A. Marcelly de Souza Santos[1]
M. R. Ferraz[2]
C. V. Teixeira[1]
F. J. B. Sampaio[1]
C. da Fonte Ramos[1]

Effects of Undernutrition on Serum and Testicular Testosterone Levels and Sexual Function in Adult Rats

Abstract

The aim of this study was to evaluate testosterone concentrations, sexual behavior, and androgen receptor protein level in the testes of rats submitted to protein- and energy-restricted diets during 30 days. Adult male Wistar rats were assigned to one of the following groups: (C) control, diet with 23% of protein; (PR) protein-restricted, diet with 8% of protein; (ER) energy-restricted, diet with 23% of protein in restricted quantities. Mount number, ejaculation latencies and copulatory efficiency were evaluated to determine sexual behavior. At the end of the experiment, the animals were sacrificed to determine serum and testicular testosterone concentrations as well as testicular androgen receptor protein level. Compared to the C group, the ER group presented a significant decrease in body (36%), testis (20%) and epididymis (14%) weights in serum (78%) and testicular (68%) testosterone concentrations as well as in copulatory efficiency (26%). On the other hand, the ER group presented a significant increase in mount number (114%) and ejaculatory latency (62%). The androgen receptor protein levels were significantly reduced in both PR and ER groups (41% and 74%, respectively). This is the first paper to demonstrate that the effect of undernutrition on reproduction is not related to reduced protein intake but caloric restriction. Also, in caloric restriction, there is a relationship between sex behavior, androgen receptors, and testosterone concentration.

Key words
Malnutrition · Rats · Androgen receptor · Testosterone · Sex behavior

Introduction

Androgens are important to sperm production in the testes, sperm maturation in the epididymis, and development and function of accessory glands [1]. Androgen receptors (AR) are members of the steroid/thyroid hormone receptor gene superfamily and, like other steroid receptors, are intracellular receptors that function as ligand-dependent transcription factors [2,3]. Steroid receptors have also been shown to be regulated by their own ligands in a process termed autoregulation [4].

In many androgen target tissues, androgens promote downregulation of AR mRNA levels [5–8], although androgenic upregulation of receptor mRNA has been observed in a few tissues [9–11]. The physiological significance of androgen receptor autoregulation has not been established; however, cellular AR concentration correlates relatively well with the extent of androgen responsiveness, suggesting that autoregulation of AR may influence hormonal sensitivity [12].

The sexual behavior of male rat is characterized by repeated approaches and mounts on the female. It has been demonstrated that sexual behavior is produced by interaction among 4 distinct

Affiliation
[1] Urogenital Research Unit, State University of Rio de Janeiro, Rio de Janeiro, Brazil
[2] Department of Pharmacology and Psicobiology, State University of Rio de Janeiro, Rio de Janeiro, Brazil

Correspondence
Dra. C. da Fonte Ramos · Urogenital Research Unit · UERJ · Av. 28 de Setembro, 87 · fundos · FCM · terreo, 20551-030, Rio de Janeiro, RJ, Brazil · Phone: +55(21)2587-6499 · Fax: +55(21)2587-6121 · E-Mail: cramos@uerj.br

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mechanisms – initiation factor, intromission count factor, hit rate factor, and copulatory rate factor [13]. Further studies have shown that mount and intromission latencies are related to the initiation factor, allowing evaluation of the arousal component of sexual behavior; intromission count factor-related mount and intromission number and hit rate factor-related copulatory efficiency allow to evaluate the erectile response. Finally, ejaculatory latency and intercopulatory intervals are both related to the copulatory rate factor, allowing the evaluation of the ejaculatory component of sexual behavior [14].

Undernutrition is known to have a wide variety of effects on endocrine systems [15 – 17]. Regarding the reproductive system, it has been shown that food restriction can inhibit both the maintenance and onset of reproductive capability [18].

In adult rats, food restriction can reduce body weight [19] as well as testes, epididymis, and prostate weights [20]. The serum concentration of LH, FSH and testosterone are also reduced by undernutrition [19 – 25]. Malnutrition has been shown to lead to morphological gonadotrophic alterations that are typical of those found in cells whose secretory activities are suppressed [23].

The majority of research presented in the areas of nutrition, reproduction and reproductive behavior is limited to females. Wade et al. [26] showed that undernutrition could change the sexual behavior of female hamster (inhibition of lordosis) and it can be related to the reduction observed in the number of estrogen receptors in the ventromedial hypothalamus. Dong et al. [27] showed that the number of androgen receptors is increased in the pituitary. Fernandez et al. [28] showed that early undernutrition produces sexual behavior dysfunction in male rats, which could be reversed by feeding oil-enriched diets.

Despite the fact that undernutrition-related reproductive suppression in rats is a well-documented phenomenon, we have as yet no knowledge about how undernutrition affects the androgen receptors protein level in the reproductive tissues and the specific arousal and consummatory components of adult male rat sexual behavior. These data would be very important to understand the potential effects of undernutrition on reproductive function in humans.

Therefore, to understand how undernutrition can affects the sexual function, the present study aims to compare the effects of protein and energy malnutrition in the testicular androgen receptors protein level, in serum and testicular testosterone concentrations, and in the sexual behavior of adult male rats.

Materials and Methods

Adult male Wistar rats were kept in a room with controlled temperature (25 ± 1 °C) and artificial dark-light cycle (lights on from 7:00 a.m. to 7:00 p.m.). The use and handling of experimental animals followed the principles described in the Guide for the Care and Use of Laboratory Animals [29], and the project was approved by the local Ethical Committee for the care and use of laboratory animals.

Table **1** Composition of control and protein-restricted diets

	Control §	*Protein-Restricted* *
Ingredients (g/Kg)		
Soybean + Amino acids	230.0	80.0
Corn starch	676.0	826.0
Soybean oil	50.0	50.0
Vitamin mix †	4.0	4.0
Mineral mix †	40.0	40.0
Macronutrient composition (%)		
Protein	23.0	8.0
Carbohydrate	66.0	81.0
Fat	11.0	11.0
Total energy (KJ/Kg)	17038.7	17038.7

§ Standard diet for rats (Nuvilab-Nuvital ltd., Paraná, Brazil).
* The protein-restricted diet was prepared in our laboratory by using the control diet with some of its protein content replaced by corn starch. The amount of the latter was calculated to replace the same energy content of the control diet.
† Vitamin and mineral mixtures were formulated to meet the American Institute of Nutrition AIN-93G recommendation for rodent diets [30].

The animals were randomly assigned to one of the following groups, with 10 animals in each group: (C) control group, with free access to a standard laboratory diet containing 23% protein; (PR) protein-restricted group, with free access to an isoenergetic and protein-restricted diet containing 8% protein; and (ER) energy-restricted group, receiving standard laboratory diet restricted to 50% of that consumed by the PR group. The low-protein diet was prepared in our laboratory and its composition is shown in Table **1**. Vitamin and mineral mixtures were formulated to meet the American Institute of Nutrition AIN-93G recommendation for rodent diets [30].

The animals received the protein and energy restricted diets for 30 days. To evaluate the nutritional state, the food consumption and body weight were monitored throughout the experiment. During this period, sexual behavior was analyzed 3 times, the last on day 30. At the end of the experiment, all the animals were slaughtered under thiopental anesthesia (0.15 ml/100 g body weight), always in the morning. The blood was collected by cardiac puncture and the serum kept at – 20 °C. The testes were excised, dissected, weighted, and kept at – 70 °C for subsequent androgen receptor determination by Western blot.

0.5 g of each testis were homogenized in 500 µl TEG buffer (50 mM TRIS pH 7.4, 1.5 mM EDTA, 50 mM NaCl, glycerol 10%, 5 mM DTT, 10 µg/ml leupeptin). The homogenate was centrifuged at 100,000 g for 120 min at 4 °C. The proteins extracted from the supernatants were measured using Bradford's method [31] and analyzed by Western blot technique. 80 µg of total protein was loaded in each lane of 8% polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membrane, and the detection of specific proteins was performed using AR-specific antibody (Rabbit polyclonal 200 µg/ml, Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase IgG was used as a secondary antibody, followed by autoradiography using ECL detection reagents supplied by Amersham (Braunschweig,

Germany). Bands were computer-scanned and their relative intensities determined by densitometry using Scion Image software.

The testosterone serum concentration was determined using specific radioimmunoassay for each hormone. The intra- and interassay variation coefficient was 4.6% and 7.5% for testosterone, and 6.4% and 5.9% for estradiol, respectively. The intratesticular testosterone concentration was performed after testes homogenization in 10 ml of TEG buffer.

Sexual behavior

Each rat was tested for sexual capability 3 times, with an interval of 7 days between each test. Sexual behavior observations were performed between 4:00 a.m. and 7:00 a.m. during the lights-off period in the cycle. Male rats were placed in a standard cage (80 × 80 × 120cm). After 10 minutes for adaptation to a standard wooden cage with a transparent front side (80 × 80 × 120 cm), a sexually receptive female rat was introduced in the cage and the matting test started. The following measures were recorded or calculated: ejaculatory latency, time from the first intromission to ejaculation; mount number, the number of the mounts without intromission prior to ejaculation; copulatory efficiency, a measure of intermissive success (calculated as percentage of mounts in which the male gained vaginal insertion). The observations of sexual behavior were performed by the same investigator, and the recorded tapes of the observational periods were reviewed subsequently by the research group for validation of the results. Females rats were brought into estrous through subcutaneous injections of 100 µg/kg of estradiol benzoate dissolved in corn oil 72 h and 48 h before testing, and 500 µg/kg medroxyprogesterone acetate 5 h before testing.

Open field test

Immediately before mounting tests, each rat was tested in open field arena to evaluate locomotion and exploratory behavior [32].

The data was reported as mean ± standard deviation. Mount number were analyzed by Kruskal-Wallis test followed by Mann-Whitney U-test. All other parameters were analyzed by one-way ANOVA followed by the Newman-Keuls test [33]. The level of significance was set at $p < 0.05$.

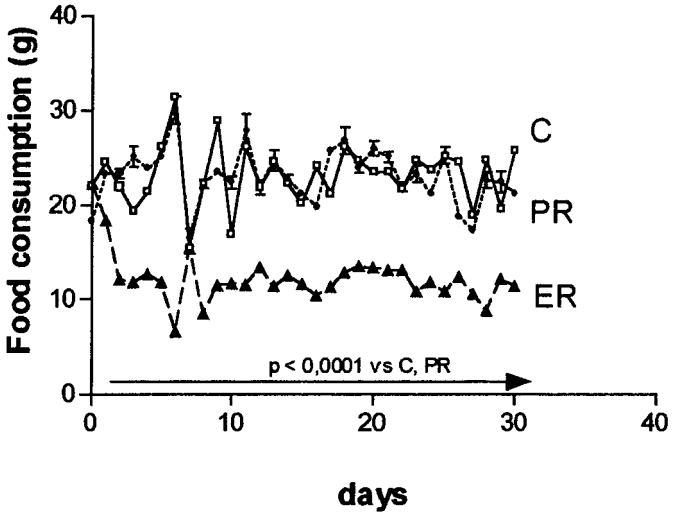

Fig. **1** Food consumption in adult rats in the control group (C), protein-restricted group (PR), and energy-restricted group (ER) during the entire treatment period. Values are given as the mean ± standard deviation of 8 animals.

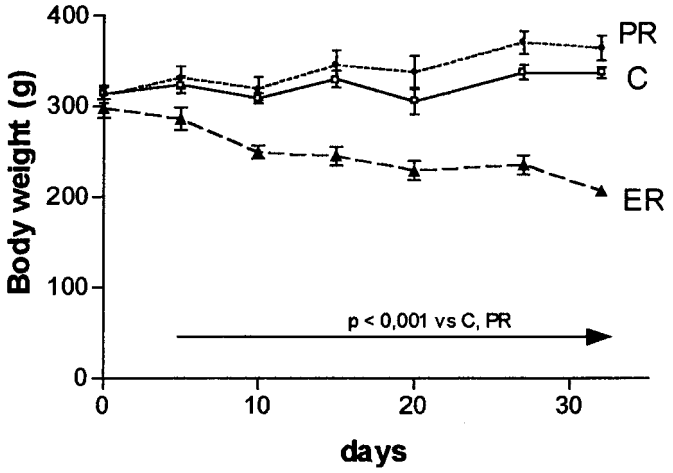

Fig. **2** Body weight of adult rats in the control group (C), protein-restricted group (PR), and energy-restricted group (ER) during the entire treatment period. Values are given as the mean ± standard deviation of 8 animals.

Results

Fig. **1** shows food consumption of the groups throughout the experiment. The PR and C groups consumed the same quantity of food, while the ER group consumed 50% less than C and PR groups as described in the **Materials and Methods** section.

The body weight of the ER group was significantly decreased compared to the C and PR groups ($p < 0.001$). The protein-restricted diet did not alter the body weight of the animals (Fig. **2**).

The absolute testis and epididymis weights were significantly decreased in the ER group ($p < 0.05$), while the weight of these organs relative to the body weight were significantly increased ($p < 0.05$). The protein-restricted diet did not alter the organ weights of the animals (Fig. **3**).

Fig. **4** shows the sexual response of the groups during the third mounting test. The ER group showed a significant increase in mount number and ejaculatory latency and a reduction in copulatory efficiency ($p < 0.05$). The animals submitted to a protein-restricted diet did not show any significant changes in sexual behavior. Neither protein nor energy diet changed locomotion and exploratory behavior.

The serum and testicular testosterone concentration were only significantly reduced in the ER group, $p < 0.05$ (Fig. **5**).

Fig. **6** shows that the androgen receptors were significantly reduced in both PR and ER groups ($p < 0.05$).

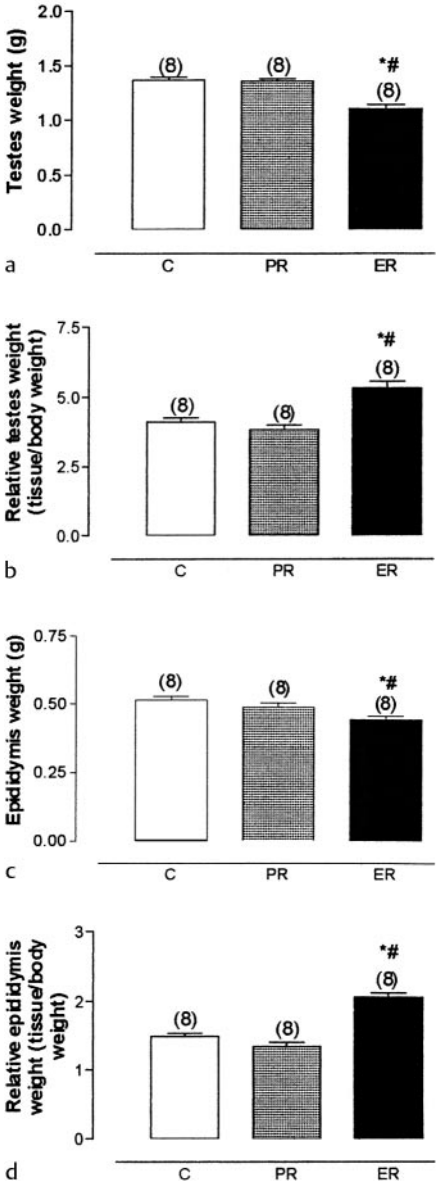

Fig. **3** Absolute testis (**a, b**) and epididymis (**c, d**) weights and their relation to the body weight of adult rats at 30 days of treatment in the control group (C), protein-restricted group (PR), and energy-restricted group (ER). Values are given as the mean ± standard deviation. The number of animals studied is shown in parentheses. * $p < 0.05$ *vs.* C; # $p < 0.05$ *vs.* PR.

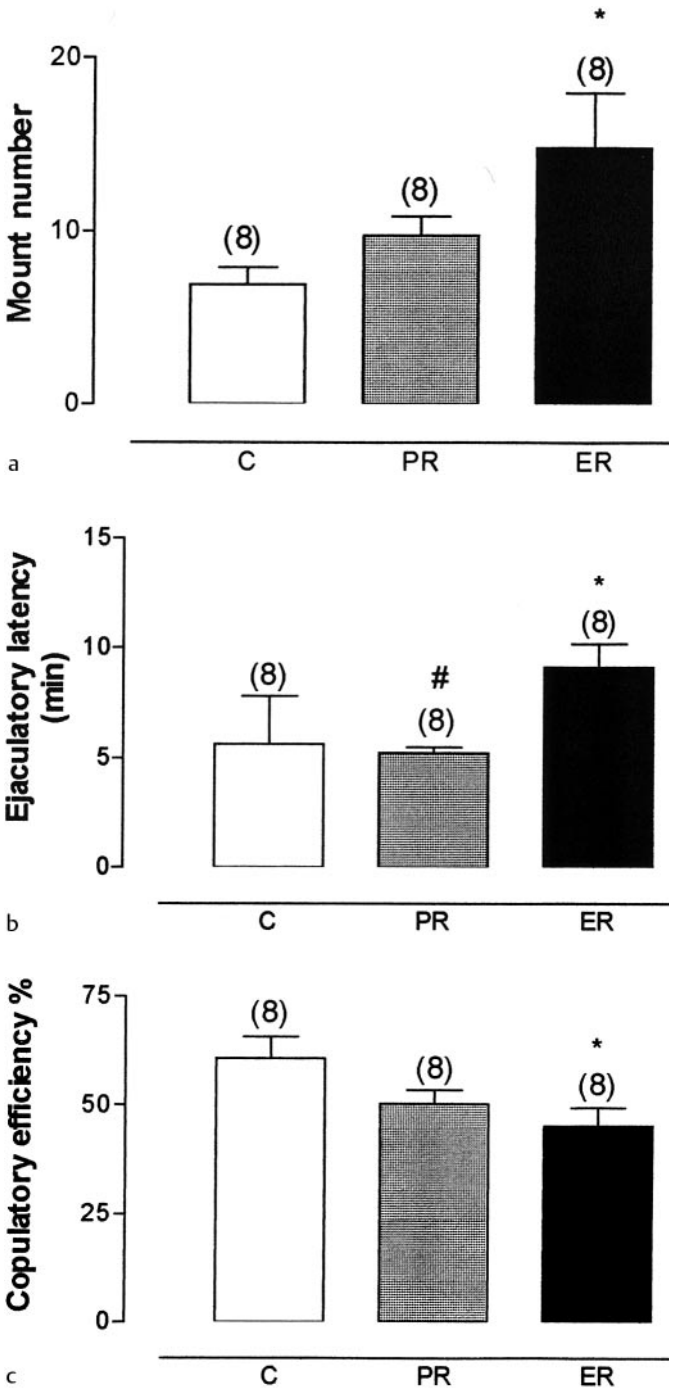

Fig. **4** Sexual behavior of adult rats at 30 days of treatment. The mount number (**a**), ejaculatory latency (**b**), and copulatory efficiency (**c**), in the control group (C), protein-restricted group (PR), and energy-restricted group (ER). Values are given as mean ± standard deviation. The number of animals studied is shown in parentheses. * $p < 0.05$ *vs.* C; # $p < 0.05$ *vs.* PR.

Discussion

Although the individual components of this study do not appear to be novel, the combination of all measures along with sexual behavior study appears to be unique. So, the present study provides new insight into the effects of energy restriction on the androgen receptors protein level in the testes and on the sexual behavior of adult male rats.

In this study, we have shown that the effects on reproduction are not related to reduced protein intake since all parameters studied in this group, except for the androgen receptor protein level, were unchanged. The protein-restricted diet did not alter food consumption, body or organ weights of the animals. The ingestion of protein-restricted diets seems to be associated with a reduction in food consumption with consequent reduction in body and organ weights in lactating [16, 34, 35] and pre-pubertal rats [23, 36 – 38].

The animals submitted to energy-restricted diet presented a reduction in body weight corresponding to the low ingestion. Chick et al. [19] also showed that adult animals submitted to energy restriction presented a reduction in body weight. Therefore, these results show that the effects of protein- and energy-restricted diets depend on the animals' age.

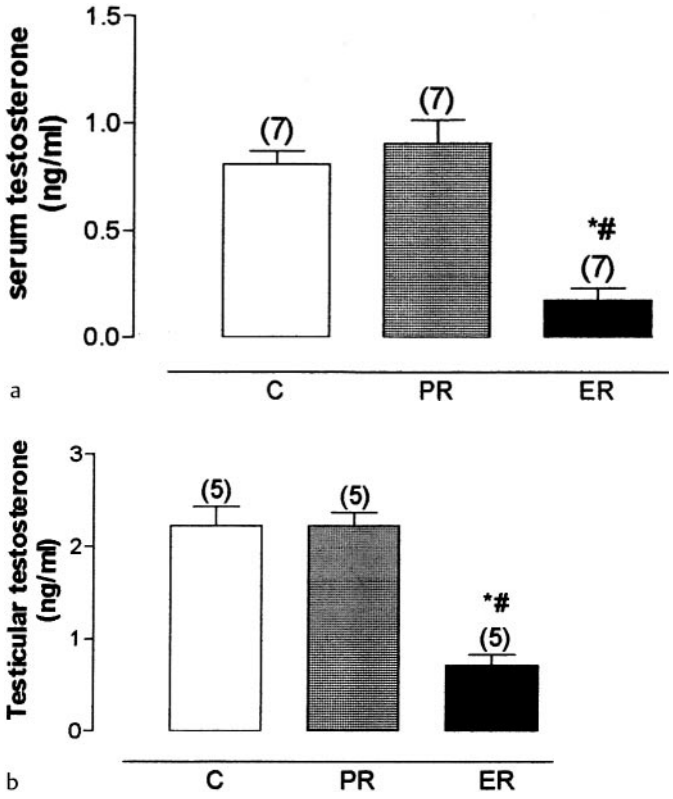

Fig. **5** Serum (**a**) and testicular (**b**) concentrations of testosterone of adult rats at 30 days of treatment in control group (C), protein-restricted group (PR), and energy-restricted group (ER). Values are given as mean ± standard deviation. The number of animals studied is shown in parentheses. * $p < 0.05$ *vs.* C; # $p < 0.05$ *vs.* PR.

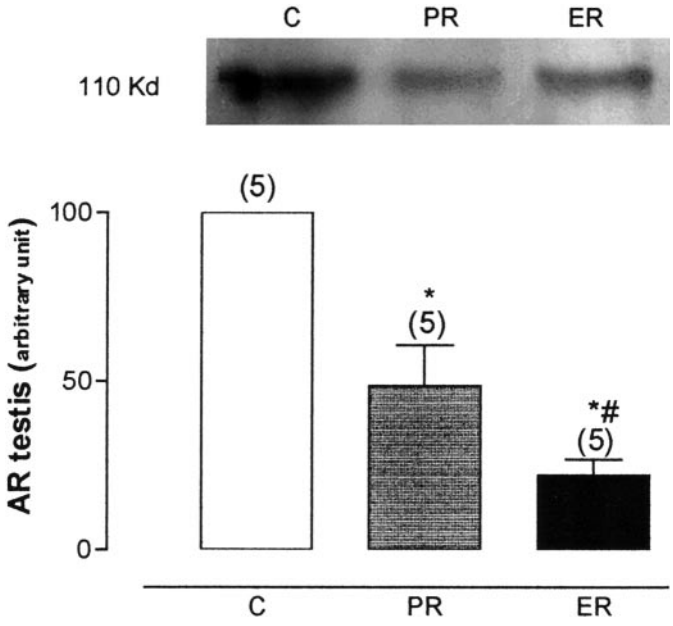

Fig. **6** Western blot analysis of testicular androgen receptor protein level of adult rats at 30 days of treatment in the control group (C), protein-restricted group (PR), and energy-restricted group (ER). 80 µg of total protein was added in each lane. Values are given as the mean ± standard deviation. The number of animals studied is shown in parentheses. * $p < 0.05$ *vs.* C; # $p < 0.05$ *vs.* PR.

Testes and epididymal weights were significantly reduced, while the relative weights of these organs increased in the energy-restricted group. So, we can conclude that the reduction in testis and epididymal weights is due the reduction in body mass observed in this group. However, we cannot discard the possibility of low testosterone concentrations causing the weight fall. Independently of the reason of this observation, these findings show that the energy restriction did not directly affect the testes, rather the entire organism.

The serum and testicular testosterone concentrations were only decreased in the energy-restricted group, and these findings are consistent with previous studies [19–21,24]. Some authors have showed that energy restriction leads to a low serum LH concentrations [19,22,25,27]. So, the reduction observed in testosterone concentration in the energy-restricted group may be due to the lack of pituitary stimulus, leading to low testicular synthesis and therefore low serum concentration of testosterone.

Several studies have suggested that sexual response is produced by interaction among distinct mechanisms probably involving different neural components [14]. The male rat sexual behavioral analysis showed that the energy-restricted diet significantly decreased both erectile response and ejaculatory components, but not the arousal component. These effects could be related to an inhibition on penile erection mechanisms [14,39] as sexual drive was not significantly modified by the energy-restricted diet. On the other hand, the delay in ejaculation could also be due to difficulty in achieving vaginal intromissions. However, we do not discard the hypothesis of a specific inhibition in ejaculatory component. The protein-restricted diet did not change the male rat sexual response compared to control rats. Although sexual drive appears to be increased, it is not significant, so these data are consistent with normal serum and testicular concentration of testosterone. In conclusion, the dramatic reduction in testosterone levels could be related to a decreased ability to achieve penile erection among rats on the energy-restricted diet, and the lack of significant effects on sexual behavior and on testosterone levels in protein-restricted diet rats appears to support this hypothesis.

In the energy-restricted group, there was a lack of all components of the diet including lipid derivatives, which could explain the alterations in the body and organ weights, testosterone concentration, androgen receptors, and sexual behavior. So, these findings show that the effect on reproduction is not related to a reduced protein intake, but to caloric restriction.

Curiously, in the protein-restricted group, the androgen receptor protein level was the only parameter that was altered by the treatment. Some authors showed that steroid receptors can be regulated by their own ligands, a process termed autoregulation [4]. Androgens promote downregulation of AR mRNA levels in the canary liver [11] and in the ventral prostate of rats [40], although androgenic upregulation of receptor mRNA has been observed in the canary kidney [11] and in the smooth-muscle cells of the rat's penis [9]. At protein level, androgens also promote down-regulation in the prostate and seminal vesicle [40] and up-regulation in the androgen-independent prostate cancer cell line [6]. We did not find any study on this topic in relation to testis.

We showed that in the protein-restricted group, androgen receptor protein levels were reduced, while serum concentrations of testosterone were normal. In the energy-restricted group, both serum testosterone and androgen receptor protein level were reduced, which could suggest that an autoregulation for the androgen receptor in the testes does not exist. However, it is known that dihydrotestosterone has four times more affinity to the androgen receptor than testosterone [41]. So, we can not discard a possibility of receptor autoregulation through this hormone. It had been shown that specific aliphatic unsaturated fatty acids can inhibit human or rat microsomal 5 alpha reductase activity [42]. It is possible that this enzyme could have its activity reduced in face of low protein intake, which is the common characteristic of both diets leading to a reduction in the synthesis of dihydrotestosterone and consequently reduction in the androgen receptor protein level, independently of testosterone concentration. However, further studies are necessary to evaluate the autoregulation of this receptor in the testis, specially evaluating the 5 alpha reductase mRNA and activity.

Estrogen receptors, specially ERβ, are widely expressed in the male reproductive tract [43 – 45]. Furthermore, three out of the four mouse models deficient in estrogen (action or synthesis) show infertility [46 – 48]. This suggests that the local aromatization of testosterone to estrogen is an alternative route by which testosterone might regulate male reproductive function [49]. Turner et al. [50] showed that only AR immunostaining in the testis was subject to modulation by either testosterone or estrogen. Transfection studies have demonstrated that the estradiol-ER complex can inhibit AR-mediated transcriptional activity [51]. The accumulating data would suggest that, *in vivo*, gene expression is under multihormonal control such that activation or suppression will depend on a variety of factors including the hormonal environment of the cell, the complement of the individual steroid receptors and their isoforms [51 – 53], and the availability of multiple co-regulatory proteins [54].

This is the first study to show that the effect of undernutrition on reproduction is not related to reduced protein intake but caloric restriction. Also, there is a relationship between sex behavior, androgen receptors and testosterone concentration under caloric restriction.

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