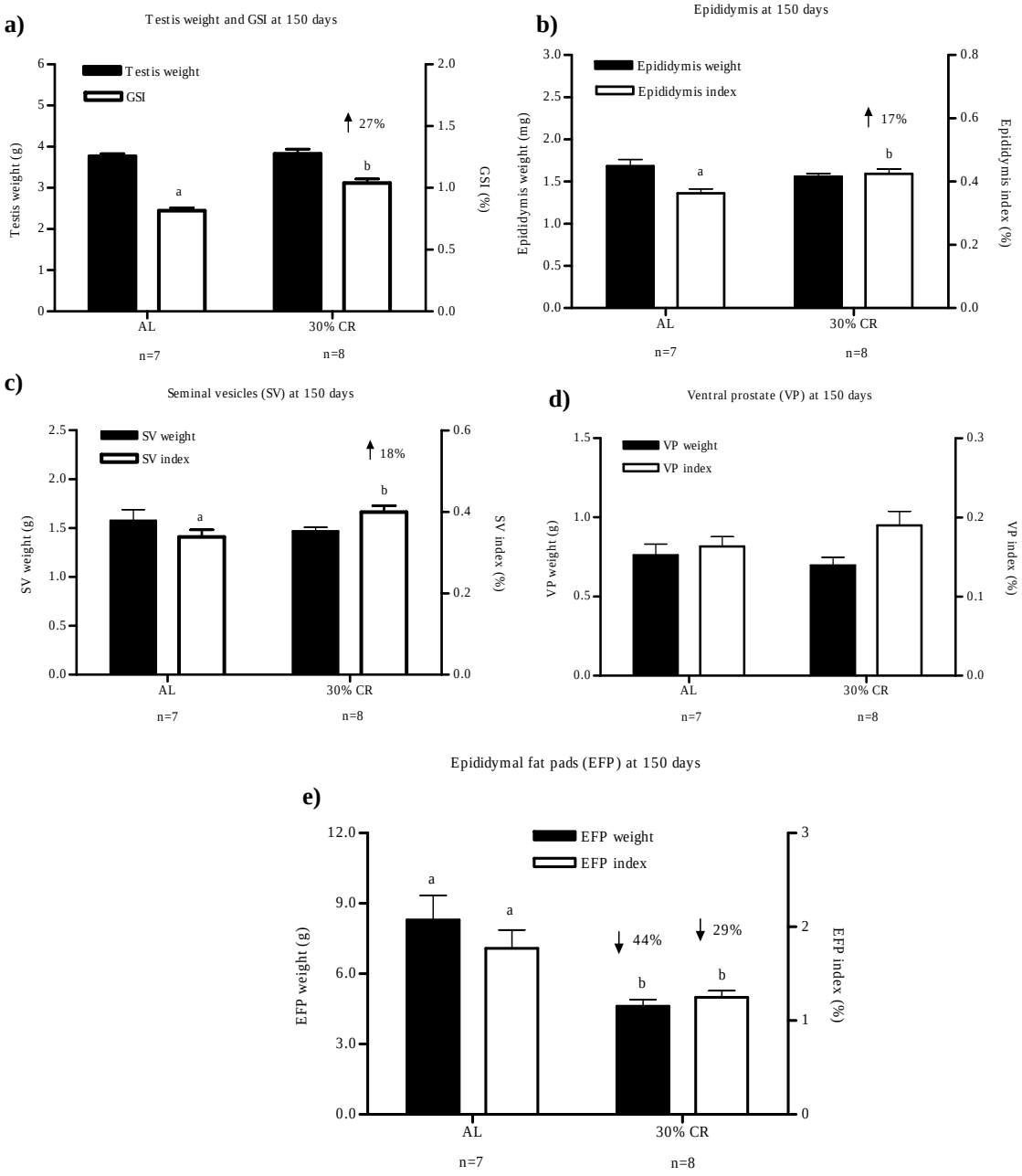


Figure 3. Effects of 30%CR on absolute and relative weights (indices) of several reproductive organs in male Wistar rats at the age of 150 days. Testis weight and GSI (A); epididymis (B); seminal vesicle (C); prostate (D); and epididymal fat pad (E). Bars represent means ± SEM, and those that do not share the same letter are statistically different at $P < 0.05$. Numbers preceded by arrows above the CR bars represent the percentage of reduction or increase in relation to the AL group.

Table 2. Summary of the effects of 30%CR on selected organ weights in male Wistar rats at 50 and 150 days.

Organ	50-day-old rats		150-day-old rats	
	Absolute weight	Relative weight	Absolute weight	Relative weight
Testis	=	↑ (GSI)	=	↑ (GSI)
Epididymis	=	=	=	↑
Seminal vesicle	↓	=	=	↑
Ventral prostate	↓	=	=	=
Epididymal fat pad	↓	↓	↓	↓

↑ means significant increase by 30%CR; ↓ means significant decrease by 30%CR; = means not significantly impacted by 30%CR.

Table 3. Effects of 30%CR on plasma levels of cholesterol and testosterone in male Wistar rats at 50 and 150 days of age.

Age	Cholesterol (mg/dl)		Testosterone (ng/dl)	
	AL	30%CR	AL	30%CR
50 d	77 ± 7 (8)	92 ± 12 (8)	141 ± 42 (8)	73 ± 23 (8)
150 d	80 ± 5 (7)	70 ± 5 (8)	643 ± 140 (7)	670 ± 189 (8)

( ) number of animals tested; Numbers represent mean ± SEM; Animals received food *ad libitum* (AL) or were restricted (30%CR).

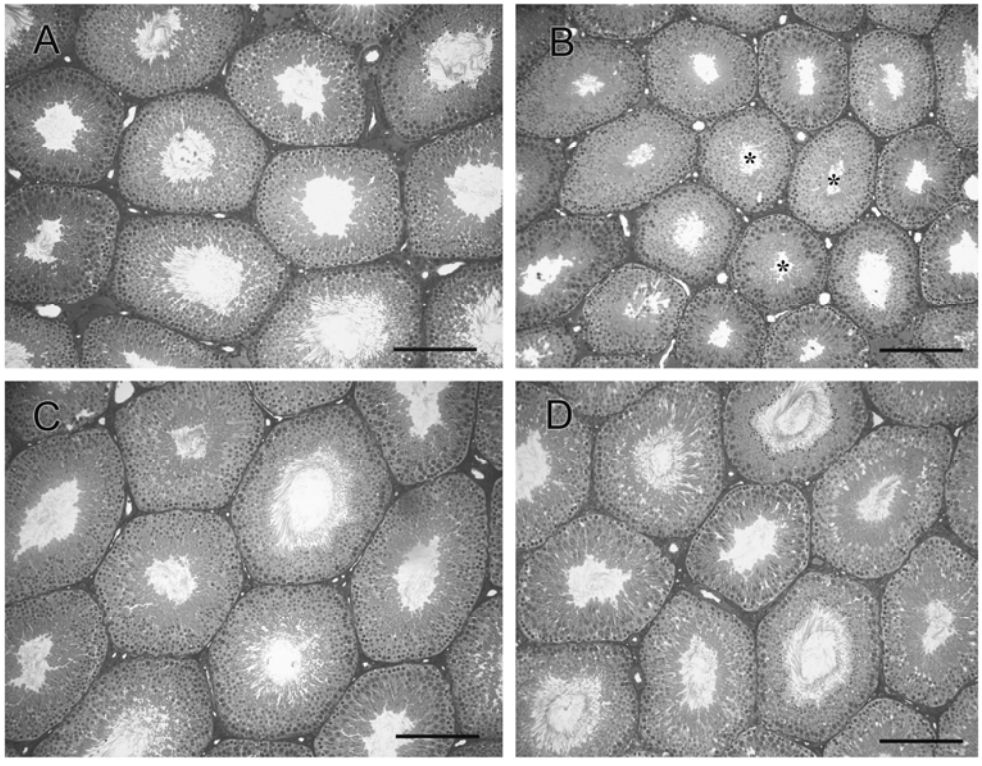


Figure 4. Seminiferous tubule cross sections of male Wistar rats at the ages of 50 (A and B) and 150 days (C and D). Images illustrate the results from the morphometric analyses. Note the reduction in the lumen (asterisks) of 50-day-old CR rats compared to AL at the same age. Bar = 200 µm.

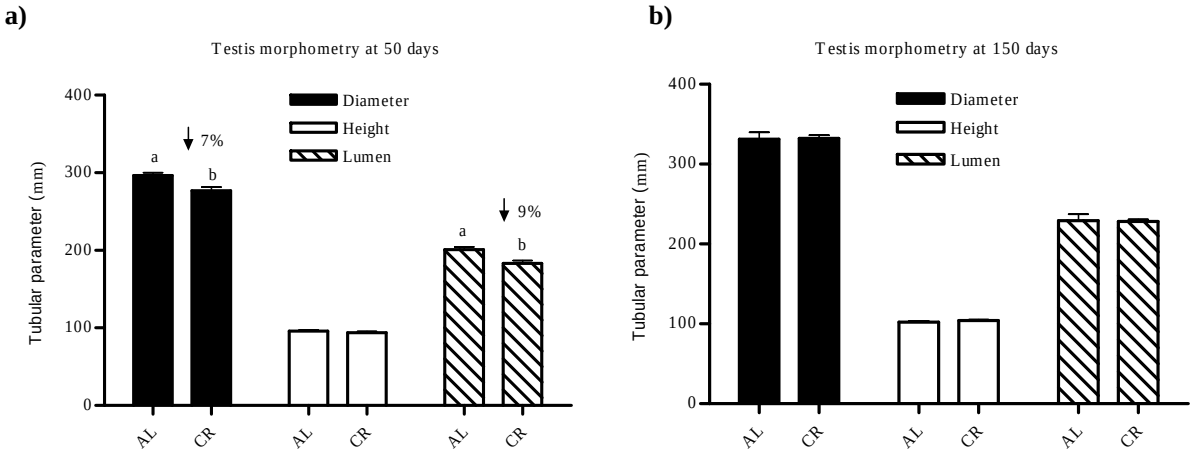


Figure 5. Effects of 30%CR on seminiferous tubule morphometry of male Wistar rats. Tubular diameter, seminiferous epithelial height and luminal diameter at the ages of 50 (A) and 150 days (B). Bars represent means ± SEM, and those that do not share the same letter are statistically different at $P < 0.05$. Numbers preceded by arrows above the CR bars represent the percentage of reduction or increase in relation to the AL group.

Discussion

The present study assessed the effects of CR on several parameters, such as onset of puberty, reproductive organ weight, testis morphometry, fertility and testosterone and cholesterol plasma levels. Although slightly delayed, the day of onset of BPS was not statistically different between CR and AL animals. Contrarily, Léonhardt *et al*. (2003) demonstrated a significant delay in BPS in Wistar rats submitted to calorie restriction during intrauterine life, whereas Delemarre-van de Waal et al. (2002) observed similar delay, but in rats submitted to early postnatal food restriction.. Hence, our results contrast with those and several other reports in the literature (Merry and Holehan, 1979; Holehan and Merry, 1985; Nelson *et al*., 1995; McShane and Wise, 1996; Lane *et al*., 2001; Masoro, 2001; Ottinger *et al*., 2005; Zeinoaldini *et al*., 2006), and might imply that the day of onset of 30%CR was not sufficient to disturb the age of BPS in the Wistar rats studied.

Negative effects of CR on reproductive performance have been widely reported (Holehan and Merry, 1985; Chapman and Partridge, 1996; Masoro, 2001). Nevertheless, in the present study, 30%CR did not impact breeding performance; furthermore, CR animals showed a trend to improved fertility compared to AL animals. This "positive" effect of CR was observed in several studies, which report an extension of reproductive lifespan, in spite of depression of reproduction to some extent (Holehan and Merry, 1985; McShane and Wise, 1996).

The effect of CR on body weight (BW) is often observed in the literature (see review in Fontana and Klein, 2007), and the current study corroborates with that finding. Along with the reduction in BW, at 50 days we observed a reduction in absolute weights of seminal vesicles, prostate and epidydimal fat pad, but testis and epididymis absolute weights were not impacted by 30%CR. At 150 days, only the epidydimal fat pad absolute and relative weights were decreased, while the relative weights of the testis and epididymis were increased. Contrasting in part with our results, Martin *et al*. (2007) subjected Sprague-Dawley rats to 20%CR and 40%CR, and reported an increase in absolute and relative weight of the testes, in spite of a significant reduction in BW. The observation of increased relative weights of the testes at both ages studied, and of epididymides and seminal vesicles in 150-day-old rats, might be related with the trend of better fertility parameters found in the present study.

A very important finding in our investigation was the consistent reduction of both absolute and relative weight of epididymal fat pads at both ages assessed. As it is already known, epididymal fat pads represent a reliable predictor of body fat (Eisen and Leatherwood, 1976; Rogers and Webb, 1980), and their reduction by CR is indicative of improved body condition (see review in Fontana and Klein, 2007).

At both ages evaluated in the present study, peripheral levels of cholesterol and testosterone were not significantly affected by CR. As it is usual for rodents, testosterone levels are notably variable, probably due to the pulsatile fashion of LH secretion (Bartke *et al*., 1973; Bartke and Dalterio, 1975). Regarding cholesterol levels, there are reports on a reduction of this parameter after CR treatment (see review in Masoro, 2002). Despite this, we did not find a decrease in cholesterol levels in the CR rats evaluated in our study.

To our knowledge, there are no reports in the literature on the effect of CR on testicular morphometry. The smaller tubular and luminal diameters observed in 50-day-old rats under CR may reflect a slight retardation in testicular development caused by this treatment, particularly on the aspects related to Sertoli cell maturation and fluid secretion (lumen formation) that are under androgen regulation (Sharpe, 1994; Auharek and França, 2010). As observed in our study, there was a trend for lower testosterone plasma levels in CR rats. Interestingly, CR animals seemed to recover later on, since by 150 days, no differences were noted for tubular diameter and lumen size between treated and control groups. Furthermore, ventral prostate weight was reduced by CR at age 50 days, and was not different between CR and controls at age 150 days, corroborating our assumption of accommodation/catch-up in reproductive parameters after a longer period of CR.

In conclusion, the present investigation covered the effects of 30%CR on several biometric, reproductive, and morphometric parameters in male Wistar rats from weaning to ages 50 and 150 days. To summarize, the CR applied led to a reduction in BW and epididymal fat pad weight, which is a good predictor of life-long improved body conditions, while it did not impact BPS, fertility performance or testosterone and cholesterol levels. Overall, the relative weights of several parameters evaluated were improved by CR in adult rats. Therefore, we suggest that the effects of 30%CR on male Wistar rats are non-deleterious for reproduction and have potential benefits for health.

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