

## RESEARCH

# Low-protein diet in puberty impairs testosterone output and energy metabolism in male rats

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## Abstract

We examined the long-term effects of protein restriction during puberty on the function of hypothalamic–pituitary–adrenal (HPA) and hypothalamic–pituitary–gonadal (HPG) axes in male rats. Male Wistar rats from the age of 30 to 60 days were fed a low-protein diet (4%, LP). A normal-protein diet (20.5%) was reintroduced to rats from the age of 60 to 120 days. Control rats were fed a normal-protein diet throughout life (NP). Rats of 60 or 120 days old were killed. Food consumption, body weight, visceral fat deposits, lipid profile, glycemia, insulinemia, corticosteronemia, adrenocorticotrophic hormone (ACTH), testosterone and leptinemia were evaluated. Glucose–insulin homeostasis, pancreatic-islet insulinotropic response, testosterone production and hypothalamic protein expression of the androgen receptor (AR), glucocorticoid receptor (GR) and leptin signaling pathway were also determined. LP rats were hypophagic, leaner, hypoglycemic, hypoinsulinemic and hypoleptinemic at the age of 60 days ( $P < 0.05$ ). These rats exhibited hyperactivity of the HPA axis, hypoactivity of the HPG axis and a weak insulinotropic response ( $P < 0.01$ ). LP rats at the age of 120 days were hyperphagic and exhibited higher visceral fat accumulation, hyperleptinemia and dyslipidemia; lower blood ACTH, testosterone and testosterone release; and reduced hypothalamic expression of AR, GR and SOCS3, with a higher pSTAT3/STAT3 ratio ( $P < 0.05$ ). Glucose–insulin homeostasis was disrupted and associated with hyperglycemia, hyperinsulinemia and increased insulinotropic response of the pancreatic islets. The cholinergic and glucose pancreatic-islet responses were small in 60-day-old LP rats but increased in 120-day-old LP rats. The hyperactivity of the HPA axis and the suppression of the HPG axis caused by protein restriction at puberty contributed to energy and metabolic disorders as long-term consequences.

## Key Words

- ▶ metabolic programming
- ▶ insulin resistance
- ▶ insulinotropic
- ▶ pancreatic islets
- ▶ hypothalamic–pituitary–gonadal axis

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## Introduction

Insulin resistance and obesity are complex metabolic disorders that exhibit several etiologies. Metabolic disorders in adult life are associated with disturbances in neuroendocrine pathways that are malprogrammed during critical stages of life (Ravelli et al. 1999, de Oliveira et al. 2013). The worldwide pandemic of obesity and type 2 diabetes mellitus (T2DM), especially in developing countries, is associated with caloric energy restriction during critical stages of development (Barker 2004, Uauy et al. 2011); this restriction contributes to metabolic syndrome.

Hyperactivity of the hypothalamic–pituitary–adrenal (HPA) axis (Wang 2005) and hypoactivity of the hypothalamic–pituitary–gonadal (HPG) axis (Mauvais-Jarvis 2011) contribute to metabolic disturbances and lead to insulin resistance, T2DM and obesity. In males, testosterone deficiency may promote insulin resistance and increase the risk of T2DM (Kapoor et al. 2005, Grossmann et al. 2010) and visceral obesity (Jones 2010b, Hamilton et al. 2011). Cortisol/corticosterone excess also contributes to insulin resistance and visceral obesity (de Guia & Herzig 2015). A maternal low-protein diet is also associated with hypotestosteronemia (Chamson-Reig et al. 2009) and hypercorticosteronemia (Reyes-Castro et al. 2012) in offspring, which link these hormone alterations with nutritional disturbances in critical stages of life.

Protein restriction during (de Oliveira et al. 2013) or just after puberty (Malta et al. 2014) malprograms rats to exhibit higher visceral fat depots, insulin secretion and inappropriate autonomic nervous system responses in adulthood. Indeed, young adolescent women exposed to moderate or severe malnutrition during the Dutch famine episode in 1944–1945 exhibited high T2DM risk in adulthood (van Abeelen et al. 2012). Together, these data emphasize that metabolisms of both animals and human are vulnerable to protein-calorie restriction during puberty, leading to long-term consequences. Rising levels of gonadal steroid hormones during puberty program the sensitivity of the adult HPA axis to gonadal steroids in adulthood (Evuvarherhe et al. 2009). Notably, a reciprocal interaction between these endocrine systems is evident. Sex steroid hormone output and HPG function are inhibited during stress conditions, and HPA function depends on gonadal–adrenal interaction in the paraventricular nucleus of the hypothalamus (Viau 2002, Retana-Marquez et al. 2003).

The present study hypothesized that protein restriction during puberty would negatively affect male

rat testosterone production, which is associated with dysfunction in the HPA axis and may malprogram body composition and glucose homeostasis in adulthood. Therefore, we aimed to assess HPA and HPG axis markers, including body composition, the hypothalamus–leptin signaling pathway, lipid profile and function of the pancreatic islets, in rats fed a low-protein diet throughout puberty followed by a period of dietary reestablishment. We thus aimed to elucidate reciprocal interactions between the HPA and HPG neuroendocrine systems involved in metabolic syndrome later in life.

## Materials and methods

### Diet treatment and animal groups

The Ethical Committee for Animal Experiments of the State University of Maringá, which adheres to Brazilian Federal Law, approved this protocol. Rats were maintained in groups of 5 per cage under controlled conditions (temperature:  $22 \pm 2^\circ\text{C}$ ; photoperiod: 07:00–19:00h; and water and food *ad libitum*) throughout the protocol.

Male Wistar rats aged 30 days were randomly assigned to two different groups ( $n=40$  rats per group). Control rats were fed a normal-protein diet *ad libitum* (20.5% protein, Nuvital, Curitiba/PR, Brazil) throughout the experimental protocol (normal-protein group, NP), and the protein-restricted rats (low-protein group, LP) received an isocaloric low-protein diet (4% protein) from the age of 30 to 60 days. Diets were composed as previously described (Malta et al. 2016) and as shown in Table 1. The salt and vitamin mixture used in the manufactured diet follows AIN-93 recommendations (Reeves et al. 1993).

**Table 1** Composition of the low- and normal-protein diets.

Diet components	Normal protein (20.5%)		Low protein (4.0%)	
	(g/kg)	(kJ/kg)	(g/kg)	(kJ/kg)
Sucrose	127.2	2.129	200.0	3.347
Cornstarch	527.5	8.828	642.5	10.753
Casein (88% protein)	233.3	3.905	45.5	0.761
Mix of mineral salts*	32.0	–	32.0	–
Mix of vitamins*	16.0	–	16.0	–
Soybean oil	48.0	1.807	48.0	1.807
Fish oil	16.0	0.602	16.0	0.602
Total	1000.0	17.272	1000.0	17.272

The dietary component values are presented as g/kg of diet and the energy in kJ/kg.

\*The salt and vitamin mixture used in the manufactured diet followed the AIN-93 recommendation (Reeves et al. 1993).

One batch of rats ( $n=20$ ), sampled equally from both groups, was killed at the age of 60 days and the other ( $n=20$ ) at the age of 120 days to investigate long-term effects of protein restriction. Tissues and blood samples were collected, and body length and the Lee index were evaluated (Bernardis & Patterson 1968).

### Food consumption and body weight assessment

Food consumption and body weight were assessed every two days throughout the experiment. Absolute food intake was calculated as the difference between the total food administered two days before ( $D_{\text{initial}}$ ) and the amount of food remaining ( $D_{\text{final}}$ ), divided by the number of days and the number of rats per cage:  $(FI_{(g)} = (D_{\text{initial}} - D_{\text{final}}) / 2 / 5)$  (Vicente *et al.* 2004, de Oliveira *et al.* 2011). Spilled food was measured, but it was not significant compared to the amount consumed. Relative food intake was calculated as the value of absolute food intake divided by the mean body weight of the 5 rats in each cage. Daily body weight gain was calculated as the subtraction of each body weight value from the body weight value on the prior day, from 30 until 120 days of age.

### Intravenous glucose tolerance test (ivGTT)

Rats were anesthetized with a ketamine and xylazine mix (3 and 0.6 mg/100 g of body weight, respectively) at the age of 60 or 120 days and were subjected to a surgical procedure to implant a silicone cannula into the right jugular vein. An ivGTT was performed after a 12-h fast (19:00–07:00 h) in conscious rats ( $n=10$ ), as previously described (de Oliveira *et al.* 2011). Glycemia was measured using the glucose oxidase method (Trinder 1969) with a commercial kit (Gold Analisa; Belo Horizonte/MG, Brazil). Insulinemia was measured by the radioimmunoassay (RIA) method (Scott *et al.* 1981) in a Wizard2 Automatic Gamma Counter (PerkinElmer) with  $^{125}\text{I}$ -labeled recombinant human insulin (PerkinElmer). The intra- and inter-assay coefficients of variation for insulin detection were 9.8 and 12.2%, respectively, and the insulin level detection limit was 1.03 pmol/L.

Insulinemia and glycemia values at baseline and following ivGTT were used to calculate the insulin sensitivity index (ISI), which generates a reasonable approximation of whole-body insulin sensitivity. ISI was calculated as follows:  $ISI = 10^4 / \sqrt{(\text{fasting glycemia} \times \text{fasting insulinemia}) \times (AUC_{\Delta\text{Glycemia}} \times AUC_{\Delta\text{Insulinemia}})}$  (Matsuda & DeFronzo 1999).

### Pancreatic islet isolation and insulin secretion

Pancreatic islets from 60- and/or 120-day-old rats ( $n=6$  per group) were isolated using the collagenase technique. Insulin secretion stimulation was adapted to a baseline glucose concentration and stimulated with different glucose concentrations, as previously described (de Oliveira *et al.* 2013, Malta *et al.* 2016). Islets from another group of rats ( $n=6$  per group) were incubated for 60 min in Krebs-Ringer solution containing stimulatory acetylcholine concentrations ( $\mu\text{mol/L}$ ): 0.1; 1.0; 10.0; 100.0 and 1000.0 in the presence of 8.3 mmol/L glucose and 10  $\mu\text{mol/L}$  neostigmine to avoid acetylcholinesterase action. Supernatants from the incubations were collected to examine cholinergic muscarinic function and were stored at  $-20.0^\circ\text{C}$  for further insulin quantification using RIA (Scott *et al.* 1981).

Insulin is expressed as pmol/L for every 4 isolated pancreatic islets.

### Testosterone production

Testosterone production was assessed from the right testicles of 60- and/or 120-day-old rats ( $n=6$  per group). Testicles were decapsulated and sliced into four pieces weighing approximately 75 mg. The slices were incubated with or without 100 IU/mL human chorionic gonadotrophin (hCG; Sigma-Aldrich) for 90 min in 1.5 mL of oxygenated Gibco Cell Culture Medium M199 (Life Technologies) at  $34^\circ\text{C}$  (Noriega *et al.* 2009). Samples were centrifuged at 3000 g for 5 min at  $4^\circ\text{C}$ , and the supernatant (1000  $\mu\text{L}$ ) was collected and stored at  $-80^\circ\text{C}$  for subsequent quantification of testosterone release using an enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, Plymouth Meeting, PA, USA). Testosterone is expressed as pmol/L per gram of testicle tissue.

### Fat store assessment

Rats were killed at the end of all experimental procedures, and retroperitoneal and mesenteric fat pad stores were removed and weighed to evaluate body composition.

### Western blotting

The expression of leptin receptor (ObR-b), the signal transducer and activator of transcription 3 (STAT3), phosphorylated-STAT3 (pSTAT3), Janus kinase 2 (JAK2), phosphorylated-JAK2 (pJAK2), the suppressor of cytokine

signaling 3 (SOCS3), androgen receptor (AR) and glucocorticoid receptor (GR) proteins in hypothalamus homogenates obtained from 60- and 120-day-old rats ( $n=6$ ) were measured using Western blotting, as previously described (Miranda *et al.* 2017).

The following primary antibodies (Santa Cruz Biotechnology) were used: anti-OBR-b, anti-JAK2, anti-pJAK2, anti-STAT3, anti-pSTAT3 and anti-SOCS3 (all at 1:500) and anti-AR, anti-GR and anti- $\beta$ -actin (all at 1:1000). Polyvinylidene difluoride filters were washed three times with 0.1% Tween-TBS, followed by a 60-min incubation with appropriate secondary antibodies conjugated to biotin (Santa Cruz Biotechnology). Membranes were incubated with streptavidin conjugated with horseradish peroxidase (HRP) (Caltag Laboratories, Burlingame, CA, USA). Immunoreactive proteins were visualized using an enhanced chemiluminescence (ECL) prime kit and Image Quant LAS (GE Healthcare). Protein bands were quantified using densitometry in ImageJ 1.4 (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).  $\beta$ -actin protein content (Santa Cruz Biotechnology, diluted 1:1000 in TTBS) was used for normalization. Representative Western blotting images were obtained from the same membranes.

### Lipid profile and leptin, corticosterone, adrenocorticotrophic hormone and testosterone plasma level measurements

Total blood samples were collected from 12-h fasted (20:00–08:00 h) 60- and 120-day-old rats ( $n=10$ ), centrifuged and maintained at  $-80^{\circ}\text{C}$  to assess lipid profile and hormone levels.

Plasma levels of triglycerides, total cholesterol and high-density lipoprotein (HDL)-cholesterol were assessed using a colorimetric method and commercial kits (Gold Analisa; Belo Horizonte, MG, Brazil). Circulating very low-density lipoprotein (VLDL)- and low-density lipoprotein (LDL)-cholesterol levels were quantified using the Friedewald calculation:  $\text{VLDL-cholesterol} = \text{triglycerides}/5$  and  $\text{LDL-cholesterol} = \text{total cholesterol} - (\text{HDL-cholesterol} + \text{VLDL-cholesterol})$ , respectively.

The Castelli index, which predicts the atherogenic index associated with lipid profile, assesses the risk for development of atherogenesis and coronary artery disease (Criqui & Golomb 1998, Olamoyegun *et al.* 2016). The following established formula was used to calculate atherogenic indexes: Castelli index I =  $\text{total cholesterol}/\text{HDL-cholesterol}$ , and Castelli index II =  $\text{LDL-cholesterol}/\text{HDL-cholesterol}$ .

A commercial ELISA kit was used to quantify plasma levels of leptin, corticosterone, testosterone (Enzo Life Sciences) and adrenocorticotrophic hormone (ACTH) (MyBioSource, San Diego, CA, USA). The intra- and inter-assay coefficients of variation were 5.9% and 7.2%, respectively, for leptin, 7.7% and 9.7% for corticosterone, 9.5% and 11.7% for testosterone, and 3.7% and 4.7% for ACTH. Hormone level detection limits were (in pmol/L) 4.20 for leptin, 74.46 for corticosterone, 1967.49 for testosterone and 0.22 for ACTH.

### Statistical analyses

Data are shown as the means  $\pm$  s.e.m., and results were subjected to Student's *t*-tests or one-way ANOVAs when appropriate. The association between plasma corticosterone and plasma testosterone levels was calculated using Pearson's correlation and linear regression analyses. Values of *P* less than 0.05 were considered statistically significant. Tests were performed using GraphPad Prism version 6.0 for Windows (GraphPad Software).

## Results

### Biometric and fasting biochemical and hormonal parameters

LP rats at the age of 60 days were leaner than NP rats and exhibited decreased body weight ( $-72.2\%$ ,  $P < 0.001$ ), body length ( $-30.6\%$ ,  $P < 0.001$ ) and mesenteric ( $-39.3\%$ ,  $P < 0.01$ ) and retroperitoneal fat pads ( $-68.0\%$ ,  $P < 0.001$ , Table 2). LP rats at the age of 120 days exhibited a different phenotype, with only a slight reduction in body weight ( $-3.4\%$ ,  $P > 0.05$ ) and body length compared to NP rats ( $-1.8\%$ ,  $P < 0.01$ ), and higher mesenteric ( $+19.8\%$ ,  $P < 0.01$ ) and retroperitoneal fat pads ( $+29.1\%$ ;  $P < 0.001$ , Table 2).

The body weight gain of LP rats was reduced by 87.7% over the protein restriction period ( $P < 0.001$ , inset of Fig. 1A), and food intake decreased 30.7% ( $P < 0.001$ , inset of Fig. 1B) compared to NP rats. Notably, body weight gain ( $+94.1\%$ ,  $P < 0.001$ , inset of Fig. 1A) and food intake ( $+29.7\%$ ;  $P < 0.001$ , inset of Fig. 1B) in LP rats reverted throughout the period of dietary reestablishment.

LP rats at the age of 60 days were hypoglycemic ( $-27.9\%$ ,  $P < 0.01$ ), hypoinsulinemic ( $-46.7\%$ ,  $P < 0.05$ ), hypoleptinemic ( $-81.6\%$ ,  $P < 0.01$ ) and hypotestosteronemic ( $-10.8\%$ ,  $P < 0.05$ ); additionally, they exhibited higher levels of corticosterone ( $+53.2\%$ ,  $P < 0.01$ ) and ACTH ( $+57.4\%$ ;  $P < 0.01$ , Table 2). The ISI

**Table 2** The effects of protein-calorie restriction during adolescence on biometric and biochemical parameters as short- (60 days old) and long-term (120 days old) consequences in male rats.

Biometric and biochemical parameters	60 days old	120 days old	60 days old	120 days old
	NP	LP	NP	LP
Body weight (g)	267.20 ± 7.69	74.30 ± 3.90***	403.10 ± 5.00	389.40 ± 4.76 <sup>ns</sup>
Body length (cm)	19.97 ± 0.15	13.85 ± 0.30***	23.23 ± 0.11	22.82 ± 0.14*
Mesenteric fat pad (g/100g bw)	0.61 ± 0.06	0.37 ± 0.03**	0.81 ± 0.03	0.97 ± 0.04**
Retroperitoneal fat pad (g/100g bw)	0.78 ± 0.08	0.25 ± 0.03***	1.41 ± 0.08	1.82 ± 0.07***
Glycemia (mmol/L)	5.95 ± 0.11	4.29 ± 0.40**	4.84 ± 0.12	5.41 ± 0.05**
Insulinemia (pmol/L)	30.27 ± 5.96	16.12 ± 2.78*	36.27 ± 5.16	57.43 ± 6.12*
ISI	12.59 ± 1.20	30.77 ± 2.43***	10.64 ± 0.84	7.56 ± 0.60**
Leptinemia (pmol/L)	37.78 ± 7.12	6.97 ± 0.42**	53.21 ± 7.30	109.36 ± 23.58*
Corticosteronemia (µmol/L)	1.26 ± 0.13	1.94 ± 0.08**	1.68 ± 0.07	1.54 ± 0.11 <sup>ns</sup>
ACTH (pmol/L)	41.06 ± 3.96	64.64 ± 8.25**	62.92 ± 2.30	46.17 ± 5.56**
Testosteronemia (pmol/L)	6.57 ± 0.26	5.86 ± 0.17*	6.872 ± 0.28	5.75 ± 0.29*

Data are expressed as the means ± s.e.m. of rats ( $n=20$  for biometric and  $n=10$  for biochemical parameters and ISI calculation) from 4 different litters. Significant differences between NP and LP groups at 60 or 120 days old were compared using Student's *t* test.

\* $P<0.05$ , \*\* $P<0.01$  and \*\*\* $P<0.001$ . ns, not significant

was increased in 60-day-old LP rats (+144.4%;  $P<0.001$ , Table 2). In contrast, 120-day-old LP rats exhibited diminished ISI values (−28.9%;  $P<0.01$ , Table 2), hyperglycemia (+10.6%,  $P<0.01$ ), hyperinsulinemia (+58.3%,  $P<0.05$ ) and hyperleptinemia (+105.5%,  $P<0.05$ ). Corticosteronemia was unaltered ( $P=0.329$ ), ACTH plasma levels decreased (−26.6%,  $P<0.01$ ) and testosteronemia was reduced (−16.3%;  $P<0.05$ , Table 2).

Lipid profiles of the rats at the age of 60 and 120 days are provided in Table 3. LP rats at the age of 60 days exhibited reduced triglycerides (−39.3%,  $P<0.01$ ), increased total cholesterol (+39.8%,  $P<0.01$ ), decreased VLDL-cholesterol (−33.4%,  $P<0.001$ ), increased LDL-cholesterol (+125.7%,  $P<0.001$ ) and increased HDL-cholesterol (+30.1%,  $P<0.001$ ). Castelli indexes I and II increased 10.5% and 119.6%, respectively, in 60-day-old LP rats ( $P<0.01$ , Table 3). LP rats at 120 days old exhibited an increase of +26.8% for triglycerides, +15.5% for total cholesterol, +30.7% for VLDL-cholesterol and +41.4% for LDL-cholesterol, as well as a reduction of 13.1% in HDL-cholesterol values compared to NP rats ( $P<0.05$ , Table 3). Castelli indexes I and II increased +16.2% and 58.4%, respectively, in 120-day-old LP rats ( $P<0.01$ , Table 3).

### Glucose-insulin homeostasis

The 60-day-old LP rats were hypoinsulinemic (−32.0%;  $P<0.05$ , inset of Fig. 2A) and hypoglycemic (−14.3%;  $P<0.05$ , inset of Fig. 2B) according to the ivGTT compared to the NP rats. LP rats were hyperinsulinemic (+36.1%;  $P<0.05$ , inset of Fig. 2C) and exhibited similar glucose levels ( $P=0.122$ , inset of Fig. 2D) as NP rats at 120 days old.

### Glucose and/or acetylcholine insulinotropic response

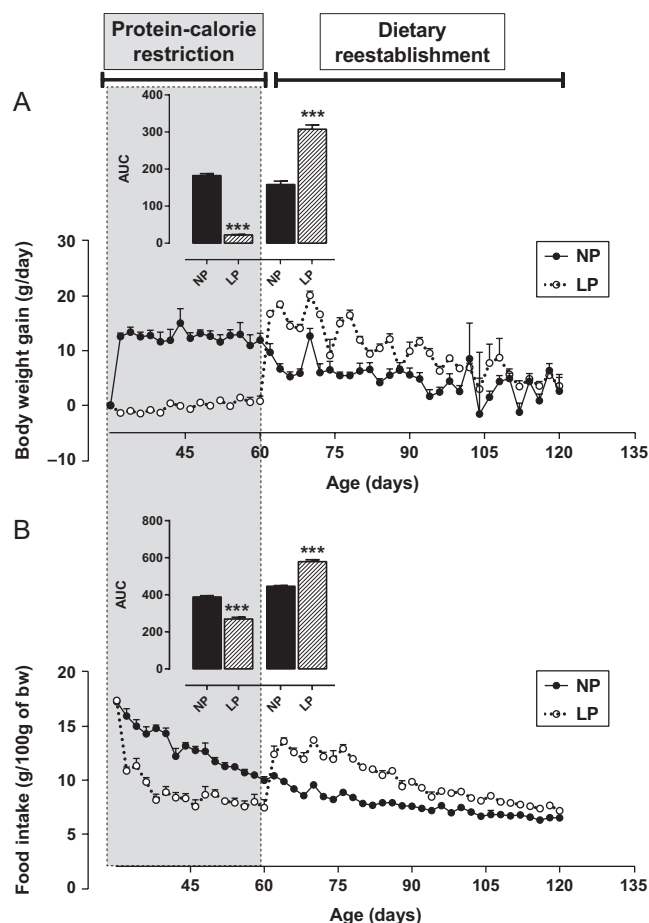
Increasing concentrations of glucose or acetylcholine increased insulin secretion in a dose-dependent manner in both 60-day-old rat groups (Fig. 3). However, the increment of insulin secretion from islets of LP rats was smaller than that of NP rats under glucose ( $P<0.05$ , Fig. 3A) and acetylcholine ( $P<0.05$ , Fig. 3B) stimulation. Conversely, islets from 120-day-old LP rats were more responsive to increasing glucose and acetylcholine concentrations ( $P<0.05$ , Fig. 3C and D).

### Testosterone production/secretion and plasma corticosterone and testosterone correlation

Testosterone secretion in 60-day-old LP rats was reduced by 73.9% ( $P<0.001$ , Fig. 4A). The increment of testosterone production following hCG stimulation was 16.9% in NP rats and 65.4% in LP rats ( $P<0.001$ , Fig. 4A).

Testosterone release from 120-day-old LP rats exhibited a smaller reduction of −12.0% ( $P<0.01$ , Fig. 4B). However, this parameter did not differ for NP rats under hCG stimulation ( $P=0.716$ , Fig. 4B). The magnitude of hCG-stimulated testosterone production in adult rats was +10.7% in NP rats and +19.0% in LP rats compared to production without hCG ( $P<0.01$ , Fig. 4B).

Corticosteronemia showed a significant negative correlation with testosteronemia for both animal groups aged 60 days, with higher magnitude for LP rats (NP:  $r=-0.803$ ,  $P<0.05$  and LP:  $r=-0.971$ ,  $P<0.001$ ; Fig. 4C). At the age of 120 days, the blood values of corticosterone displayed a slight significant negative correlation with



**Figure 1**

Body weight gain (A) and relative food intake (B). Data are presented as the means  $\pm$  s.e.m. of 20 rats from 4 different litters. The left panels, as an inset to each figure, depict the area under the curve (AUC) for both periods of dietary treatment. Bars on the left in each panel depict the AUC for body weight gain and relative food intake during the protein-calorie restriction period (30–60 days old). Bars on the right depict the AUC during the period of dietary reestablishment (60–120 days old). \*\*\* $P < 0.001$  using Student's *t* test.

testosterone for LP rats ( $r = -0.682$ ,  $P < 0.05$ ; Fig. 4D) but did not for NP rats ( $r = 0.165$ ,  $P = 0.671$ ; Fig. 4D).

### Hypothalamic androgen and glucocorticoid receptors

AR protein expression in the hypothalamus was less in 60-day-old LP rats than in NP rats ( $-45.8\%$ ;  $P < 0.05$ , Fig. 5A), and this parameter remained reduced in 120-day-old LP rats ( $-25.3\%$ ,  $P < 0.05$ , Fig. 5A). Expression of hypothalamic GR protein decreased by 79.5% ( $P < 0.001$ , Fig. 5B) in 60-day-old LP rats and 44.3% ( $P < 0.05$ , Fig. 5B) in 120-day-old LP rats compared to NP rats.

### Hypothalamic leptin pathway

As depicted in the Fig. 6A, pubertal protein restriction did not alter hypothalamic ObR-b protein expression in rats aged 60 ( $P = 0.974$ ) or 120 days ( $P = 0.289$ ). The pJAK2/JAK2 ratio in hypothalamic tissue was unaltered in rats aged 60 and 120 days ( $P > 0.05$ , Fig. 6B). The pSTAT3/STAT3 ratio was not altered in 60-day-old LP rats ( $P = 0.443$ , Fig. 6C) but increased 3-fold in 120-day-old LP rats ( $P < 0.001$ , Fig. 6C). SOCS3 protein expression was decreased by 26.5% in 60-day-old LP rats ( $P < 0.05$ , Fig. 6D) and by 49.8% in 120-day-old LP rats ( $P < 0.05$ , Fig. 6D).

## Discussion

The current study demonstrated that dysfunction of the HPA axis together with HPG axis impairment due to pubertal malnutrition was associated with physiological changes in adiposity-related signals, leptin and insulin, according to nutritional status. These results support the

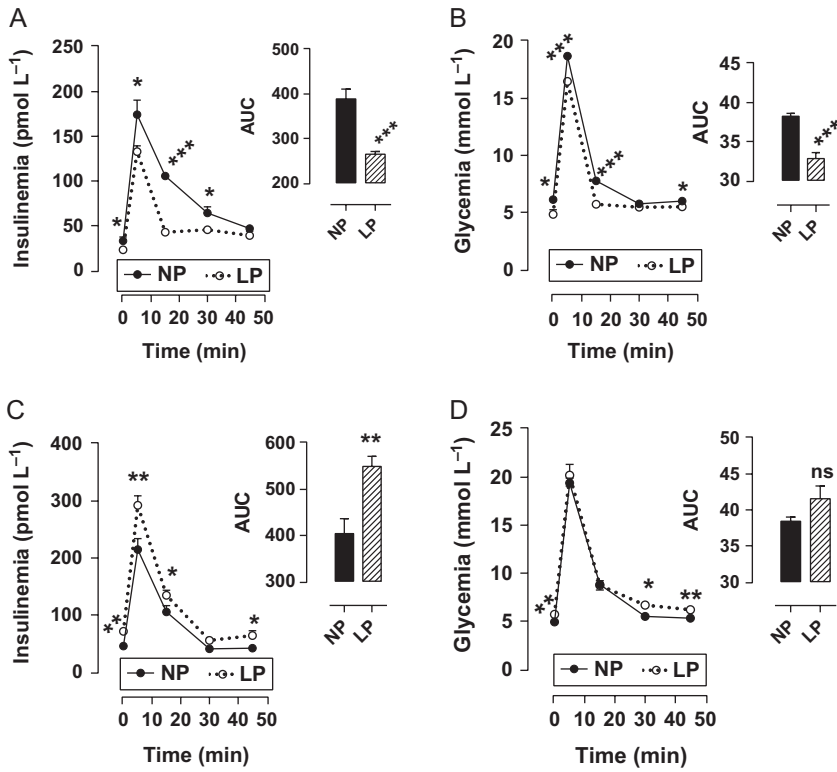
**Table 3** The effects of protein-calorie restriction during adolescence on the lipid profile as short- (60 days old) and long-term (120 days old) consequences in male rats.

Lipid profile ( $\mu\text{mol/L}$ )	60 days old		120 days old	
	NP	LP	NP	LP
Triglycerides	754.0 $\pm$ 70.7	457.5 $\pm$ 26.6**	718.1 $\pm$ 27.8	910.4 $\pm$ 46.9**
Total cholesterol	1831.0 $\pm$ 72.7	2560.0 $\pm$ 150.6**	1887.0 $\pm$ 38.9	2180.0 $\pm$ 93.3*
VLDL-cholesterol	317.1 $\pm$ 20.9	211.3 $\pm$ 10.7***	323.1 $\pm$ 10.7	422.4 $\pm$ 15.7***
LDL-cholesterol	297.7 $\pm$ 37.3	671.9 $\pm$ 71.5**	384.2 $\pm$ 15.2	543.4 $\pm$ 38.8**
HDL-cholesterol	1281.0 $\pm$ 45.8	1666.0 $\pm$ 58.2***	1388.0 $\pm$ 54.6	1206.0 $\pm$ 58.1*
Castelli index I	1.43 $\pm$ 0.02	1.58 $\pm$ 0.03***	1.54 $\pm$ 0.01	1.79 $\pm$ 0.06**
Castelli index II	0.22 $\pm$ 0.02	0.49 $\pm$ 0.04***	0.30 $\pm$ 0.02	0.50 $\pm$ 0.05**

Data are expressed as the means  $\pm$  s.e.m. of rats ( $n = 10$ ) from 4 different litters. The significant differences between NP and LP groups at the age of 60 or 120 days old were measured using Student's *t* test.

\* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ .

LP, Low-protein diet-treated group; NP, Normal-protein diet-treated group.

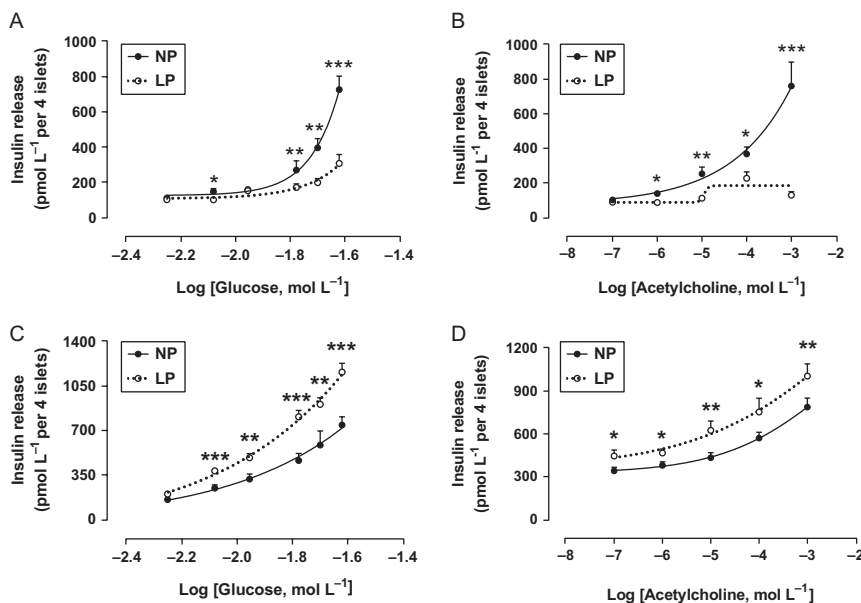


**Figure 2** Insulinemia and glycemia during the intravenous glucose tolerance test (ivGTT). Data are presented as the means  $\pm$  s.e.m. of 10 rats from 4 different litters. Insulinemia (A) and glycemia (B) during the ivGTT at the end of protein-calorie restriction (60 days old). Insulinemia (C) and glycemia (D) during the ivGTT after the period of dietary reestablishment (120 days old). The upper panel of each figure represents the area under the curve (AUC). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  by Student's *t* test.

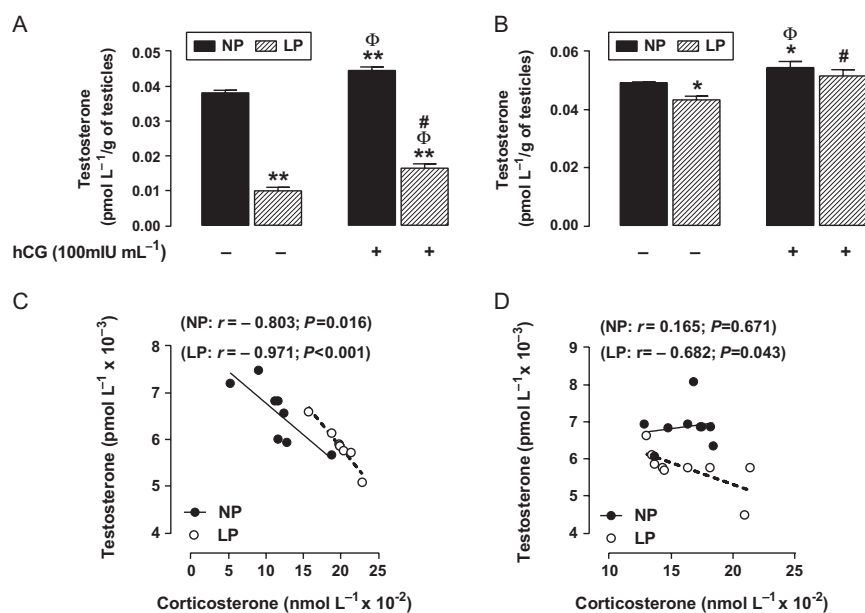
hypothesis that hyperactivity in the HPA axis, as well as hypoactivity of the HPG axis in puberty, is critical for inducing metabolic dysfunction in males during adulthood (de Oliveira *et al.* 2013, Malta *et al.* 2014, 2016). It is likely that human illnesses such as Cushing's syndrome and hypogonadotropic hypogonadism are associated with functional alterations in cortisol, leptin and insulin and that these alterations induce obesity,

cardiovascular disease and T2DM (Reynolds *et al.* 2001, Grumbach 2002, Prodam *et al.* 2013, Reynolds 2013).

We further speculate that protein restriction during puberty can impair neuroendocrine pathways in the hypothalamus that secondarily may program insulin resistance, dyslipidemia and pancreatic-islet insulinotropic disrupted responses in adult LP rats; however, further experiments are necessary to clarify



**Figure 3** Short- and long-term effects of pubertal protein-calorie restriction on insulin secretion. Data are presented as the means  $\pm$  s.e.m. of insulin secretion from the pancreatic islets of 6 rats from 3 different litters. Insulinotropic effect of different glucose (A and C) and acetylcholine (B and D) concentrations refers to data obtained from rats at the end of protein-calorie restriction (60 days old) and after dietary reestablishment (120 days old). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  using Student's *t* test.

**Figure 4**

Short- and long-term effects of pubertal protein-calorie restriction on testosterone output and the correlation between corticosteronemia and testosterone. Data are presented as the means  $\pm$  S.E.M. of testosterone output for isolated testicles of 6 rats from 3 different litters.

Testosterone output without (-) and with (+) hCG 100 IU/mL at the end of protein-calorie restriction (60 days old, A) and after the dietary reestablishment (120 days old, B). Correlation between plasma levels of corticosterone and testosterone in rats of 60 days old (C) and those of 120 days old (D). Symbols over the bars depict significant differences between NP and LP values of testosterone output with and without hCG, as determined using a one-way ANOVA, in which \* $P < 0.01$ , \*\* $P < 0.001$  compared to NP without hCG;  $\Phi P < 0.001$  compared to LP without hCG; and # $P < 0.001$  compared to NP with hCG.

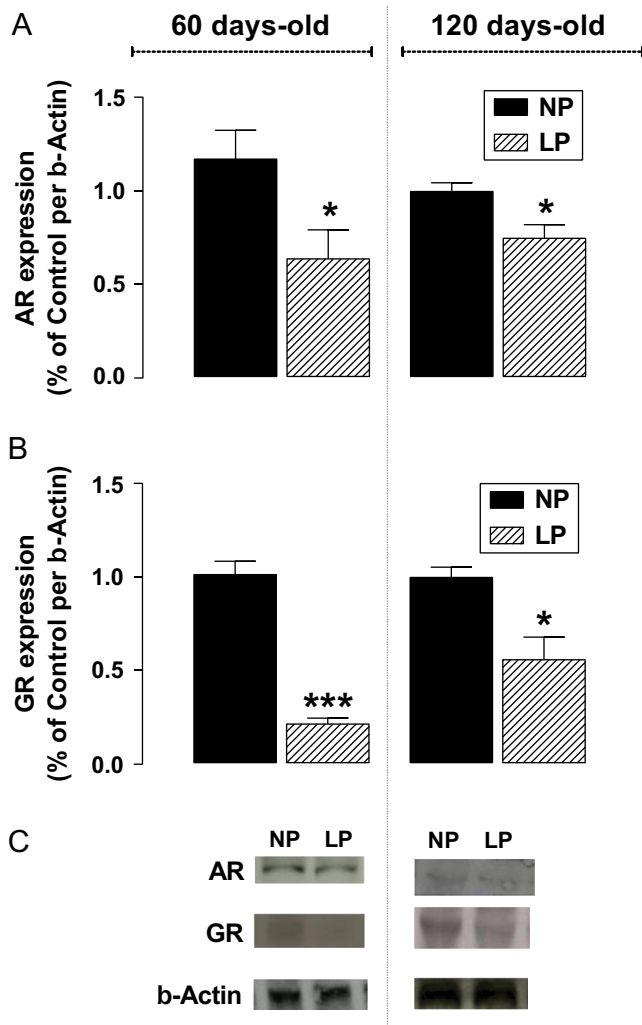
this possibility. Pancreatic islets from 60-day-old LP rats exhibited impaired insulin secretion during glucose and acetylcholine stimulation, leading to a low insulinotropic-cholinergic response at this age. In contrast, dietary reestablishment reverted it to pancreatic islet over function in 120-day-old LP rats, which suggests a greater responsiveness to glucose and cholinergic signaling. Hyperactivity of the parasympathetic tonus in this rat model during adulthood is associated with insulin resistance and obesity (de Oliveira *et al.* 2013), and it modulates pancreatic function as a compensatory response for the high metabolic demand, which increases the risk of T2DM onset. In addition, an LP diet decreases vagal tonus as a direct effect (Leon-Quinto *et al.* 1998) and as an indirect and long-term consequence (de Oliveira *et al.* 2011); this decrease is associated with pancreatic beta-cell dysfunction.

The role of corticosterone in the HPG axis under stress conditions inhibits reproductive function (Viau 2002). This interaction is strongly associated with the regulation of energy homeostasis, especially during puberty (Gomez & Dallman 2001). Circulating corticosteroid levels decrease the pituitary response to gonadotrophin-releasing hormone (GnRH), which reduces luteinizing hormone (LH) release and negatively affects gonadal hormone production (Tilbrook *et al.* 2000, Viau 2002). Sixty-day-old LP rats exhibited higher blood levels of corticosterone and ACTH, which were associated with lower testosterone and testosterone secretion. Therefore, protein restriction during puberty seems to exert an inhibitory effect on the HPG axis that can be mediated through hyperactivity

of the HPA axis. Notably, testosterone production in LP rats at the end of protein restriction was reduced and remained reduced and lower in magnitude in 120-day-old rats after dietary reestablishment, even with hCG in the media. The magnitude of hCG-induced testosterone production was 3.9-fold higher in 60-day-old LP rats and 1.8-fold higher in 120-day-old LP rats compared to NP rats. Therefore, we suggest that the testicles of LP rats exhibited a normal response to gonadotropins, and these changes occur in the hypothalamus or pituitary. One limitation of our study was that we did not measure plasma LH or hypothalamic GnRH. However, at the age of 60 days, hypercorticosteronemia was inversely correlated with testosterone in LP rats; this finding suggests that hyperactivity of the HPA axis at this age causes persistent inhibition of HPG function in LP rats that remains into adulthood. In fact, the strength of this correlation was not observed in adulthood, since ACTH was also lower, and corticosterone was unchanged. HPA activation during the acute stress of protein scarcity is prominent in the malprogramming of metabolism, and a hyperactive HPA axis may decrease HPG function and impair reproductive capacity to preserve energy stores (Gomez & Dallman 2001).

Leptin, insulin, corticosterone and testosterone were altered in 60-day-old LP rats, which suggests complex cross-talk between these hormones. These hormones affect body mass and energy metabolism and imprint an altered regulatory pattern as a long-term consequence. As previously reported, hypoglycemia induces afferent signals that reach the brainstem, especially





**Figure 5**  
Hypothalamic measurement of AR (A) and GR (B) protein expression. Data are presented as the means  $\pm$  s.e.m. of AR and GR protein expression in hypothalamus samples of 6 rats from 3 different litters. Representative blots of AR, GR and  $\beta$ -actin (control load) are shown in (C). Mean values from the hypothalamic protein expression of AR and GR from LP rats at the end of protein-calorie restriction (60 days old) or after dietary reestablishment (120 days old) were significantly different from those in the NP group: \* $P < 0.01$  and \*\*\* $P < 0.001$  using Student's *t* test.

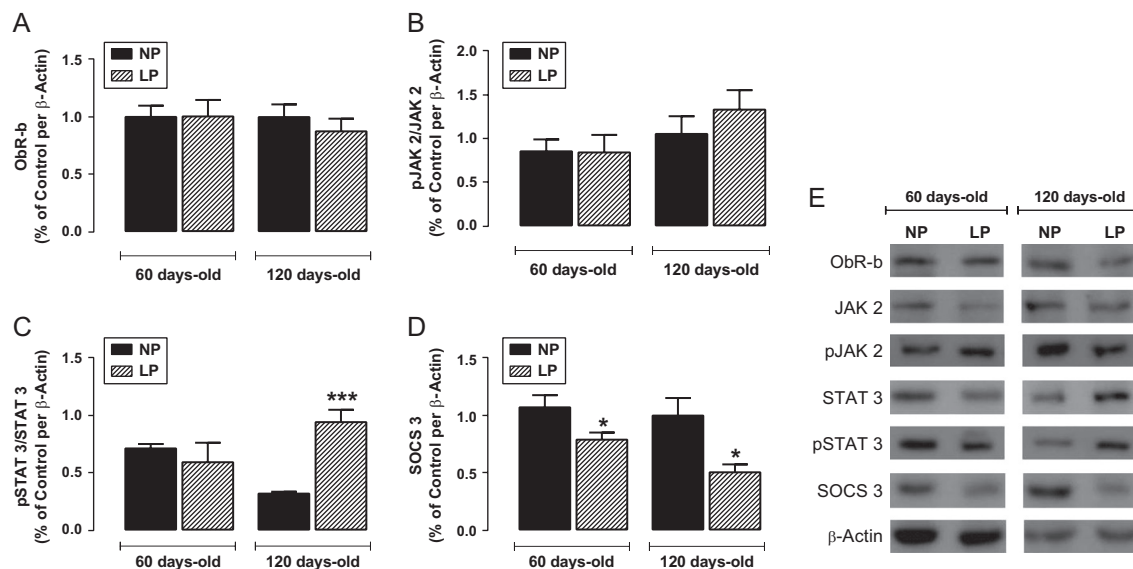
the nucleus of the tractus solitarius, which activates hypothalamic paraventricular neurons to produce/ secrete corticotrophin-releasing hormone (CRH) and activate the HPA axis (Marty *et al.* 2007). An inhibitory effect of leptin on CRH and ACTH production/secretion in stress conditions was reported previously (Heiman *et al.* 1997, Morton *et al.* 2015). Thus, hyperactivity of the HPA axis in 60-day-old LP rats may be explained by both hypoglycemia and hypoleptinemia. Hyperglycemia and hyperleptinemia were found in 120-day-old LP rats after dietary reestablishment, which also decreased ACTH.

Leptin hypofunction in humans and rodents, which is found in malnutrition, is associated with reduced HPG function (Grumbach 2002). However, even with hyperleptinemia at 120 days of age, those animals had hypotestosteronemia, suggesting a hypothalamic leptin resistance that was not confirmed by evaluation of the leptin signaling pathway in the hypothalamus.

Dyslipidemia, insulin resistance and accumulated higher visceral fat stores in the 120-day-old LP rats may be associated with hypotestosteronemia (Cameron *et al.* 2016). Castelli indexes I and II were increased, suggesting a high-risk factor for cardiovascular diseases in 120-day-old LP rats. Hypotestosteronemia promotes visceral obesity and insulin resistance in males, and it is a risk factor for cardiovascular diseases associated with T2DM in humans and rodents (Kapoor *et al.* 2005, Grossmann *et al.* 2010, Jones 2010a, b). The physiological role of testosterone on body and energy homeostasis was highlighted in obese men exposed to testosterone therapy (Saad *et al.* 2016). A lack of testosterone signaling by ARs in adipocytes promotes a metabolic syndrome pattern in mice, with glucose-insulin disruption and susceptibility to high-fat diet-induced visceral obesity (McInnes *et al.* 2012). High cortisol and LDL-cholesterol levels were also specifically associated with hypertension in obese children and adolescents (Prodham *et al.* 2013). Alterations in lipid profile may be related to the lower testosterone levels that were observed during the two periods evaluated, because both groups of LP rats were dyslipidemic in rats aged 60 and 120 days.

GR expression in hypothalamus was lower at 60 days of age, probably due to a down-regulatory role of hypercorticosteronemia in these animals, which helps explain why they still exhibited increased ACTH despite higher corticosteronemia. Conversely, if GR was constitutively lower due to protein-calorie restriction, then higher corticosteronemia would have been necessary to suppress ACTH. AR expression in the hypothalamus was decreased due to protein restriction, and lower testosterone production led to a marked decrease in testosterone action. Notably, these receptors remained decreased after dietary reestablishment, despite some recovery. These changes did not sufficiently alter the action of the hormone ligands, except in the case of GR, where the partial recovery may be responsible for the suppression of ACTH and corticosterone normalization.

Leptin and insulin signaling in hypothalamic neuronal circuitry strictly control food intake and energy balance (Schwartz *et al.* 2000, Woods *et al.* 2008, Anderson *et al.* 2016). Changes in the protein expression of leptin signaling in the hypothalamus cannot explain why these

**Figure 6**

Hypothalamic measurement of protein expression of the leptin signaling pathway. Data are presented as the means  $\pm$  s.e.m. of ObR-b (A), pJAK2/JAK2 (B), pSTAT3/STAT3 (C) and SOCS3 (D) protein expression in hypothalamus samples of 6 rats from 3 different litters of each group. Representative blots of ObR-b, pJAK2, JAK2, pSTAT3, STAT3, SOCS3 and  $\beta$ -actin (control load) are shown in (E). Significant differences between the LP and NP values of hypothalamic protein expression were determined using Student's *t* test. \* $P < 0.01$  and \*\*\* $P < 0.001$ .

animals ate less during protein restriction. LP rats were hyperphagic at dietary reestablishment, because the only alteration observed was lower SOCS3 at both ages and an increase in pSTAT3/STAT3 ratio at 120 days old. Other hormones, such as insulin, also regulate these proteins, and changes in leptinemia did not explain these alterations. Changes other than leptin or insulin may exert direct actions, or other leptin and insulin signaling pathways may promote hyperphagia and/or attenuate catabolic-pathway activation in adult LP rats (Schwartz *et al.* 2000, Chong *et al.* 2015).

Leptin regulates the HPG axis and increases testosterone, which regulates leptin synthesis in white adipocytes (Ramos & Zamoner 2014). We suggest that hypotestosteronemia and hypercorticosteronemia play a critical role in malprogramming, which affects the hypothalamic control of energy homeostasis in adulthood.

Our data support the hypothesis that protein restriction during puberty impairs functional interaction between the HPA and HPG axes and leads to dyslipidemia, visceral fat accumulation, hyperinsulinemia and insulin resistance associated with a disrupted insulinotropic response in the pancreatic islets. These results highlight the fact that persistent neuroendocrine changes imprinted by stress conditions during puberty lead to metabolic syndrome as a long-term consequence in LP rats. These results suggest a risk factor for development of long-lasting obesity, cardiovascular diseases and T2DM.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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#### Author contribution statement

J C O, P C F M, E G M and P C L conceived and designed the study; J C O, R A M and L F B collected and analyzed data and wrote the manuscript; A M P M, I P M, C C S F, R M G, T A R and A M collected and analyzed data; J C O, R A P and E P S C contributed to Western blot analyses; J C O, P C F M, E G M, R A M and L F B contributed to discussion and reviewed the manuscript. All authors have read and approved the final manuscript.

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