British Journal of Nutrition, page 1 of 12 doi:10.1017/S0007114524002149

Neonatal undernutrition induced by litter size expansion alters testicular parameters in adult *Wistar* rats

Ana Camila Ferreira Menezes[1‡], Ana Luiza Machado Wunderlich[2‡], Karen Gomes Luiz[1], Giovanna Fachetti Frigoli[1], Ivana Regina D Costa[1], Larissa Rugila dos Santos Stopa[2], Camila Franciele Souza[3], Rhauany Pelisson Guergolette[2], Polyana Keiko Shishido[2], Ana Beatriz Oliveira Aquino[4], Simone Forcato[3], Daniela Cristina Ceccatto Gerardin[2,3,4†], Cássia Thaïs Bussamra Vieira Zaia[2,3,4], Ernane Torres Uchoa[2,3,4]* and Glaura Scantamburlo Alves Fernandes[1,2]

[1]*State University of Londrina, Biological Sciences Centre, Department of General Biology, Laboratory of Toxicology and Metabolic Disorders of Reproduction, Londrina, Brazil*
[2]*Postgraduate Program in Physiological Sciences, State University of Londrina, Londrina, Brazil*
[3]*Multicentre Postgraduate Program in Physiological Sciences, State University of Londrina, Londrina, Brazil*
[4]*Department of Physiological Sciences, State University of Londrina, Londrina, Brazil*

(Submitted 27 December 2023 – Final revision received 16 August 2024 – Accepted 5 September 2024)

Abstract

Several models of maternal undernutrition reveal impairment of testicular development and compromise spermatogenesis in male offspring. The expansion of the litter size model, valuable for studying the impact of undernutrition on early development, has not yet been used to evaluate the consequences of early undernutrition in the adult male reproductive system. For this purpose, pups were raised in either normal litter (ten pups/dam) or large litter (LL; sixteen pups/dam). On postnatal day 90, sexual behaviour was evaluated or blood, adipose and reproductive tissues were collected for biochemical, histological and morphological analysis. Adult LL animals were lighter and thinner than controls. They showed increased food intake, but decrease of retroperitoneal white adipose tissue weight, glycaemia after oral glucose overload and plasma concentration of cholesterol. Reproductive organ weights were not altered by undernutrition, but histopathological analysis revealed an increased number of abnormal seminiferous tubules and number of immature spermatids in the tubular lumen of LL animals. These animals also showed reduction in total spermatic reserve and daily sperm production in the testes. Undernutrition decreased the number of Sertoli cells, and testosterone production was increased in the LL group. Mitochondrial activity of spermatozoa remained unchanged between experimental groups, suggesting no significant impact on the energy-related processes associated with sperm function. All animals from both experimental groups were considered sexually competent, with no significant difference in the parameters of sexual behaviour. We conclude that neonatal undernutrition induces histological and physiological testicular changes, without altering sperm quality and sexual behaviour of animals.

Keywords: **Malnutrition: Developmental programming: Reproduction: Testes: Sexual behaviour**

The nutritional status during critical windows in early development is thought to program the onset of major diseases in adulthood[(1)]. The term programming is used to describe the process by which exposure to specific environmental stimuli or insults can trigger adaptations that result in permanent changes to the physiology of the organism, which then permit the development of the neonate to their current environment[(2–4)]. In line with this, early-life undernutrition has been associated with several postnatal risk factors with an increased propensity for the development of cardiovascular diseases, type 2 diabetes and hypertension, all of them related to metabolic syndrome[(5)].

Abbreviations: DAB, 3,3′diaminobenzidine tetrahydrochloride; GTT, glucose tolerance test; LL, large litter; NL, normal litter; PND, postnatal day; UEL, State University of Londrina.

* **Corresponding author:** Dr Ernane Torres Uchôa, email euchoa@uel.br

† Deceased (02/03/2024)

‡ These authors contributed equally to this work.

Within the context of the Developmental Origin of Health and Disease, the manipulation of litter size (change in the number of pups per litter during lactation) has been instrumental in demonstrating the biological plausibility of the programming theory and to study the short- and long-term effects of neonatal nutrition[6]. The importance of early nutrition was initially demonstrated by manipulating litter sizes in rats such that body weight of weanling pups tended to vary inversely with the number of the littermates, due to the competition for the supply[7]. In accordance, recent studies have shown that lactation undernutrition, induced by increasing litter size, can have long-lasting effects on body weight[8,9], adiposity and some mediators involved in energy homeostasis[1,8,10,11].

In addition, in males, maternal food restriction during postnatal development led to disturbed reproductive performance of the progeny and damaged testicular structural development[12], mainly related to decreased diameter of the seminiferous tubules in rats at weaning[13]. Moreover, maternal nutrient restriction during late gestation and/or lactation was associated with reduction in circulating concentrations of follicle-stimulating hormone and compromised spermatogenesis in adult male rats[14]. This impairment of spermatogenesis was associated with a reduction in Sertoli cell number, observed both in Sprague-Dawley rats[15] and sheep submitted to fetal to pubertal undernutrition[16]. According to the study of Bansal-Rajbanshi and Mathur[17], in the testes of undernourished rat pups, the cell generation cycle of spermatogonial germ cells and supporting cells (future Sertoli cells) on day 9 of age showed marked prolongation of DNA synthetic phase, indicating a depression in DNA synthesis in undernutrition.

These data are an important observation in the context of developmental programming, as Sertoli cells number is maximised at puberty and is the major determinant of adult sperm production, which suggests that the proliferation and differentiation of Sertoli cells are vulnerable to early-life undernutrition, leading to long-lasting impacts on the adult male reproductive system[14]. However, as far as it is known, no information regarding the influence of postnatal undernutrition, by increasing the litter size, on the male reproductive system has been reported so far. Thus, this study aimed to verify the effects of metabolic programming, through the litter size expansion model, on biometric, metabolic and reproductive parameters of adult male rats.

Material and methods

Animals

Experiments were performed in male *Wistar* rats obtained from the mating of male and female rats from the Central Animal Care Facility of the State University of Londrina (UEL). Day 1 of pregnancy was determined by the presence of sperm on the vaginal smear of the female on the morning following mating. Pregnant dams were then housed in individual cages until the litter's birth. At birth, postnatal day (PND) 0, male and female pups were allocated to either control litters (normal litter – NL), with ten pups, or to large litters (LL), with sixteen pups. Male-to-female ratio was 1:1 in all litters. When necessary, pups from different litters (pups born on the same day) were used to reach the total number of pups in the litter and the proportion of males and females. The surplus pups were euthanised by decapitation. Only male rats were evaluated in this study, and each experimental group contained pups from at least five different litters. After weaning, on PND 21, male animals were housed in groups of four to five rats of the same experimental litter in each cage.

Animals were housed at the Sectoral Animal Care Facility of the Department of Physiological Sciences/UEL and kept under controlled and adequate conditions of light (12 h light/dark cycle) and temperature (22 ± 2°C), with free access to tap water and pelleted rat chow (Nuvilab CR1, Nuvital®). Experiments were performed on PND 90 at the Departments of Physiological Sciences and General Biology/UEL. All experimental procedures were approved by the Ethics Committee of Animal Use for experimentation – UEL (OF. CIRC. CEUA 13/2021 – CEUA nº 18310.2019.03).

Assessment of neonatal undernutrition on biometric and metabolic parameters

Animals were weighed at birth, on PND 0 and on PND 3, 7, 10, 14, 17 and 21. After weaning, animals were weighed every 5 d until PND 90. Body weight was expressed in grams (g). Food intake was measured every 5 d from weaning until PND 90, considering the difference between the supply and the leftover food. Food intake was expressed as g/100 g of body weight.

The Lee index, expressed as $g^{1/3}$/cm, was evaluated at weaning and at the end of experimental protocol, on PND 90. The Lee index was calculated considering body weight and naso-anal distance; the cubic root of body weight in grams was divided by naso-anal length in centimetres[18].

Assessment of neonatal underfeeding on glucose tolerance test

In a first set of animals, on PND 89, after 6 h of food deprivation, a drop of tail blood was collected at 14.00 for basal glucose determination using the Accu-Chek® Active test strip (Roche, Taquara, RJ, Brazil) and a device for blood glucose determination. Next, animals received an intraperitoneal injection of glucose (1·0 g/kg body weight) at 25 % concentration and then blood glucose measurements were taken from the test strip 15, 30, 60 and 120 min after glucose overload. Animals were replaced back in the cages at the end of the test[19].

Assessment of neonatal underfeeding on metabolic parameters and tissues weight

In this first set of animals, on PND 90, after 6 h of food deprivation, animals were euthanised by decapitation at 14.00, and trunk blood was immediately collected in heparinised tubes and centrifuged at 14 000 × g per 20 min at 4°C to obtain the plasma. Plasma was stored at −20°C and used for biochemical analysis.

Measurement of cholesterol, TAG, corticosterone and testosterone plasma levels. The spectrophotometric determination of cholesterol and TAG plasma concentrations was

performed using the BioLiquid Cholesterol Commercial Kit (Laborclin, PR) and BioLiquid TAG GPO-Trinder Commercial Kit (Laborclin, PR), respectively, according to the manufacturer's protocol, and values were expressed as mg/dl. The fluorometric method, based on the fluorescence of corticosterone in sulphuric acid, was used to determine corticosterone plasma concentration[20]. The analysis was performed on a fluorometer (Victor3TM, PerfinElmer) with excitation at 477 nm, emission 520 nm and sensitivity 11. Concentration of corticosterone was expressed in μg/dl. Plasma testosterone levels were assessed by a competitive enzyme immunoassay kit (EIA-1559; DRG Instruments GmbH), according to manufacturer's instructions. The minimum and maximum detection levels were 0·2 and 16 ng/ml, and the intra assay coefficients of variation were 3·2 %. Testosterone was expressed as ng/ml.

Collection of tissue and organs. Immediately after decapitation, visceral white adipose tissues, comprising the epididymal and retroperitoneal subdivisions, brown adipose tissue, testis and epididymis, were carefully removed and weighed. Tissue weights were expressed as g/100 g of body weight. Spermatozoa from the vas deferens were collected to determine sperm motility and morphology and mitochondrial activity.

Assessment of neonatal underfeeding on testes and epididymis histological parameters in adulthood

Histological processing. The left testes were removed and fixed in a methacarn solution (10 % acetic acid, 60 % methanol and 30 % chloroform) for 3 h at 30°C, as described by Ogo *et al.*[21] The testes were embedded in Paraplast® (SIGMA Life Science). Three non-consecutive sections (5 μm thick) per animal, separated by 100 μm distance, were obtained, mounted on glass slides and stained with haematoxylin and eosin. The sections were examined under light microscopy for histopathological and morphometric analysis, as described by Fernandes *et al.*[22]

Histopathological analysis in testis. For this analysis, one hundred random testicular cross sections of the seminiferous tubules per animal were observed using an Opton microscope (10× or 40× objective). The seminiferous tubules were divided into normal or abnormal. The abnormal tubules were subdivided into immature germ cells in the lumen; acidophilic cells and vacuolisation[23].

Seminiferous tubule diameters and seminiferous epithelium height. Ten random testicular cross-sections of the seminiferous tubules, per animal, in stage IX of the seminiferous epithelium cycle, were examined. Seminiferous tubule diameters were measured using Opton photomicroscope (40× objective) and BELview software (version 6.2.3.0 for Windows). Likewise, the seminiferous epithelium height was measured using the same tubules and methodology mentioned above. In each seminiferous tubule, the mean of four measures for diameters and height was calculated and used in the statistical analysis[23].

Number of Leydig and Sertoli cells. The number of Leydig cell nuclei was counted in ten random fields of interstitial tissue in each testes section per rat using a light microscope at 40× objective. The Leydig cell morphology was identified as described by Teerds and Huhtaniemi[24]. The number of Sertoli cell nuclei was determined in twenty cross-sections of the seminiferous tubules per testes in each rat, using a light microscope at 40× objective[25].

Kinetics of spermatogenesis. To evaluate the spermatogenic process, 100 random seminiferous tubules per animal were classified into four categories: Stages I–VI, VII–VIII, IX–XIII and XIV of the germinal epithelium cycle, according to Leblond and Clermont[26], under a light microscope (Opton) at 10× objective.

Assessment of neonatal underfeeding on spermatic parameters in adulthood

Daily sperm production per testis, sperm number and transit time in the epididymis. To evaluate daily sperm production, the left testes were decapsulated, weighed and homogenised, as described previously by Siervo *et al.*[23]. After dilution of the homogenate, a small sample volume was transferred to a Neubauer chamber (four fields per animal) for counting of homogenisation-resistant spermatids (that corresponds to the stage 19 of spermatogenesis) in the testes and spermatozoa in epididymis. To calculate daily sperm production, the concentration of spermatids per testes was divided by 6·1, which is the number of days for which the mature spermatids are present in the seminiferous epithelium. To calculate sperm transit time in the epididymis, the right epididymis was decapsulated, weighed and homogenised in 5 ml of NaCl 0·9 % containing Triton X-100 0·5 % (Sigma-Aldrich Co.®), according to the method described by Robb *et al.*[27], with adaptations described by Siervo *et al.*[23]. After ten-fold dilution of the homogenate, a small sample volume was transferred to a Neubauer chamber, and late spermatozoa were counted (four fields of view per animal). To calculate sperm transit time through the epididymis, the number of sperm in each portion was divided by the daily sperm production.

Sperm motility. Sperm motility was evaluated according to methods described by Siervo *et al.*[23], with adaptations. Briefly, the left vas deferens was washed with 0·8 ml of phosphate-buffered saline (137 mM NaCl, 2·68 mM KCl, 8·03 mM Na2HPO4 1·47 mM, pH 7·2) enriched with 0·75 % Bovine Serum Albumine at 34°–37°C to obtain spermatozoa. At the same temperature, a Makler counting chamber (Sefi-Medical, Haifa, Israel) was loaded with a 10 ml aliquot of the sperm solution previously prepared. Sperm motility was assessed by visual estimation (100 spermatozoa per animal) under a light microscope (Motic®) at 10× objective and was performed by the same person throughout the study. Spermatozoa were classified as motile or immotile.

Sperm morphology. The contents of the vas deferens were removed via internal rinsing with 1·0 ml of 10 % formol saline. Histological slides were prepared from this solution and

British Journal of Nutrition

observed using an Opton photomicroscope (40× objective). Two hundred spermatozoa were analysed per animal.

Morphological analysis was classified into three general categories: normal morphology, head abnormalities (without characteristic curvature or isolated form, i.e. no tail attached) and tail abnormalities (broken, rolled into a spiral and isolated, i.e. no head attached). This analysis was performed as described by Fernandes *et al.*[22]

Mitochondrial activity analysis of spermatozoa. To evaluate the mitochondrial activity of spermatozoa, a method based on the selective incorporation of the 3,3′diaminobenzidine tetrahydrochloride (DAB) dye by the active mitochondria present in the sperm intermediate piece was used[28]. Sperm were collected from the vas deferens in phosphate-buffered saline. A 100 µl aliquot of the sperm was added to 200 µl of the DAB dye solution. After incubating this solution for 1 h at 37°C, a smear was prepared and fixed in phosphate-buffered saline with formaldehyde (10 %). Two hundred cells were counted and classified into: (1) DAB class I: 100 % of the intermediate piece stained; (2) DAB class II: more than 50 % of the intermediate piece stained; (3) DAB class III: less than 50 % of the intermediate piece stained. The result was expressed as percentage.

Assessment of neonatal underfeeding on sexual behaviour in adulthood

Fifteen days before sexual behaviour analysis, on PND 75, a second set of animals from NL and LL groups were transferred to an inverted 12 h light/dark cycle room with controlled temperature and noise. The observations started 2–3 h after the onset of darkness and were recorded by a video camera, linked to a monitor in an adjacent room, under dim red light.

Copulatory behaviour. For the evaluation of copulatory behaviour, male was inserted into the observation cage for 10 min and then a female in natural estrous was introduced into the same cage. The latencies and number of intromissions and ejaculations were observed for 30 min, as described previously in Gerardin *et al.*[29]. If the male did not mount within 10 min, the evaluation was interrupted and repeated another day with another female. Males were considered sexually inactive if there was no intromission in the initial 10 min after two attempts on different days. Moreover, males were considered sexually competent when they exhibited all the parameters analysed to evaluate sexual behaviour (mount, intromission and ejaculation)[30].

Sexual incentive motivation. The same male evaluated for copulatory behaviour was submitted to the sexual incentive motivation test, as previously described by Costa *et al.*[31]. In this test, a rectangular arena with dimensions 50 × 50 × 100 cm (height × width × length) was used, with two openings that lead to two small arenas of 25 cm^2. The small arenas were diagonally opposed to each other, and the communication with the main arena was closed with a wire mesh. For the test, an estrous female was placed in one of the small arenas (female zone), and a sexually active male was placed in the other arena (male zone). The floor of the main arena had two 25 cm^2 divisions (zones) in front of each small arena opening, named female and male incentive zones, respectively. The experimental male was placed in the centre of the main arena and observed for 20 min. The number of visits and the total time spent visiting male/female zone was quantified, and a preference score was calculated as (time spent in female zone/total time spent in both incentive zones) × 100[32].

Statistical analysis

Initially, an exploratory analysis was conducted to evaluate normal distribution (Shapiro–Wilk test) and homogeneity of variance (Levene's test) of each variable. Variables that presented normal distribution and homogeneity of variance were analysed by Student's *t* test. Conversely, for other variables the Mann–Whitney U was performed. Repeated measures (RM) ANOVA, followed by Sidak for multiple measures post-test, was performed to evaluate food intake and glucose tolerance test (GTT). Differences were considered significant if $P < 0{\cdot}05$.

Results

Effects of neonatal undernutrition on biometric and metabolic parameters

Statistical analysis showed that LL animals had lower body weight gain during the lactational period ($t(45) = 4{\cdot}669$, $P < 0{\cdot}0001$) and in adulthood ($t(22) = 2{\cdot}717$, $P = 0{\cdot}0126$) than the NL group, as seen in Fig. 1(a) and (b), whereas naso-anal length was smaller only at weaning ($t(31) = 6{\cdot}454$, $P < 0{\cdot}0001$; PND 90: $t(18) = 1{\cdot}741$, $P = 0{\cdot}0987$) (Fig. 1(c)). The Lee index was decreased at weaning ($U = 0$, $P < 0{\cdot}0001$) and on PND 90 ($U = 15{\cdot}5$, $P < 0{\cdot}014$) in the LL group in comparison with the rats from normal litters (Fig. 1(d)). Mixed and repeated-measure factorial ANOVA of food intake showed that there was an interaction between days (25–85) and groups (NL and LL) on food intake ($F_{12{\cdot}192} = 5{\cdot}223$; $P < 0{\cdot}0001$). LL rats presented increased food intake on PND 30 ($P < 0{\cdot}0001$) and 35 ($P < 0{\cdot}01$). These data were integrated into a food intake AUC, which also showed that animals of the LL group had an increase of food intake ($t(16) = 2{\cdot}367$, $P = 0{\cdot}0309$), in comparison with the NL group (Fig. 1(e)).

Mixed- and repeated-measure factorial ANOVA of GTT showed that there was an interaction between time after glucose overload (0–120) and groups (NL and LL) on glycaemia ($F_{4{\cdot}80} = 2{\cdot}658$; $P = 0{\cdot}0387$). The LL group showed lower blood glucose at 15 ($P < 0{\cdot}01$) and 30 min ($P < 0{\cdot}05$) after oral glucose overload (Fig. 2(a)). This GTT response was integrated into the GTT AUC, which showed decreased GTT AUC in the LL group compared with NL one ($t(20) = 2{\cdot}616$, $P = 0{\cdot}0165$) (Fig. 2(a)). Plasma concentrations of cholesterol ($t(18) = 2{\cdot}201$, $P = 0{\cdot}0410$) and testosterone ($U = 14$, $P = 0{\cdot}0052$) were lower and higher, respectively, in LL animals when compared with NL, as shown in Fig. 2(b) and (e), respectively. There were no significant differences in plasma concentrations of corticosterone

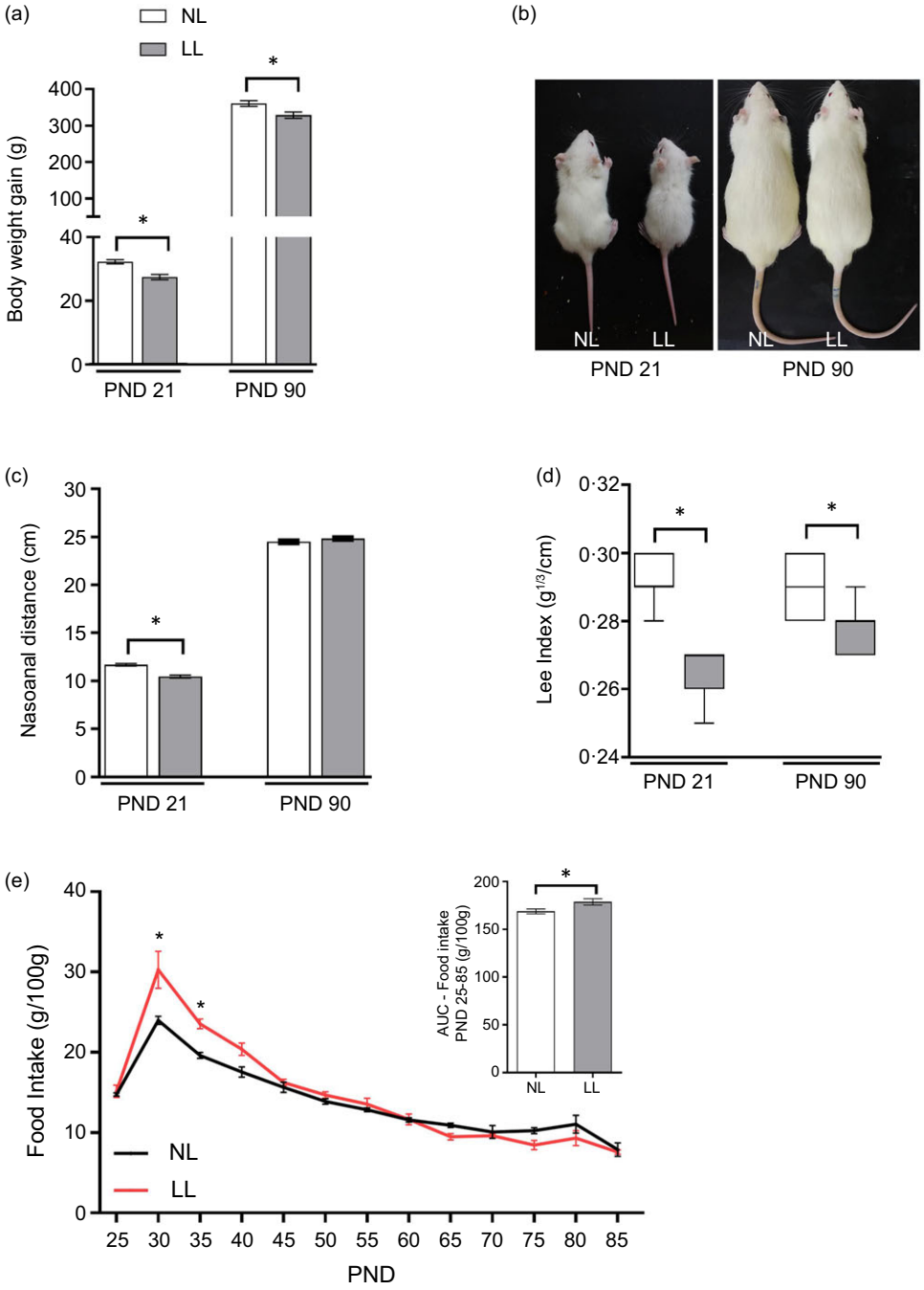

Fig. 1. Body weight gain, naso-anal distance, Lee index and food intake of male *Wistar* rats reared in normal (NL) and large litters (LL). (a) Body weight gain (g). (c) Naso-anal distance (cm). (d). Lee index ($g^{1/3}$/cm). (e) Food intake (g/100 g). Data are expressed as mean (SEM) for the Student's *t* test (body weight gain, naso-anal distance, AUC of food intake) and RM ANOVA (for food intake curve), or as median (Q1–Q3) for the Mann–Whitney test (Lee index). *$P < 0{\cdot}05$ *v.* NL (*n* 10–12 animals/group). B. Representative image of one animal from each litter on PND 21 and 90.

(t(19) = 0·2118, $P = 0{\cdot}8345$) and TAG (t(19) = 0·1327, $P = 0{\cdot}8959$) between groups (Fig. 2(c) and (d)).

The weights of adipose tissues and reproductive organs of the adult males are presented in Table 1. LL animals showed decreased weight of retroperitoneal white adipose tissue in comparison to the NL animals. The weights of epididymal white adipose tissue, brown adipose tissue and reproductive organs were similar between the experimental groups.

Effects of neonatal undernutrition on testes histopathological parameters, cell count and kinetics of spermatogenesis in male adult rats

Histopathological analysis of testes from undernourished animals revealed a decrease in the number of normal tubules and an increase in the number of abnormal seminiferous tubules (t(8) = 3·584, $P = 0{\cdot}007$; Fig. 3(a)) compared with the control group. Structural changes, such as the presence of immature

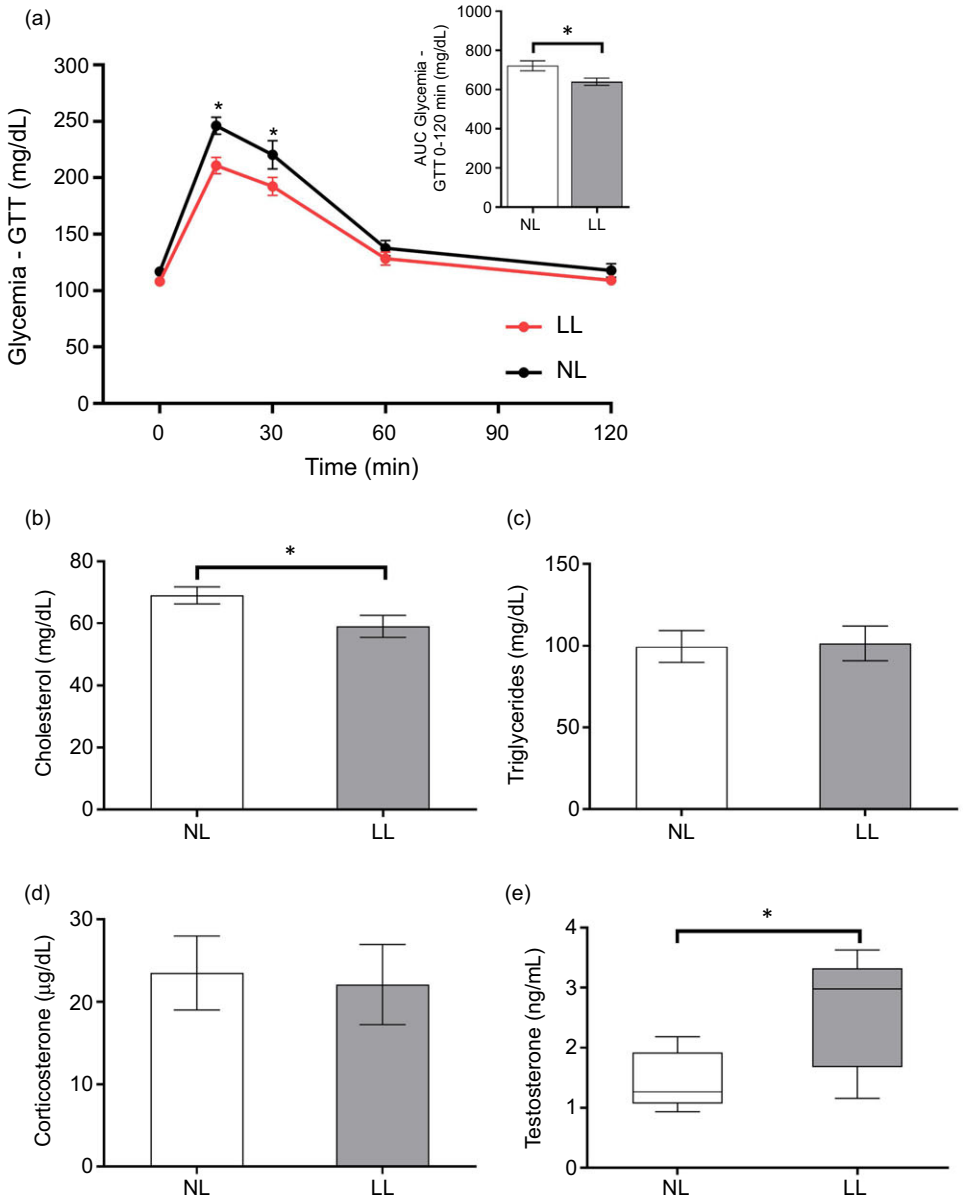

Fig. 2. Glycaemia after glucose tolerance test (GTT) and plasma levels of cholesterol, TAG, corticosterone and testosterone of adult male *Wistar* rats reared in normal (NL) and large litters (LL). (a) Glycaemia after GTT (mg/dl). Plasma levels of cholesterol (mg/dl) (b), TAG (mg/dl) (c), corticosterone (µg/dl) (d) and testosterone (ng/dl) (e). Data are expressed as mean (SEM) for RM ANOVA (for GTT) and Student's *t* test (for GTT AUC, cholesterol, TAG, corticosterone), or as median (Q1–Q3) for the Mann–Whitney test (for testosterone). *$P < 0{\cdot}05$ *v.* NL (*n* 10–12 animals/group).

germ cells in the lumen, were increased in LL animals, compared with the NL group ($t(8) = 3{\cdot}345$, $P = 0{\cdot}01$; Fig. 3(b)), while acidophilic cells were not found, and there was no difference in the vacuolisation between groups ($U = 0{\cdot}11$, $P > 0{\cdot}99$) (Table 2). Moreover, there was a reduction in the diameter of seminiferous tubules ($t(6) = 3{\cdot}378$, $P = 0{\cdot}0149$) and the seminiferous epithelium height ($t(6) = 2{\cdot}866$, $P = 0{\cdot}0286$) in LL animals compared with the NL group, as shown in Table 2.

The analysis also showed that neonatal undernutrition did not alter the number of Leydig cells ($t(7) = 0{\cdot}279$, $P = 0{\cdot}788$; Fig. 3(c)), but led to a decrease in the number of Sertoli cells ($t(8) = 5{\cdot}964$, $P = 0{\cdot}0003$; Fig. 3(d)) per seminiferous tubules in the animals from the LL group when compared with the NL group. There was no significant change in the kinetics of spermatogenesis, as exhibited in Table 3.

Effects of neonatal undernutrition on spermatic parameters in male adult rats

The sperm count parameters are shown in Table 4. Parametric analysis showed that neonatal undernutrition reduced the absolute number of spermatids and spermatozoa in the testes of LL animals compared with the NL group (absolute: $t(8) = 3{\cdot}903$, $P = 0{\cdot}0045$). However, the absolute number of spermatids and spermatozoa in the caput ($U = 10$, $P = 0{\cdot}691$) and cauda ($t(8) = 1{\cdot}374$, $P = 0{\cdot}206$) of the epididymis remained

British Journal of Nutrition

Table 1. Weights of adipose depots and reproductive organs (g/100 g body weight) of adult male *Wistar* rats raised in normal (NL) and large litters (LL) (Mean values with their standard errors of the mean)

	NL (11)		LL (10)			
	Mean	SEM	Mean	SEM	*P* value	T value
Retroperitoneal WAT	0·837	0·08	0·582	0·06*	0·0191	2·562, df = 19
Epididymal WAT	1·372	0·10	1·142	0·10	0·1298	1·592, df = 17
BAT	0·055	0·003	0·056	0·003	0·8358	0·210, df = 19
Testes	0·422	0·01	0·447	0·01	0·1323	1·573, df = 19
Epididymis	0·586	0·05	0·593	0·06	0·7948	0·264, df = 19
Prostate	0·565	0·10	0·651	0·15	0·1410	1·536, df = 19

BAT, brown adipose tissue; WAT, white adipose tissue.
Data are expressed as mean (SEM) and were analysed by the Student's *t* test. *$P < 0·05$ NL *v.* LL.
Numbers in brackets represent the number of animals per group.

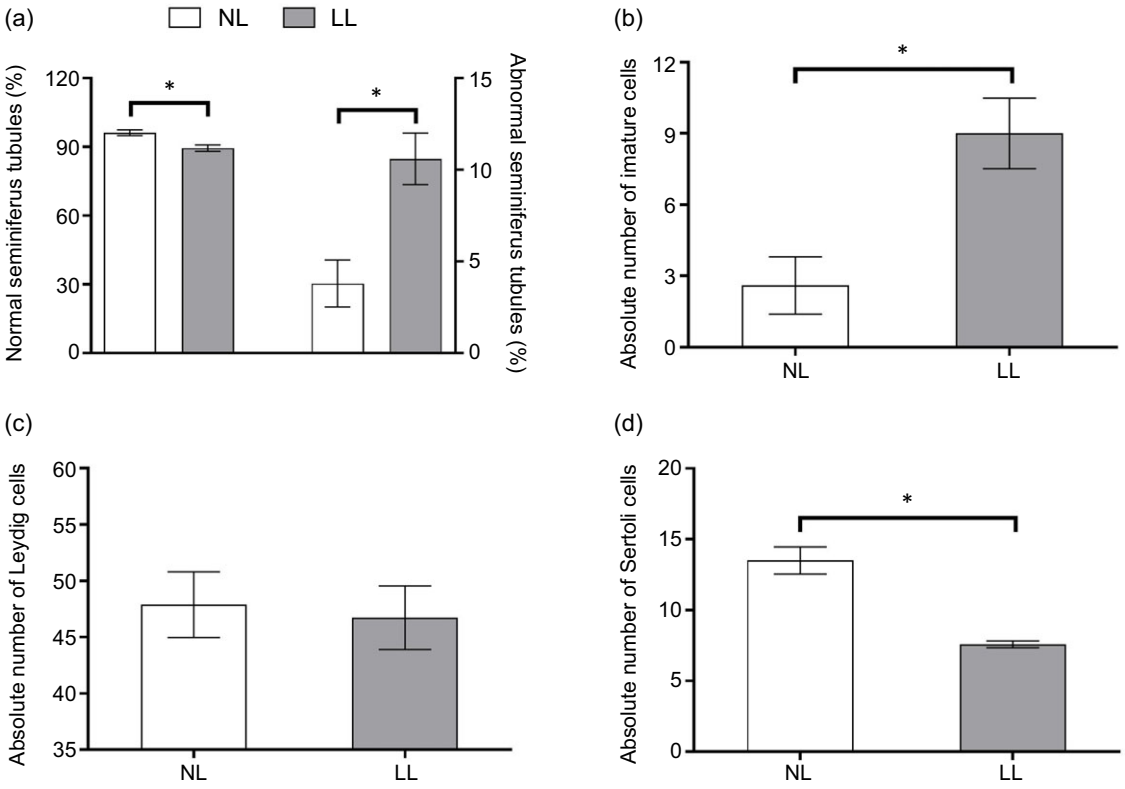

Fig. 3. Effect of litter size on testicular histopathological analysis and cell count in adult male *Wistar* rats reared in normal (NL) and large litters (LL). (a) Percentage of normal and abnormal seminiferous tubules. (b) Absolute number of immature cells. (c) Absolute number of Leydig and (d) Sertoli cells in the seminiferous tubules. Data are expressed as mean (SEM) and were analysed by the Student's *t* test. *$P < 0·05$ *v.* NL (*n* 5 animals/group).

Table 2. Testicular morphometry of adult male *Wistar* rats reared in normal (NL) and large litters (LL) (Mean values with their standard errors of the mean; median values and Q1–Q3)

Parameters	Groups			
	NL		LL	
	Median	Q1–Q3	Median	Q1–Q3
Vacuolisation†	1·00	0·00–2·00	1·00	0·50–2·00
	Mean	SEM	Mean	SEM
Seminiferous tubule diameter (μm)‡	244·6	8·30	209·3	6·35*
Height of the germinal epithelium (μm)‡	69·75	3·31	59·61	1·25*

Data presented as ‡mean (SEM) for Student's *t* test, or †median (Q1–Q3) for Mann–Whitney test. *$P < 0·05$ *v.* NL (*n* 5 animals/group).

Table 3. Spermatogenesis kinetics of adult male *Wistar* rats reared in normal (NL) and large litters (LL) (Mean values with their standard errors of the mean; Median values and Q1–Q3)

	NL		LL			
	Median	Q1–Q3	Median	Q1–Q3	*P* value	T/U value
I–VI†	49·50	45–54·50	57·00	46–65·00	0·165	1·444 (df = 19)
	Mean	SEM	Mean	SEM		
VII–VIII*	31·80	2·08	27·20	2·11	0·159	1·552 (df = 8)
IX–XIII*	16·00	1·23	17	3·82	0·809	0·249 (df = 8)
	Median	Q1–Q3	Median	Q1–Q3		
XIV†	0·00	0·00–0·50	0·00	0·00–0·50	> 0·9	12·50

Stages I–XIV of the germinal epithelium cycle.
Data are expressed as *mean (SEM) for Student *t* test or †median (Q1–Q3) for Mann–Whitney test, (*n* 5 animals/group).

Table 4. Sperm parameters in the testis and epididymis of adult male *Wistar* rats reared in normal (NL) and large litters (LL) (Mean values with their standard errors of the mean; Median values and Q1–Q3)

Parameters	NL		LL	
	Mean	SEM	Mean	SEM
Testes				
Sperm absolute number ($\times 10^6$)†	129·70	1·84	111·60	4·25*
Sperm relative number (per g $\times 10^6$)†	78·71	2·11	63·75	6·22
DSP ($\times 10^6$)†	21·26	0·30	18·3	0·69*
Epididymal caput/corpus				
Sperm absolute number ($\times 10^6$)‡				
Median	71·18		67·12	
Q1–Q3	57·63–160·2		60·31–104·2	
Sperm relative number (per g $\times 10^6$)†	357·60	94·69	276·0	54·74
Spermatic transit ($\times 10^6$)†	4·79	1·23	4·37	0·644
Epididymal cauda				
Sperm absolute number ($\times 10^6$)†	85·05	20·86	118·70	12·77
Sperm relative number (per g $\times 10^6$)†	402·50	108·8	479·20	55·26
Spermatic transit ($\times 10^6$)†	3·95	0·91	6·56	0·84

DSP, daily sperm production.
Data are expressed as †mean (SEM) for Student *t* test or ‡median (Q1–Q3) for Mann–Whitney test, (*n* 5 animals/group). *$P < 0·05$.

unchanged (Table 4). These data are consistent with reduced daily sperm production (t(8) = 3·903, $P = 0·004$, Table 4), and unchanged sperm transit in the caput/corpus and cauda of the epididymis observed in LL animals (caput/corpus: t(8) = 0·304, $P = 0·769$; cauda: t(8) = 2·094, $P = 0·069$; Table 4), compared with the NL group. However, it is important to note that although there was no significant difference in the cauda of the epididymis, the LL group showed a 66 % increase in sperm transit compared with the NL group (Table 4).

The percentage of normal sperm morphology was statistically equivalent between groups (U = 24, $P = 0·7756$; Fig. 4(a)), and there was no change in the amount of motile sperm (t(15) = 0·872, $P = 0·397$; Fig. 4(b)) in LL animals, compared with the control group. Moreover, the analysis of mitochondrial activity also remained unchanged, as exhibited in Table 5.

Effects of neonatal undernutrition on sexual behaviour

Tables 6 and 7 present data from the evaluation of the sexual behaviour of LL and NL adult rats. All animals from both experimental groups were considered sexually competent, and there was no significant difference in the parameters of sexual behaviour between the LL and the NL groups (Table 6). Also, the sexual incentive motivation test did not differ between the groups (Table 7).

Discussion

Neonatal undernutrition, employing expanded litter size, resulted in a lighter (body weight), shorter (length) and thinner (Lee index) phenotype of 21-day-old rats in comparison with the control group. At 90 d of life, undernourished animals were still lighter and thinner, and body length was similar compared with control. Rödel *et al.*[33] already reported the negative correlation between litter size and body mass at weaning, reinforcing that undernutrition during the lactation period affects the development and metabolism of the progeny, successively impacting growth, length and BMI of the offspring. The immediate increase of food intake in the post-weaning period and increased food intake AUC observed in underfed animals corroborate results found by Remmers, Fodor and Delemarre-van de Wall[11], who reported that LL male animals had a higher energy intake shortly after weaning, which may have permitted their partial catch-up growth, as seen in the current study. Also, the difference in body weight observed between litter sizes may reflect the significant difference in fat mass, as undernourished animals showed decreased weight of retroperitoneal adipose tissue compared with control. According to Engelbregt *et al.*[10], animals raised in large litter present a lower percentage of fat mass and a higher percentage of lean mass, which is compatible with underfed animals being lighter and thinner. This may also be related to the decrease in the size of white adipose cells seen in undernourished rats[34], reinforcing the idea that certain circumstances, such as early food restriction, force the use of fat stocked as droplets to obtain energy.

Evidence of this preference for fat as an energy source was also seen in the liver. This may be due, at least in part, to mitochondrial and enzymatic dysfunction[35] that predispose the offspring of undernourished mothers to store cholesterol in the liver[36], possibly leading to lower plasma concentrations of cholesterol, with no significant differences in the circulating levels of triglycerides[35], as observed in the present study and in others[37–39]. Also, decreased glycaemia in response to glucose

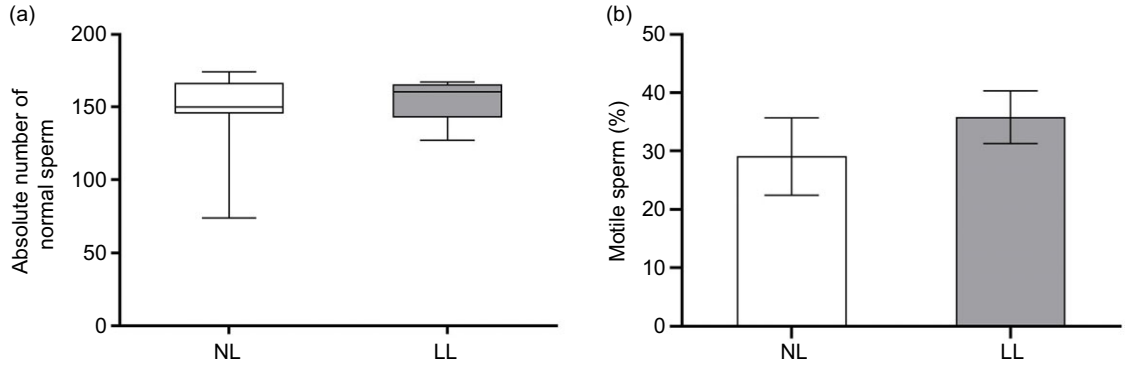

Fig. 4. Effect of litter size on sperm morphology and motility of adult male *Wistar* rats reared in normal (NL) and large litters (LL). (a) Absolute number of normal sperm and (b) percentage of motile sperm. Data are expressed mean (SEM) for Student *t* test (for absolute number of normal sperm) or median (Q1–Q3) for Mann–Whitney test (for percentage of motile sperm). $^{*}P < 0{\cdot}05$ *v.* NL (*n* 5 animals/group).

Table 5. Effect of litter size on sperm mitochondrial activity of adult male *Wistar* rats reared in normal (NL) and large litters (LL) (Mean values with their standard errors of the mean; median values and Q1–Q3)

	NL		LL			
	Mean	SEM	Mean	SEM	*P* value	T/U value
DAB class I*	164·8	2·33	159·0	3·53	0·223	1·308 (df = 9)
DAB class II*	34·8	2·33	40·7	3·37	0·204	1·370 (df = 9)
	Median	Q1–Q3	Median	Q1–Q3		
DAB class III†	0·00	0·00–0·50	0·00	0·00–0·50	> 0·9	18

DAB, 3,3′diaminobenzidine tetrahydrochloride.
Data are expressed as *mean (SEM) for Student *t* test or †median (Q1–Q3) for Mann–Whitney test (*n* 5 animals/group).

Table 6. Copulatory behaviour of adult male *Wistar* rats reared in normal (NL) and large litters (LL) (Mean values with their standard errors of the mean; median values and Q1–Q3)

Parameters	NL		LL	
	Mean	SEM	Mean	SEM
Latency to the first intromission (s)*	148·90	32·50 (10/10)	185·20	48·17 (10/10)
Number of intromissions until the first ejaculation*	13·33	1·43 (6/10)	17·75	3·49 (8/10)
Latency to the first ejaculation (s)*	882·43	192·27 (7/10)	1158·50	162·53 (8/10)
Latency of the first post-ejaculatory intromission (s)*	1091·33	248·67 (6/10)	1366·71	136·81 (7/10)
Number of post-ejaculatory intromissions*	10·40	3·30 (5/10)	8·71	2·30 (7/10)
Total number of intromissions*	16·70	3·14 (10/10)	22·90	3·05 (10/10)
	Median	Q1–Q3	Median	Q1–Q3
Number of ejaculations†	1·00	0·00–2·00 (6/10)	1·00	0·75–2·00 (8/10)

Data are expressed as *mean (SEM) for Student *t* test or †median (Q1–Q3) for Mann–Whitney test.
Number of animals that displayed the behaviour per total number of animals in the group is given in brackets.
Parameters with (s) means time on seconds spent exhibiting that specific behaviour during the observation period.

overload in the GTT test observed in undernourished animals agrees with Garcia-Souza *et al.*[39], who showed that maternal undernourishment during early postnatal life resulted in higher membrane GLUT4 expression in the white adipose tissue, as well as upregulation of the expression of insulin receptors in the skeletal muscle[40], possibly because of low insulin production[41]. Such alteration in insulin sensitivity and production in malnourished animals is often associated with an increased glucose uptake into the muscle and in the adipocytes and, eventually, can lead to insulin resistance, visceral obesity and diabetes[41].

Regarding the weight of the reproductive organs, there was no effect of litter size in these parameters, despite the significant increase in testosterone plasma levels in LL rats compared with the NL group. Similarly, Rodríguez-Rodríguez *et al.*[42] reported a tendency towards higher levels of testosterone in 21-day-old males from undernourished mothers. Interestingly, a recent study[14] found that maternal undernutrition, especially during lactation, increases testicular immunostaining for 3β-hydroxysteroid dehydrogenase, an important enzyme in steroidogenesis, suggesting an elevation of steroidogenic capability. This functional change would explain the increase in testosterone plasma

Table 7. Sexual incentive motivation of adult male *Wistar* rats reared in normal (NL) and large litters (LL) (Mean values with their standard errors of the mean; median values and Q1–Q3)

Parameters	NL (12)		LL (10)	
Time spent in male zone (s)	165·50 (104·50–217·75)		154·50 (126·50–183·25)	
	Mean	SEM	Mean	SEM
Time spent in female zone (s)	699·25	41·89	723·0	34·13
Number of visits in male zone	14·75	1·10	13·20	1·11
Number of visits in female zone	20·33	1·75	20·50	1·93
Preference score	80·48	2·21	82·50	1·51

Data of time spent in male zone are given as median (1° and 3° quartile) (Mann–Whitney test).
The other parameters were analysed using Student's *t* test and are given as the mean (SEM).
Parameters with (s) means time on seconds spent exhibiting that specific behaviour during the observation period. Numbers in brackets represent the number of animals per group.

levels without any effect on the amount of Leydig cells. Furthermore, it is tempting to suggest that the increased steroidogenesis is another possible cause for the reported decreased plasma concentrations of cholesterol in undernourished animals.

Despite the lack of change in the quality of sperm, confirmed by the analysis of motility, morphology and mitochondrial activity between the groups, the reduction in the amount of sperm produced by the LL animals agrees with the other testicular impairments, as the increased number of abnormal (smaller) seminiferous tubules and presence of germ cells (immature cells) in the lumen, as well as reduction of length or diameter of seminiferous tubules. As these tubules are the only site of spermatogenesis in the male, changes in their length or diameter can alter sperm production, altering the area available for spermatogenesis to occur[43]. Other studies have already shown that the development of the seminiferous tubules is sensitive to nutritional insults. In this context, Melo *et al.*[44] demonstrated a decrease in the size of the seminiferous tubules of animals subjected to protein restriction in the pre and postnatal periods. Other authors obtained comparable results using fetal programming through maternal protein restriction during pregnancy[45,46], and Bielli *et al.*[16] also observed the same finding in experiments with sheep. These data demonstrate that changes in maternal nutrition during pregnancy and lactation can lead to structural changes in the male gonads, which may be responsible for future changes in the functioning of this organ.

In addition to disruption in the number and morphology of seminiferous tubules, lower sperm production by the testes may also be associated with the reduction of Sertoli cells, shown in the present study, as these cells are located inside the seminiferous tubules and provide structural and nutritional support for the development of germ cells. As each Sertoli cell can support 30–50 germ cells at distinct stages of spermatogenesis[47], the decrease in their number represents a decrease in germ cells in the seminiferous tubule and, therefore, a reduction in sperm production in the seminiferous tubule organ. In this context, previous studies have already indicated that Sertoli cells may be particularly sensitive to fetal and metabolic programming because their number is highly related to testicular size and the maximum rate of germ cell production[48]. Genovese *et al.*[15] also demonstrated a reduction in the number of these cells in animals born from undernourished mothers during pregnancy and lactation. Toledo *et al.*[46] and Rodríguez-González *et al.*[12] also observed a reduction in the number of Sertoli cells after protein restriction during only one or both periods of pregnancy and lactation. Moreover, Chiarini-Garcia *et al.*[49] found that, in addition to a numerical reduction, there was also a reduction in the ability of Sertoli cells to support germ cells by more than half.

The data obtained indicate that the epididymis is unchanged by neonatal undernutrition, as seen by the preserved absolute amount of sperm and the transit time. Accordingly, maintained sperm transit explains the preserved characteristics of sperm, since it is during their passage through the epididymis that differentiation occurs, and they acquire their characteristics through the absorptive and secretory activities of the epithelium[50]. According to this, previous studies have shown that nutritional restriction, mainly protein, during the fetal period can cause changes in both the epididymis and the daily sperm production[15,51], but there were no studies so far that indicate if restricting nutritional status only in the lactation period can cause such changes.

Furthermore, the lack of effects of lactation malnutrition in the testicular weight corroborates the findings of previous studies[12,45,51]. This can be explained by the fact that the main development and enlargement of the testes occurs during the puberty period, not right after birth, so the recovery of nutrients through pelleted food can normalise the development of the organ. Indeed, this is supported by the higher food intake of undernourished animals after weaning, as observed in the current data. Mota *et al.*[52] believe that organ reduction is related to the decrease of total proteins in the body, which may or may not be derived from nutritional restriction. In addition, as the relative testicular weight (a result of dividing the testicular weight by the body weight) is a unit widely used by researchers in the area, the non-reduction of the organ may be due to the proportional decrease of both weights caused by the experimental conditions[12,53].

Finally, the absence of differences in the parameters of sexual behaviour and sexual incentive motivation, which is testosterone dependent[54], suggests that this behaviour was not affected by increased plasma levels of testosterone observed in neonatal underfed male rats. Thus, it is likely that the neural pathway controlling male sexual behaviour is not upregulated by neonatal malnutrition.

Thus, litter expansion-induced neonatal malnutrition was shown to be an adequate model for metabolic programming, with biometric and metabolic changes in adulthood of male animals, associated with reproductive impairments. Indeed, the

current work is a pioneer in demonstrating that lactational undernutrition by increasing litter size induces morphological testicular changes, accounting for a decrease in the absolute amount of sperm in the testes, without altering the sperm quality, and sexual behaviour of the animals, indicating that malnutrition in early life can cause reproductive changes in adulthood.

Conclusion

It is possible to conclude that the litter size expansion model is suitable for foetal programming experiments by malnutrition. The restriction of nutrients in the early stages of life, represented by the lactation period, may be responsible for testicular changes in adult life, which may or may not interfere with fertility.

Acknowledgments

The authors express gratitude to Dr. Daniela C. C. Gerardin, who unfortunately passed away during the preparation of this manuscript (02/03/2024), leaving us with an important scientific legacy.

This work was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (*Coordination for the Improvement of Higher Education Personnel,* CAPES – Finance Code 001), Brazil.

A. C. F. M. participated in data acquisition, analysis and interpretation and drafting the article. A. L. M. W. participated in study concept and design, performed experiments, analysis and interpretation of data and drafting the article. K. G. L., G. F. F., I. R. da C., L. R. S. S., C. F. S., R. P. G., P. K. S. and A. B. O. A. participated in acquisition of data. A. B. O. A. also participated in analysis and interpretation of data. S. F. made substantial contributions to the study conception and design. D. C. C. G., C. T. B. V. Z., E. T. U. and G. S. A. F. participated in study concept and design and administrative, technical and material support. D. C. C. G., E. T. U. and G. S. A. F. also participate in data analysis and interpretation. D. C. C. G., E. T. U. and G. S. A. F. conceived and designed research and participated in drafting and critical revision of the manuscript and study supervision.

There are no conflicts of interest.

References

1. Velkoska E, Cole TJ, Dean RG, *et al.* (2008) Early undernutrition leads to long-lasting reductions in body weight and adiposity whereas increased intake increases cardiac fibrosis in male rats. *J Nutr* **138**, 1622–1627.
2. Gluckman PD & Hanson MA (2004) The developmental origins of the metabolic syndrome. *Trends Endocrinol Metab* **15**, 183–187.
3. Gluckman PD, Hanson MA & Low FM (2011) The role of developmental plasticity and epigenetics in human health. *Birth Defects Res* **93**, 12–18.
4. Langley-Evans SC (2015) Nutrition in early life and the programming of adult disease: a review. *J Hum Nutr Diet* **28**, 1–14. Blackwell Publishing Ltd.
5. Martín-Estal I, De La Garza RG & Castilla-Cortázar I (2016) Intrauterine growth retardation (IUGR) as a novel condition of insulin-like growth factor-1 (IGF-1) deficiency. *Rev Physiol Biochem Pharmacol* **170**, 1–35. Springer Verlag.
6. Marangon PB, Mecawi AS, Antunes-Rodrigues J, *et al.* (2020) Perinatal over- and underfeeding affect hypothalamic leptin and ghrelin neuroendocrine response in adult rats. *Physiol Behav* **215**, 112793. Elsevier Inc.
7. Mccance RA & Cantab FRCP (1962) Food, growth, and time. *Lancet* **2**, 671–676.
8. Wunderlich ALM, Martins AB, de Souza CF, *et al.* (2023) Neonatal overnutrition, but not neonatal undernutrition, disrupts CCK-induced hypophagia and neuron activation of the nucleus of the solitary tract and paraventricular nucleus of hypothalamus of male Wistar rats. *Brain Res Bull* **195**, 109–119.
9. Wunderlich AL, Stopa L, Martins AB, *et al.* (2023) Neonatal overnutritional programming impairs the hypophagia and neuron activation induced by acute lipopolysaccharide in adult male rats. *Nutr Neurosci* **26**, 413–425. Taylor and Francis Ltd.
10. Engelbregt MJT, Van Weissenbruch MM, Lips P, *et al.* (2004) Body composition and bone measurements in intra-uterine growth retarded and early postnatally undernourished male and female rats at the age of 6 months: comparison with puberty. *Bone* **34**, 180–186. Elsevier Inc.
11. Remmers F, Fodor M & Delemarre-van de Waal HA (2008) Neonatal food restriction permanently alters rat body dimensions and energy intake. *Physiol Behav* **95**, 208–215. Elsevier Inc.
12. Rodríguez-González GL, Vigueras-Villaseñor RM, Millán S, *et al.* (2012) Maternal protein restriction in pregnancy and/or lactation affects seminiferous tubule organization in male rat offspring. *J Dev Orig Health Dis* **3**, 321–326.
13. Léonhardt M, Lesage J, Croix D, *et al.* (2003) Effects of perinatal maternal food restriction on pituitary-gonadal axis and plasma leptin level in rat pup at birth and weaning and on timing of puberty. *Biol Reprod* **68**, 390–400.
14. Pedrana G, Viotti H, Lombide P, *et al.* (2020) Maternal undernutrition during pregnancy and lactation affects testicular morphology, the stages of spermatogenic cycle, and the testicular IGF-I system in adult offspring. *J Dev Orig Health Dis* **11**, 473–483. Cambridge University Press.
15. Genovese P, Núñez ME, Pombo C, *et al.* (2010) Undernutrition during foetal and post-natal life affects testicular structure and reduces the number of sertoli cells in the adult rat. *Reprod Domest Anim* **45**, 233–236.
16. Bielli A, Pérez R, Pedrana G, *et al.* (2002) Low maternal nutrition during pregnancy reduces the number of Sertoli cells in the newborn lamb. *Reprod Fertil Dev* **14**, 333–337. CSIRO.
17. Bansal-Rajbanshi M & Mathur M (1985) Testicular morphology and cell proliferation kinetics of immature germ cells and Sertoli cells in suckling undernourished rats. *Cell Tissue Kinet* **18**, 183–191.
18. Bernardis LL & Patterson BD (1968) Correlation between 'Lee index' and carcass fat content in weaning and adult female rats with hypothamic lesion. *J Endocrinol* **40**, 527–528.
19. Stopa LRS, de Souza CF, Martins AB, *et al.* (2021) Neonatal overfeeding reduces estradiol plasma levels and disrupts noradrenergic-kisspeptin-GnRH pathway and fertility in adult female rats. *Mol Cell Endocrinol* **524**, 111147. Elsevier Ireland Ltd.
20. Guillemin R, Clayton GW, Smith JD, *et al.* (1958) Measurement of free corticosteroids in rat plasma: physiological validation of a method. *Endocrinology* **63**, 349–357.
21. Ogo FM, Siervo GEML, Gonçalves GD, *et al.* (2017) Low doses of bisphenol A can impair postnatal testicular development directly, without affecting hormonal or oxidative stress levels. *Reprod Fertil Dev* **29**, 2245–2254. CSIRO.

British Journal of Nutrition

22. Fernandes GSA, Arena AC, Fernandez CDB, *et al.* (2007) Reproductive effects in male rats exposed to diuron. *Reprod Toxicol* **23**, 106–112.
23. Siervo GEML, Vieira HR, Ogo FM, *et al.* (2015) Spermatic and testicular damages in rats exposed to ethanol: influence of lipid peroxidation but not testosterone. *Toxicology* **330**, 1–8. Elsevier Ireland Ltd.
24. Teerds KJ & Huhtaniemi IT (2015) Morphological and functional maturation of Leydig cells: from rodent models to primates. *Hum Reprod Update* **21**, 310–328. Oxford University Press.
25. Nassr ACC, Arena AC, Toledo FC, *et al.* (2010) Effects of gestational and lactational fenvalerate exposure on immune and reproductive systems of male rats. *J Toxicol Environ Health – Part A: Current Issues* **73**, 952–964.
26. Leblond CP & Clermont Y (1952) Definition of the stages of the cycle of the seminiferous epithelium in the rat. *Ann N Y Acad Sci* **55**, 548–573.
27. Robb GW, Amann RP & Killian GJ (1978) Daily sperm production and epididymal sperm reserves of pubertal and adult rats. *J Reprod Fertil* **54**, 103–107.
28. Hrudka F (1987) Cytochemical and ultracytochemical demonstration of cytochrome c oxidase in spermatozoa and dynamics of its changes accompanying ageing or induced by stress. *Int J Androl* **10**, 809–828.
29. Gerardin DCC, Bernardi MM, Moreira EG, *et al.* (2006) Neuroendocrine and reproductive aspects of adult male rats exposed neonatally to an antiestrogen. *Pharmacol Biochem Behav* **83**, 618–623.
30. Montagnini BG, Forcato S, Pernoncine KV, *et al.* (2021) Developmental and reproductive outcomes in male rats exposed to Triclosan: two-generation study. *Front Endocrinol (Lausanne)* **12**, 738980. Frontiers Media S.A.
31. Costa NO, Forcato S, Cavichioli AM, *et al.* (2020) In utero and lactational exposure to triclocarban: age-associated changes in reproductive parameters of male rat offspring. *Toxicol Appl Pharmacol* **401**, 115077. Academic Press Inc.
32. Ågmo A (2003) Lack of opioid or dopaminergic effects on unconditioned sexual incentive motivation in male rats. *Behav Neurosci* **117**, 55–68. American Psychological Association Inc.
33. Rödel HG, Meyer S, Prager G, *et al.* (2010) Litter size is negatively correlated with corticosterone levels in weanling and juvenile laboratory rats. *Physiol Behav* **99**, 644–650.
34. Rodríguez-Rivera C, Santín Moreda L, Alguacil LF, *et al.* (2021) Undernutrition induces major alterations in the lipid droplets of white and brown adipose tissues in Wistar rats. *Tissue Cell* **71**, 101500. Elsevier Ltd.
35. Morris TJ, Vickers M, Gluckman P, *et al.* (2009) Transcriptional profiling of rats subjected to gestational undernourishment: implications for the developmental variations in metabolic traits. *PLoS One* **4**, e7271.
36. Lecoutre S, Montel V, Vallez E, *et al.* (2019) Transcription profiling in the liver of undernourished male rat offspring reveals altered lipid metabolism pathways and predisposition to hepatic steatosis. *Am J Physiol Endocrinol Metab* **317**, 1094–1107.
37. Zambrano E, Bautista CJ, Deás M, *et al.* (2006) A low maternal protein diet during pregnancy and lactation has sex- and window of exposure-specific effects on offspring growth and food intake, glucose metabolism and serum leptin in the rat. *J Physiol* **571**, 221–230.
38. Howie GJ, Sloboda DM & Vickers MH (2012) Maternal undernutrition during critical windows of development results in differential and sex-specific effects on postnatal adiposity and related metabolic profiles in adult rat offspring. *Br J Nutr* **108**, 298–307.
39. Garcia-Souza P, Vargas Da Silva S, Félix GB, *et al.* (2008) Maternal protein restriction during early lactation induces GLUT4 translocation and mTOR/Akt activation in adipocytes of adult rats. *Am J Physiol Endocrinol Metab* **295**, 626–636.
40. Ozanne SE, Wang CL, Coleman N, *et al.* (1996) Altered muscle insulin sensitivity in the male offspring of protein-malnourished rats. *Am J Physiol* **271**, E1128–E1134.
41. del Carmen Miñana-Solis M & Escobar C (2008) Post-weaning protein malnutrition in the rat produces short and long term metabolic impairment, in contrast to earlier and later periods. *Int J Biol Sci* **4**, 422–432.
42. Rodríguez-Rodríguez P, López de Pablo AL, García-Prieto CF, *et al.* (2017) Long term effects of fetal undernutrition on rat heart. Role of hypertension and oxidative stress. *PLoS One* **12**, e0171544.
43. Fayomi AP & Orwig KE (2018) Spermatogonial stem cells and spermatogenesis in mice, monkeys and men. *Stem Cell Res* **29**, 207–214. Elsevier B.V.
44. Melo IO, Tenorio FD, da Silva Gomes JA, *et al.* (2022) Fractal methods applied to the seminiferous lumen images can quantify testicular changes induced by heat stress. Acta Histochem 124, 151949. Elsevier GmbH.
45. Ramos CDF, Da Silva AM, Costa WS, *et al.* (2006) Stereological evaluation of the seminiferous tubules of rats after maternal undernutrition during the lactation period. *Urol Int* **76**, 63–66.
46. Toledo FC, Perobelli JE, Pedrosa FPC, *et al.* (2011) In utero protein restriction causes growth delay and alters sperm parameters in adult male rats. *Reprod Biol Endocrinol* **9**, 1–9.
47. Cheng CY, Wong EWP, Yan HHN, *et al.* (2010) Regulation of spermatogenesis in the microenvironment of the seminiferous epithelium: new insights and advances. *Mol Cell Endocrinol* **315**, 49–56.
48. Sharpe RM, McKinnell C, Kivlin C, *et al.* (2003) Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. *Reproduction* **125**, 769–784. Journals of Reproduction and Fertility Ltd.
49. Chiarini-Garcia H, Parreira GG & Almeida FRCL (2011) Glycol methacrylate embedding for improved morphological, morphometrical, and immunohistochemical investigations under light microscopy: testes as a model. *Methods Mol Biol* **689**, 3–18.
50. Carlson BM (2014) *Embriologia humana e biologia do desenvolvimento (Human embryology and developmental biology)*, 5th ed. Rio de Janeiro: Elsevier.
51. Zambrano E, Rodríguez-González GL, Guzmán C, *et al.* (2005) A maternal low protein diet during pregnancy and lactation in the rat impairs male reproductive development. *J Physiol* **563**, 275–284.
52. Mota EC, Santos AM, Toste FP, *et al.* (2001) Effects of malnutrition in the testis. *Braz J Urol* **27**, 500–506.
53. Vilanova Teixeira C, Silandre D, de Souza Santos AM, *et al.* (2007) Effects of maternal undernutrition during lactation on aromatase, estrogen, and estrogen receptors expression in rat testis at weaning. *J Endocrinol* **192**, 301–311.
54. Feder HH (1984) Hormones and sexual behavior. *Ann Rev Psychol* **35**, 165–200.

British Journal of Nutrition