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Early maternal undernutrition induces sex-related metabolic changes in adult offspring

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Original Article

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Abstract

Nutritional status during the developmental periods leads to predisposition to several diseases and comorbidities, highlighting metabolic and reproductive changes throughout adult life, and in the next generations. One of the experimental models used to induce undernutrition is litter size expansion, which decreases the availability of breast milk to pups and delays development. This work evaluated the effects of maternal undernutrition induced by litter size expansion, a maternal undernutrition preconception model, on the metabolic and reproductive alterations of the offspring. For this, metabolic and reproductive parameters were evaluated in male and female offspring of female rats reared in normal (NL - 10 pups: 5 males and 5 females) and large (LL - 16 pups: 8 males and 8 females) litters. Male and female offspring of LL mothers presented higher food intake than the offspring of NL mothers. Male offspring from undernourished females showed reduced body weight from lactation to adulthood, nasoanal distance in childhood, increased nasoanal distance, and decreased Lee index in adult life, while female offspring showed decreased nasoanal distance in childhood. The male offspring from LL mothers showed increased insulin plasma levels and glucose tolerance, and reduced triglycerides plasma levels, without changes in the female offspring. These results indicate that neonatal undernutrition in females predisposes their male and female offspring to develop metabolic alterations, without reproductive repercussions, and male offspring seems to be more susceptible to present these metabolic changes than females. Thus, there are sexual differences in the metabolic responses of the offspring elicited by maternal preconceptional undernutrition.

Introduction

Undernutrition is considered by the World Health Organization (WHO) a multifactorial condition, and it is characterized by the deficiency of one or more essential nutrients, and a deficit in food quantity and/or quality. The number of people affected by this condition in the world is 821 million, with more than 18 million women with severe undernutrition, according to current data from The United Nations. Accordingly, women with undernutrition have a deficiency of several micronutrients, which can account for a high risk of developing adverse maternal, gestational, and offspring effects.[1,2]

The pregestational, intrauterine, postnatal, and pubertal periods are critical phases of development, and events in these periods can influence normal development, causing direct early and/or late impacts on the health of the offspring. Lactation is responsible for continuing the nutrition initiated in the intrauterine period, and breast milk has several impacts besides its nutritional value, playing a key role in the development and future health condition of the offspring.[3] To study lactation undernutrition and its consequences, one of the models used is the litter size expansion. In large litters, the offspring have less milk availability, inducing undernutrition[4,5] and the litter size expansion method is a classic model to investigate the effect of lactational undernutrition directly in the offspring.[6] Studies have shown that postnatal undernutrition induced by litter size expansion leads to a decrease in body weight, hypoleptinemia, and hypoinsulinemia after weaning.[6,7]

Maternal undernutrition during pregnancy (*postconception*) induced an increased risk of developing metabolic diseases and obesity in their offspring with sex-specific sensitivity, female offspring being less susceptible to these effects than male offspring.[8] However, when these females become pregnant, they experience some physiological changes that may, in turn, create an adverse intrauterine environment for the growth of their offspring, contributing to the transmission of diseases through subsequent generations.[8]

Therefore, as lactational undernutrition due to litter size expansion is known to cause metabolic and reproductive changes in rats,[9] but it is not known whether or what consequences maternal preconception undernutrition can cause in their offspring, this study aimed to evaluate the metabolic and reproductive profiles in male and female offspring of female rats reared in expanded litter size, even after an adequate nutritional diet.

Methods

Animal model

Female Wistar rats were obtained from the mating of females with males from the Central Bioterium of Londrina State University (UEL), to generate the litters. On the day of birth, postnatal day (PND) 0, the litter size was adjusted to 16 pups (large litter, LL, 8 males, and 8 females), to induce lactation underfeeding, or 10 pups (normal litter, NL, 5 males, and 5 females), the control group. After weaning, the female animals were housed in groups of 3–4 rats of the same experimental group in each cage (NL or LL). From PND 90, female rats from each group were paired to mate with a male rat with no previous manipulation, to generate the offspring of NL and LL females. On PND 4, the litter size was adjusted to 8 pups (4 males and 4 females). The surplus pups were euthanized by decapitation. After weaning, the male and female pups were housed in groups of 3–4 rats of the same experimental group in each cage (offspring of NL: oNL or offspring of LL: oLL). The animals were kept in controlled conditions of light (12 h light/dark cycle) and temperature (22 ± 2 °C), with water and feed *ad libitum*, except for the hours of feed restriction before the glucose tolerance test (GTT) and euthanasia. All experiments were performed at the Department of Physiological Sciences/UEL. The experimental procedures were approved by the Ethics Committee on Animal Use for experimentation (CEAU protocol CEUA n°18310.2019.03, OF. CEUA n°164/2019).

Experimental protocol

Effects of preconception maternal undernutrition, induced in the neonatal period by expanding litter size, on the metabolic and reproductive parameters of their male and female offspring

On PND 0, the animals were grouped into NL and LL, and on PND 21, they were weaned. From PND 90, females were placed to mate with a control and sexually experienced male rat. The next day, when pregnancy was confirmed, gestational day (GD) was considered 0, and each female was placed in an individual cage. On GD 14, after 6 hrs of food deprivation, an Oral Glucose Tolerance Test (oGTT) was performed in the pregnant females. The day of birth of the litter was considered lactational day (LD) 0 and after weaning of the pups, on LD 21, the mothers were euthanized by decapitation, when blood was collected for plasma dosages, and uterus, ovaries, the retroperitoneal and perigonadal + perirenal adipose tissues were removed and weighed. Body weight was evaluated during pregnancy and lactation periods, and food intake during lactation period (Fig. 1).

On PND 4 of offspring of NL or LL mothers, the litter size adjustment was performed to maintain 8 pups, 4 males and 4 females. Animals were weighed every 3 days until the weaning day (PND 21). On PND 21, the rats were weighed, and the nasoanal distance was measured, for Lee Index analysis.[10] After weaning, the animals were weighed every 5 days until PND 90.

In female offspring, from PND 25 onward, the vaginal opening was daily analyzed, after which, vaginal smears were daily collected until the occurrence of first estrus and the onset of regular estrous cyclicity.[11] From PND 21 to 90, body weight and food intake were evaluated every 5 days, and from PND 75 to 90, vaginal smears were again daily collected for the analysis of female estrous cycles. On PND 89, after 6 hours of food restriction, GTT was performed. From PND 90, when in proestrus, the rats were weighed, the nasoanal distance was measured, for Lee Index analysis, and subjected to feed restriction for 6 hours before euthanasia by decapitation. After that, ovaries, uterus, and retroperitoneal and perirenal+ perigonadal adipose tissues were removed and weighed (Fig. 1).

In male offspring, from PND 40 onwards, the external genitalia was daily observed until the preputial separation. From PND 21 to 90, body weight and food intake were evaluated every 5 days. On PND 89, after 6 hours of food restriction, GTT was performed. On PND 90, the rats were weighed, the nasoanal distance was measured, for further Lee Index analysis, and subjected to feed restriction for 6 hours before euthanasia by decapitation. After that, testes, and retroperitoneal and perigonadal visceral adipose tissues were removed and weighed (Fig. 1). After the decapitation of male and female offspring, blood was collected for the analysis of plasma levels of insulin, triglycerides, total cholesterol, free fatty acids, testosterone, and estradiol.

Determination of the estrous cycle

Vaginal smears were daily collected at 8 a.m. to assess the phases of the estrous cycle. The vaginal smear was collected and placed on a histological slide, which was analyzed under a light microscope, with 10× magnification for analysis of cell proportions and types.[12] After the first estrus, the assessment was maintained to check the regularization of the estrous cycle. This onset of cyclicity was considered on the last day of the second regularized cycle, when the phases followed the order: proestrus - estrus – diestrus I (metestrus) – diestrus II.[13] The estrous cycle duration was calculated as the number of days between one estrous phase to the next.[11] The coefficient (%) of the cycle phases was obtained by the ratio of the number of occurrences (days) of each phase and the total number of days evaluated (estrous cycle), multiplied by 100. Euthanasia was performed at 2 p.m. in the first occurrence of proestrus after PND 90 for evaluation of the parameters during the positive feedback induced by ovarian steroids.

Determination of preputial separation and vaginal opening

The assessment of preputial separation is a reproductive development parameter for male rats, indicating puberty onset for males. From PND 40 onwards, the external genitalia was daily observed until the preputial separation.[14,15] A reproductive development parameter for females is the vaginal opening. From PND 25 onward, the external part of the genitalia was daily observed until the complete vaginal opening. When the vaginal opening was detected, a vaginal smear was performed until the occurrence of the first estrus.[11]

Lee index

On PND 21 and 90 (for males, and when occurred the first proestrus after PND 90 for females), the Lee index was calculated by dividing the cubic root of body weight in grams by nasoanal length in centimeters.[10]

Glucose tolerance test (GTT)

On the 14th gestational day of the rats, after a 6-hour food restriction, the oGTT was performed. A drop of tail blood was

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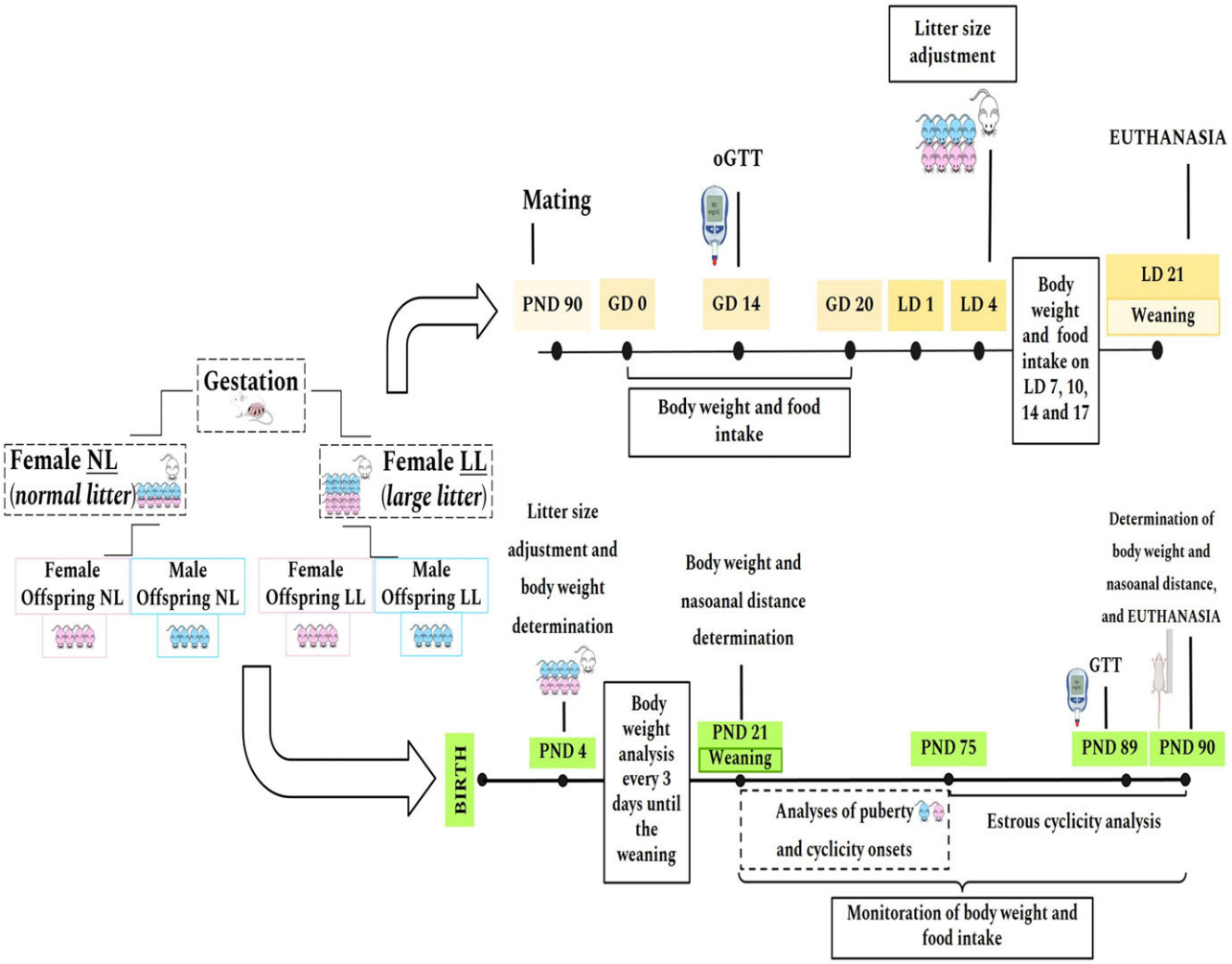

Figure 1. Experimental protocol to obtain females raised in normal litter (NL) and large litter (LL) and their offspring. GD: gestational day; LD: lactational day; oGTT: oral glucose tolerance test GTT: glucose tolerance test.

collected for basal glucose determination using the Accu-Check Advantage II test strip (Roche, Taquara, RJ, Brazil) and a device for blood glucose determination. After oral administration (gavage) of glucose (1.0 g/kg body weight) at 25% concentration was performed, blood glucose measurements were taken from the test strip 15, 30, 60, and 120 min after glucose overload. In the female and male offspring, on PND 89, the Glucose Tolerance Test (GTT) was performed. On PND 89, after 6-h food restriction, a drop of tail blood was collected for basal glucose determination using the Accu-Check Advantage II test strip (Roche, Taquara, RJ, Brazil) and a device for blood glucose determination. After intraperitoneal administration of glucose (1.0 g/kg body weight) at 25% concentration, blood glucose measurements were taken from the test strip 15, 30, 60, and 120 min after glucose overload. After 120 min, the animals returned to their cages with food and fluids *ad libitum*.

Measurement of plasma concentrations of total cholesterol, triglycerides, free fatty acids, estradiol, testosterone, and insulin

After euthanasia by decapitation, the trunk blood was collected in chilled glass tubes with heparin (Sodium Heparin - HEPAMAX-S, 10 µl of heparin per ml of collected blood) and centrifuged at 14,000 × g for 20 min at 4 °C. After centrifugation, the plasma was transferred to microtubes, which were stored at − 20 °C, for subsequent measurements of total cholesterol, triglycerides, free fatty acids, estradiol, and testosterone. Spectrophotometry was used for the determination of triglycerides (743191 - TRIGLICERIDES – BIOLIQUID – KIT 2X100 ml, kit commercial, Laborclin, PR), and total cholesterol plasma concentrations (742062, COLESTEROL DIRETO - 60 ml/20 ml – kit commercial, Laborclin, PR). The concentration of free fatty acids (FFA) was determined using a descriptive spectrophotometric method.[16] Immunoenzymatic assays (EIA) for determination of insulin (Intra-Assay-Reproducibility: CV <10%; Inter-Assay Reproducibility: CV <12%), estradiol (DRG® Estradiol ELISA; analytical sensitivity: 9.714pg/ml) and testosterone (DRG® Testosterone ELISA; analytical sensitivity: 0.083 ng/ml) . The minimum and maximum detection levels were 25 and 2000 pg/ml for estradiol and 0.2 and 16 ng/ml for testosterone and 4.69 and 300µIU/ml for insulin, according to the manufacturer's instructions.

Statistical analysis

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

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and homogeneity were analyzed by the Student's *t*-test or repeated measures ANOVA (RM ANOVA), and expressed as mean ± standard error of the mean (SEM). Conversely, Mann-Whitney *U* was performed for other variables, expressed as median, 1st and 3rd interquartile range [Q1–Q3]. Repeated measures ANOVA (RM ANOVA), followed by Sidak for multiple measures post-test, were performed to evaluate body weight, food intake, and GTT. Differences were considered significant when $p < 0.05$.

Results

Effects of postnatal undernutrition induced by litter size expansion in rats during gestation and lactation

There was no difference in the number of pups delivered by dams that formed NL and LL (Dams of NL: 12.4 ± 0.57 vs Dams of LL: 12.5 ± 0.61; $t = 0.148$, df = 12, $p = 0.8849$). Thus, the intrauterine effect of litter size was not considered in the current study, only the postnatal adjustment of litter size, which was the aim of this work. According to the ANOVA analysis of repeated measures, there was no interaction between gestational days (GD 0–20) and group (NL and LL) in body weight (F3, 39 = 0.8034, $p = 0.4996$), with effect of days gestational (F3, 39 = 624.1, $p < 0.0001$) and groups (F1, 13 = 8.164, $p = 0.0135$) (Fig. 2a). Repeated measures ANOVA also showed that there was no interaction between lactational days 7 and 17 (LD 7–17) and group (NL and LL) in body weight (F3, 45 = 0.1781, $p = 0.9107$), with effect of lactational days (F3, 45 = 5.61, $p = 0.0024$) and groups (F1, 15 = 6.914, $p = 0.019$) (Fig. 2a). LL females showed lower body weight during pregnancy and lactation. During lactation, there was no interaction between lactational days 10 and 17 (LD 10–17) and group (NL and LL) in food intake (F2, 30 = 0.1938, $p = 0.8249$), without effect of lactational days (F2, 30 = 3.231, $p = 0.0536$), but with effects of groups (F1, 15 = 7.98, $p = 0.0128$) (Fig. 2b). The large litter (LL) rats showed hyperphagia, compared to NL. There was also no difference between the groups in the weights of the ovaries [$t(18) = 0.6325$, $p = 0.5350$], uterus [$t(18) = 0.3901$, $p = 0.7010$], retroperitoneal [$t(17) = 1.975$, $p = 0.0647$] and the perigonadal + perirenal adipose tissues [$t(18) = 1.174$, $p = 0.2557$] (Fig. 2c–f).

Regarding the oGTT during pregnancy, there was no interaction between the time after glucose overload (0 to 120 min) and the groups (NL and LL) on blood glucose (F4.72 = 0.786, $p = 0.538$), with time effects (F4.72 = 64.75, $p < 0.0001$), but no group effect (F1.18 = 2.506, $p = 0.1308$) (Fig. 4g). Furthermore, there was no difference between groups in the area under the oGTT curve [$t(18) = 0.6809$, $p = 0.5046$] (Fig. 2h). There was no difference between groups in plasma concentration of triglycerides [$t(16) = 1.222$, $p = 0.2394$], and free fatty acids [$t(15) = 0.9760$, $p = 0.3446$], however, the LL rats had a lower plasmatic concentration of total cholesterol than the NL females [$t(21) = 2.329$, $p = 0.0299$]) (Fig. 2i–k).

Effects of preconception maternal undernutrition, induced in the neonatal period by litter size expansion, on the metabolic parameters of their male and female offspring

According to the repeated measures ANOVA analysis, there was interaction between days (PND 4–21) and groups (Offspring from NL mothers and Offspring from LL mothers) in body weight of male offspring from PND 4 to PND 21 (F5, 190 = 10.21, $p < 0.0001$). The body weight of the male offspring of LL mothers was lower ($p < 0.05$) on PND 14, 17 and 21 (Fig. 3a). There was no interaction between days (PND 21–90) and groups (Offspring from NL dams and Offspring from LL dams) in the body weight of male offspring from PND 21 to PND 90 (F14, 532 = 0.8727, $p = 0.589$), with effect of days (F14, 532 = 1935, $p < 0.0001$) and group (F1, 38 = 4.779, $p = 0.035$). The male offspring of LL mothers showed reduced body weight ($p < 0.05$) from PND 21 to PND 90 (Fig. 3c). Repeated measures ANOVA showed that there was no interaction between days (PND 4–21) and groups (Offspring from NL dams and Offspring from LL dams) in the body weight of female offspring from PND 4 to PND 21 (F5, 190 = 1.514, $p = 0.1871$), with days effect (F5, 190 = 1400, $p < 0.0001$), but no group effects (F1, 38 = 2.815, $p = 0.1016$) (Fig. 3b). There was also no interaction between days (PND 21–90) and groups (Offspring from NL mothers and Offspring from LL mothers) in body weight of female offspring from PND 21 to PND 90 (F14, 532 = 1.159, $p = 0.3035$), with days effect (F14, 532 = 1752, $p < 0.0001$), but without group effects (F1, 38 = 3.35, $p = 0.075$) (Fig. 3d).

There was interaction between days (PND 25–90) and groups (Offspring from NL mothers and Offspring from LL mothers) in the food intake of male offspring from PND 25 to PND 90 (F12, 228 = 3.463, $p = 0.0001$). The male offspring of LL mothers showed higher food intake ($p < 0.05$) on PND 35 and 45 than the male offspring of NL mothers (Fig. 3e). There was also interaction between days (PND 25–90) and groups (Offspring from NL mothers and Offspring from LL mothers) in the food intake of female offspring from PND 25 to PND 90 (F12, 444 = 8.347, $p < 0.0001$). The female offspring of LL mothers also showed higher food intake ($p < 0.05$) on PND 35 and 45 than the female offspring of NL mothers (Fig. 3f). Both male offspring [$t(38) = 6.281$, $p < 0.0001$] and female offspring [$t(36) = 2.574$, $p = 0.0143$] from LL mothers showed increased ($P < 0.05$) AUC of food intake compared to the offspring of NL mothers (Fig. 3g, h).

There was no difference in the weight of retroperitoneal and perigonadal adipose tissues between the offspring of NL and LL mothers, both in males {retroperitoneal [$t(38) = 1.01$, $p = 0.3188$] and perigonadal [$t(37) = 0.959$, $p = 0.3438$]}, and females {retroperitoneal [$t(37) = 0.01985$, $p = 0.9843$] and perigonadal + perirenal [$t(36) = 0.8285$, $p = 0.4129$]} (Fig. 4a–d). The male offspring of LL mothers had a reduced nasoanal distance on PND 21 [$t(36) = 3.56$, $p = 0.0011$] and an increase of this parameter on PND 90 [$t(36) = 4.331$, $p < 0.0001$] (Fig. 4g), but had a lower Lee index on PND 90 [$t(36) = 5.063$, $p < 0.0001$], with no differences in the Lee index in PND 21 [$t(36) = 1.847$, $p = 0.0733$] (Fig. 4e). The female offspring of LL mothers showed reduced nasoanal distance on PND 21 [$t(36) = 2.584$, $p = 0.014$], with no differences of this parameter on PND 90 [$t(38) = 0.4728$, $p = 0.6391$] (Fig. 4h), in addition to no repercussions on the Lee index on PND 21 [$t(36) = 1.346$, $p = 0.1868$] and PND 90 [$t(38) = 0.3393$, $p = 0.7362$] (Fig. 4f).

Male oLL had lower plasma concentrations of triglycerides than male oNL [$t(27) = 3.671$, $p = 0.0011$] (Fig. 5a), with no difference in female offspring in this parameter [$t(27) = 0.8803$, $p = 0.3865$] (Fig. 5b). There were no differences in plasma concentrations of free fatty acids in male [$t(28) = 0.8251$, $p = 0.4163$] and female [$t(27) = 1.093$, $p = 0.2839$] offspring (Fig. 5c, d), as well as total cholesterol in male [$t(28) = 0.4292$, $p = 0.6711$] and female [$t(28) = 0.4819$, $p = 0.6336$] offspring (Fig. 5e, f).

Male oLL showed an increase in plasma insulin concentration when compared to male oNL [$t(13) = 2.368$, $p = 0.0341$] (Fig. 6a), with no differences in female offspring [$t(16) = 0.6545$, $p = 0.5221$] (Fig. 6b). The ANOVA of repeated measures of the GTT in the male offspring showed that there was an interaction between the

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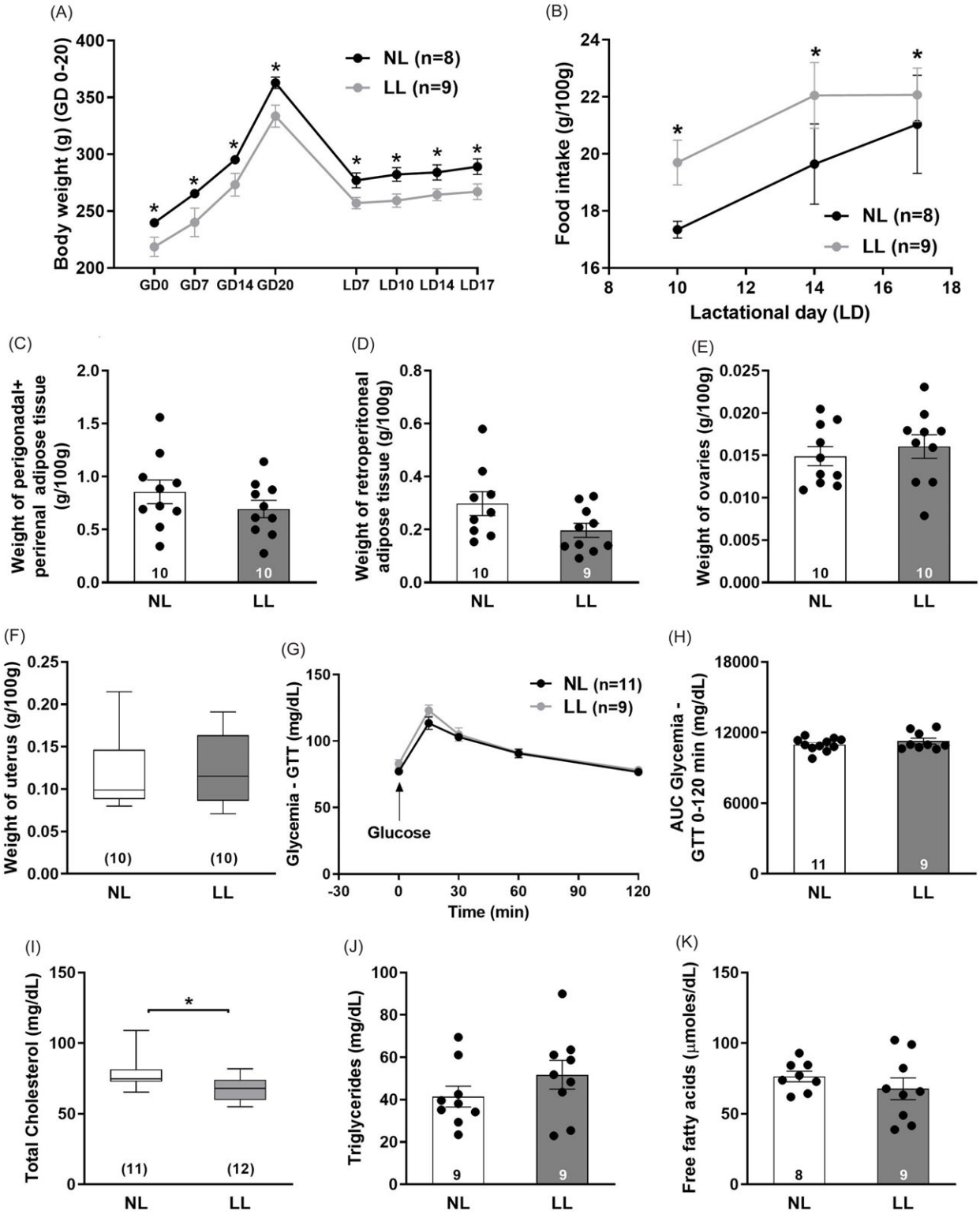

Figure 2. A) Body weight in the gestational period (GD 0 to GD 20) and lactational period (LD 7 to 17), B) food intake during the lactational period, C) weight of perigonadal+ perirenal adipose tissue, D) weight of retroperitoneal adipose tissue, E) weight of ovaries, F) weight of uterus of NL and LL females on lactational day (LD) 21, G) oral glucose tolerance test (oGTT), H) oGTT area under the curve (AUC) of NL and LL females on gestational day (GD) 14; I) plasma concentrations of total cholesterol, J) triglycerides, and K) free fatty acids of NL and LL females on LD 21. The number of animals per group is shown in the graphics. GD: gestational day; LD: lactational day. Data were analyzed by the Student's *t*-test (AUC of food intake, weights of adipose tissues and ovary, AUC of GTT, plasma concentrations of triglycerides, and free fatty acids), and MR ANOVA (body weight, food intake, and GTT), expressed as mean ± SEM, or by Mann-Whitney test (weight of uterus and plasma concentrations of total cholesterol), expressed as median [Q1–Q3]. * $p < 0.05$ NL vs LL.

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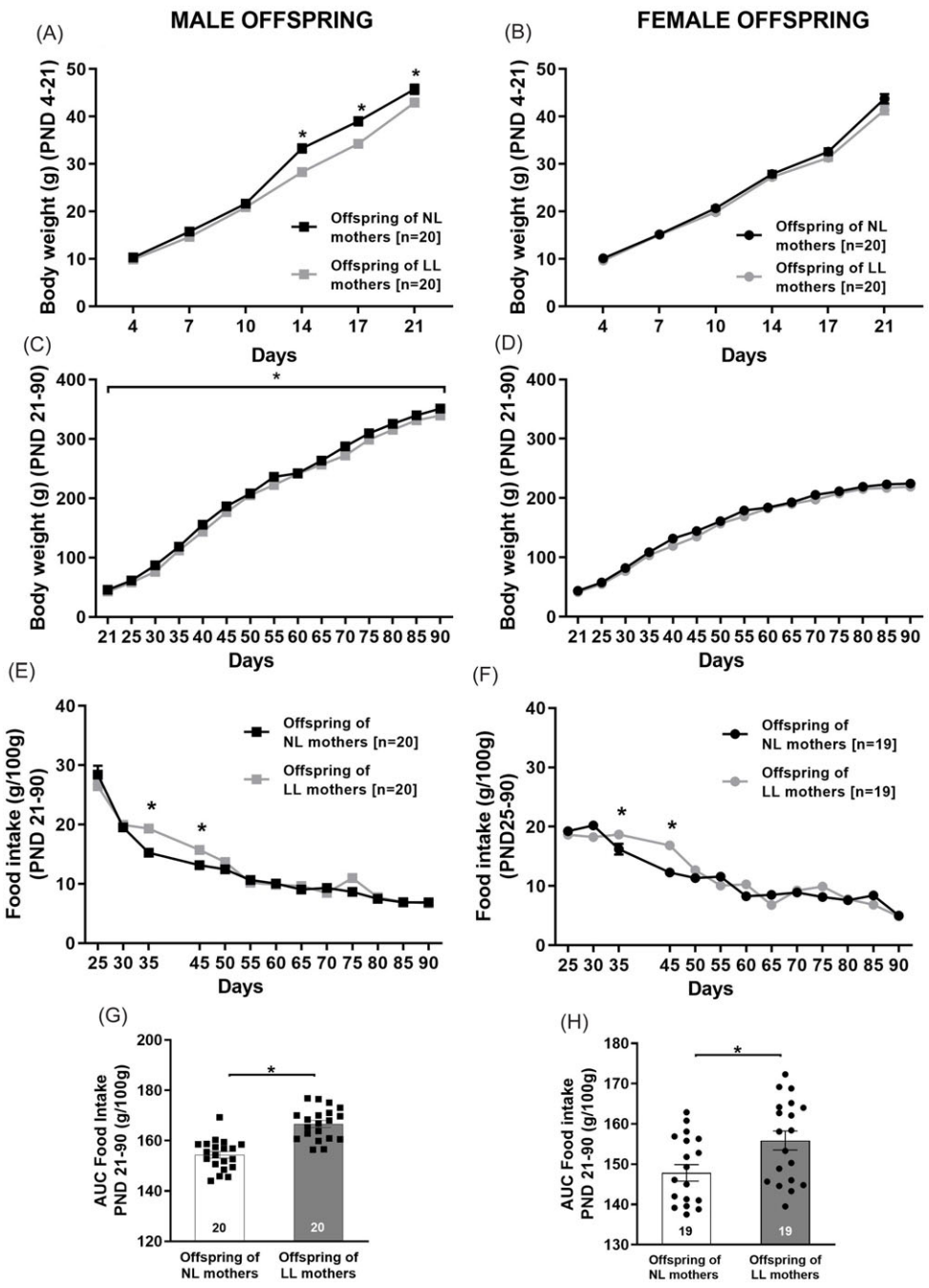

Figure 3. Body weight from DPN (postnatal day) 4 to 21 (weaning) and from DPN 21 to 90 of male (A, C) and female (B, D) offspring of females raised in normal litter (NL) and large litter (LL). Food intake and area under the curve (AUC) of food intake from PND 21 to 90 of male (E, G) and female (F, H) offspring of NL and LL female rats. Data were analyzed by the Student's *t*-test (AUC of food intake), and MR ANOVA (body weight and food intake), expressed as mean ± SEM. * $p < 0.05$ offspring from NL mothers vs offspring from LL mothers.

time after the glucose overload (0 to 120 min) and the groups (oNL mothers and oLL mothers) on glycemia (F4.128 = 5.891, $p = 0.0002$). The male oLL mothers had lower blood glucose values after 15 ($p < 0.01$) and 30 minutes ($p < 0.001$) of glucose administration than the male oNL mothers, in addition to a reduction in the area under the curve of GTT [$t(32) = 2.806$, $p = 0.0085$] (Fig. 6c, e). For the female offspring, there was no interaction between the time after glucose overload (0 to 120 min) and the groups (oNL mothers and oLL mothers) on glycemia (F4.76 = 0.5962, $p = 0$ .6665), with time effects (F4, 76 = 222.2, $p < 0.0001$), but without group effects (F1.19 = 0.0046, $p = 0.9466$), in addition to no difference in the area under curve of GTT [$t(38) = 0.0842$, $p = 0.9333$] (Fig. 6d, f).

Effects of preconception maternal undernutrition, induced in the neonatal period by large litter size, on the reproductive parameters of their male and female offspring [F2]

There was no difference between male offspring of NL and LL mothers in preputial separation [$t(38) = 0.1593$, $p = 0.8743$], testes weight [t(31) = 0.8487, $p = 0$, 4026], and testosterone plasma concentrations [$t(17) = 0.6469$], $p = 0.5263$] (Table 1). In female offspring, no difference was observed between groups in vaginal opening [$t(38) = 0.1215$, $p = 0.9039$], first estrus [$t(38) = 0.9087$, $p = 0.3692$], onset of cyclicity [$t(38) = 0.9221$, $p = 0.3623$], duration of the estrous cycle [$t(38) = 0.7113$, $p = 0.4813$], coefficient of diestrus I (metestrus) and II [$t(38) = 0.9301$, $p = 0.3582$], estrus coefficient [$t(38) = 0.8608$], $p = 0.3947$], proestrus coefficient [$t(33) = 1.169$, $p = 0$, 2507], estradiol plasma concentrations [$t(17) = 0.1036$,

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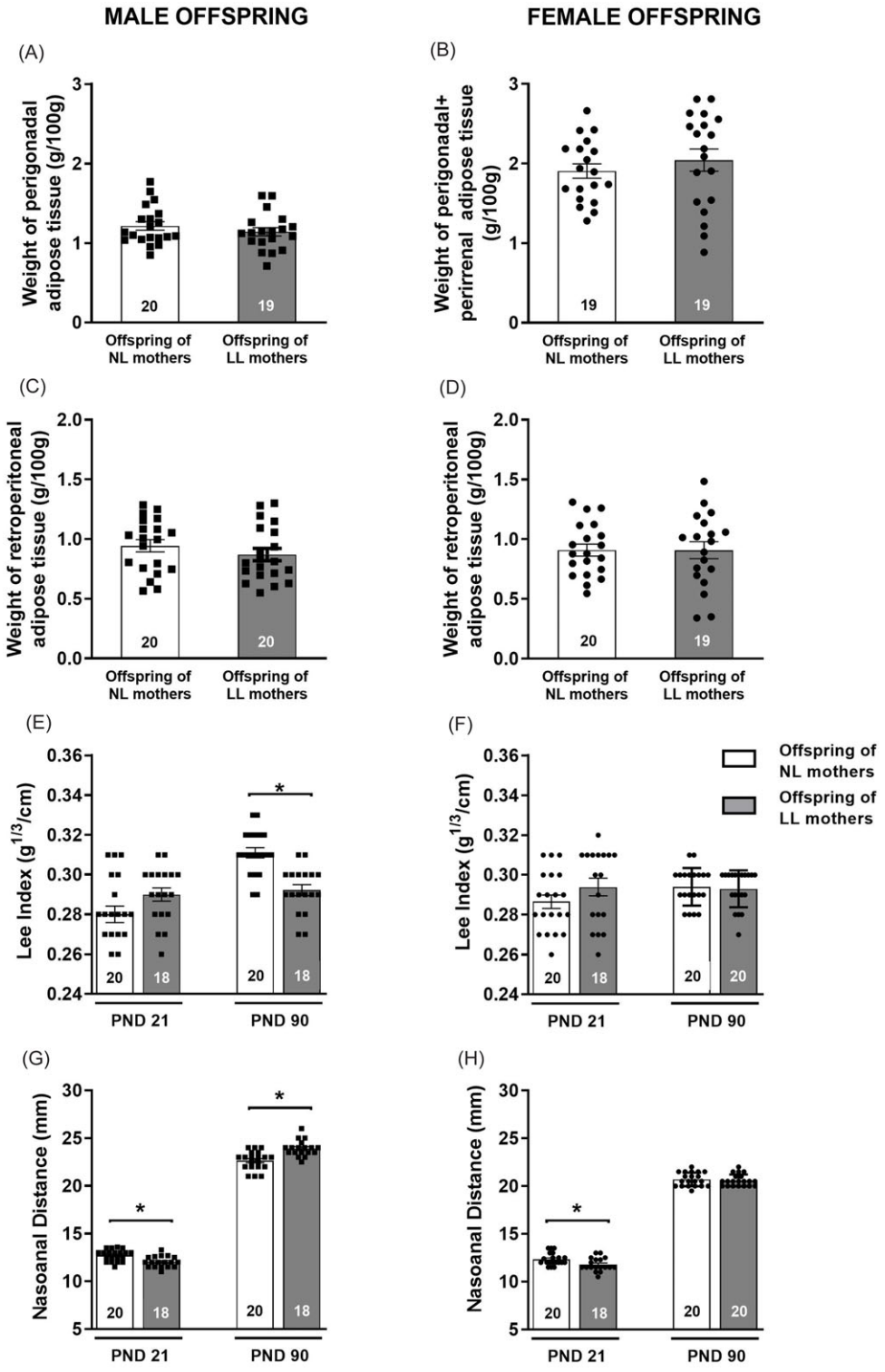

Figure 4. Weight of perigonadal and retroperitoneal adipose tissues of of male (A, C) and female (B, D) offspring of females raised in normal litter (NL) and large litter (LL). Lee index and nasoanal distance on PND 21 and 90 of male (E, G) and female (F, H) offspring of NL and LL female rats. Data were analyzed by the Student's *t*-test, and expressed as mean ± SEM. *$p < 0.05$ offspring from NL mothers vs offspring from LL mothers.

$p = 0.9187$], and ovarian [t (36) = 0.3712], $p = 0.7127$] and uterus weights [t(38) = 0.05655, $p = 0.9552$] (Table 2).

Discussion

In the current study, offspring of both sexes have shown metabolic alterations due to maternal undernutrition induced by the expansion of the litter size, and there is sexual dimorphism, observed by the reduction of body weight, despite the increase in food intake; decreased Lee index, increased nasoanal distance and plasma concentration of insulin, in addition to reduced triacylglycerol and glycemia after glucose overload in the adulthood of male offspring of underfed mothers; however, no changes were observed in reproductive parameters in both male and female offspring.

Female Wistar rats reared in large litter, the dams of the offspring used in the current work, showed reduced body weight during pregnancy and lactation periods, despite hyperphagia

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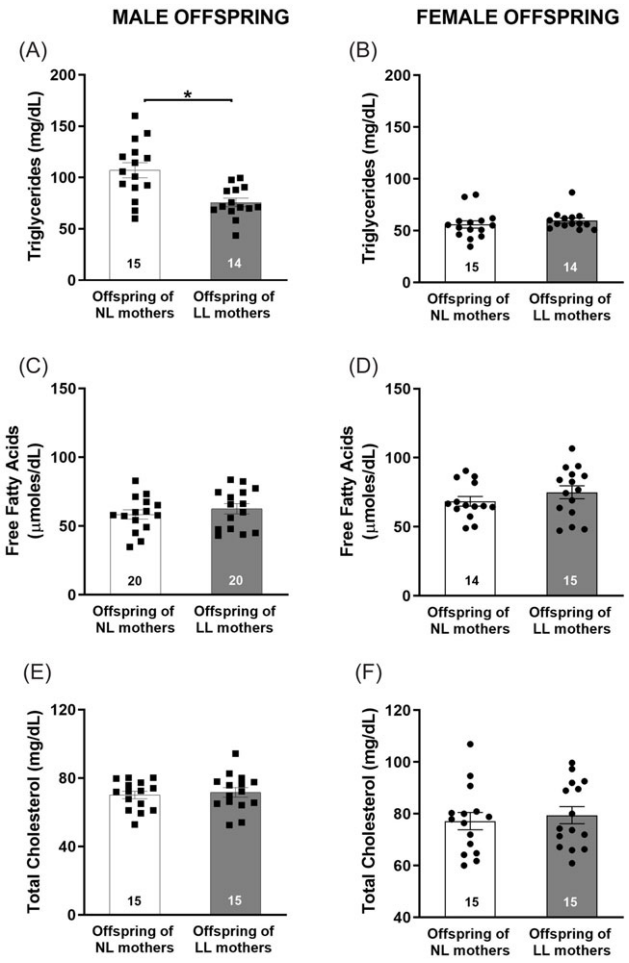

Figure 5. Plasma concentrations of triglycerides (mg/dL), free fatty acids (µmoles/dL) and total cholesterol (mg/dL) of male and female offspring of NL and LL rats. Data were analyzed by the Student's *t*-test, and expressed as mean ± SEM. * $p < 0.05$ offspring from NL mothers vs offspring from LL mothers.

during lactation. Indeed, it is well established in the literature that maternal undernutrition, both in human and rodent models, causes alterations in the programming of the offspring and metabolic diseases.[17-19] According to this, maternal undernutrition in the post-conceptional periods, such as pregnancy and/or lactation, and their effects on the offspring have been largely explored, showing that these offspring present lower body weight from weaning to adulthood and higher food intake, in addition to increased plasma levels of triglycerides and total cholesterol when compared to animals of normal offspring.[16,20-22] However, there are no studies, as far as it is known, demonstrating the effects of maternal undernutrition in the preconception periods on the metabolic and reproductive profiles of their offspring.

Accordingly, the present work shows, for the first time, that some responses observed in dams with lactational undernutrition also occur in their offspring, since offspring of both sexes showed reduced longitudinal growth during lactation and hyperphagia after weaning. According to this, research on humans shows that undernourished children have *catch-up* growth, involving increased food intake.[23] In addition, increased release of neuropeptide Y (NPY), an orexigenic neuropeptide,[24] has been reported in LL animals.[7] Thus, hyperphagia of LL offspring may also be related to increased activity of orexigenic pathways. Furthermore, reduced linear length at weaning in male and female offspring of LL dams, evaluated by nasoanal distance, suggests that maternal undernutrition may induce changes in the levels and/or action of growth hormone (GH),[25] insulin and thyroid hormone during lactation, the main hormones involved in the regulation of growth.[26,27] GH is considered the regulator of postnatal growth and somatic development[25,28], and maternal undernutrition during lactation caused decreased body length associated with reduced GH expression at weaning in rats.[29] Growth hormone therapy, before or after weaning, has been used to treat postnatal growth failure in offspring after maternal undernutrition.[28] On the other hand, maternal caloric restriction during gestation induced a reduction of insulin plasma levels at birth, but not during lactation or at weaning in the offspring.[29] Furthermore, maternal undernutrition during lactation caused decreased plasma levels of T4 and increased plasma levels of T3 throughout lactation and at the weaning of the offspring.[30] Thus, it is tempting to suggest that decreased activity of the somatotropic axis is likely to mediate the reduction in longitudinal growth during lactation in the offspring of underfed dams.

Howie et al.[31] demonstrated that the duration and degree of undernutrition are determining factors for the development of offspring, and their effects are sex-specific. The current results show that the metabolic effects of maternal undernutrition induced by litter size expansion on the offspring are also sex-dependent, as seen by reduced body weight from lactation until adulthood, increased insulin plasma levels, associated with reduced plasma levels of triglycerides and glycemia after glucose overload, in addition to increased longitudinal length in adulthood in male offspring, but not in females.

The increased plasma levels of insulin, reduced glycemia after glucose overload, and AUC of GTT in male offspring of LL dams, without difference in female offspring, is in accordance with other studies that have reported that female offspring of dams with undernutrition induced by protein restriction during lactation have lower insulin levels in adulthood than male offspring, indicating that female hormones can regulate plasma insulin levels in adulthood in female offspring of dams with undernutrition by protein restriction, suggesting a sex-specific hormonal shift.[9,32] Thus, increased insulin plasma concentrations in male offspring of LL dams might have prevented high surges of glycemia after glucose overload. Additionally, the higher insulinemia of male offspring of undernourished mothers could also explain the lower plasma concentration of triglycerides observed in this group, since it is known that insulin inhibits the production of very low-density lipoproteins (VLDL) by the liver, which contains triglycerides produced by hepatocytes from fatty acids acquired from the circulation or synthesized from glucose or other non-lipid precursors by *de novo* lipogenesis.[33] Reinforcing these data, a study carried out by Fuente-Martín and collaborators[34] demonstrated that maternal nutrition, fetal growth, and postnatal growth have been associated with possible changes in insulin sensitivity, with some of these effects being sexually dimorphic.

Interestingly, despite reduced body weight throughout life, male offspring of LL dams showed increased nasoanal distance in adulthood, evidencing a *catch-up* in longitudinal growth in male offspring, and this effect was also observed in the female offspring, as the offspring of LL mothers reached the longitudinal growth of offspring of NL mothers in adulthood. Thus, the thin phenotype of LL dams is likely to be passed to the male offspring, which also presents increased longitudinal length, which might be ascribed to a *catch-up* in hormones involved in the regulation of growth after weaning and/or from puberty. This hypothesis is reinforced by

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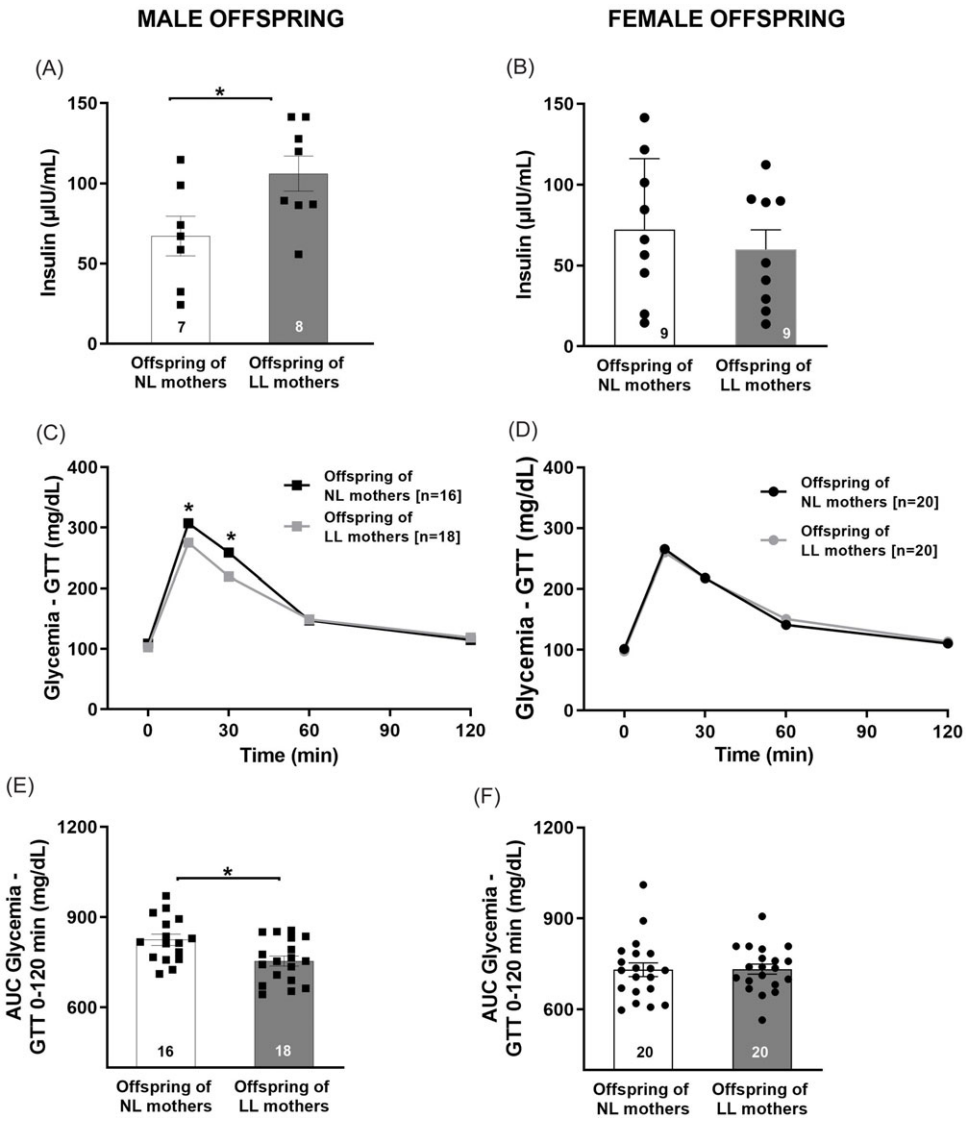

Figure 6. Plasma concentrations of insulin (µIU/ml), glucose tolerance test (GTT; mg/dL), and area under the curve (AUC) of the GTT of male and female offspring of NL and LL rats. Data were analyzed by the Student's *t*-test (plasma concentrations of insulin, and AUC of GTT), and MR ANOVA (GTT), expressed as mean ± SEM. * $p < 0.05$ offspring from NL mothers vs offspring from LL mothers.

Moura et al.,[29] who demonstrated that maternal energy restriction during lactation caused decreased body length associated with reduced GH expression at weaning in rats, but enhanced body length associated with increased GH expression in the adulthood of the offspring.

Sex differences in different metabolic challenges have been increasingly shown in the literature. Male rats were more susceptible to the anabolic effects of glucocorticoids than females,[12] and male mice were more susceptible to developing obesity than females in response to a hyperlipidic diet.[35] In addition, males were more susceptible than females to early dietary deficiencies, radiation and handling stresses.[36] A study by Bañoz-Gómez and collaborators[9] suggests that female estrogens protect against drastic changes in female metabolic regulation. Importantly, sex-related differences in energy balance in response to caloric restriction were shown by Valle et al.,[37] who concluded that female rats seem to decrease energy expenditure by protecting metabolically active organs to a greater extent than male rats during caloric restriction and that this sex-dependent response may be critical to achieving conservation of energy in females, promoting their survival and of the species. Considering metabolic programming, a recent study observed a sex-specific response in the offspring of females with obesity induced by lactation overfeeding, in which only male offspring showed cardiac dysfunction.[38] This study also demonstrated that both male and female offspring of dams with lactational obesity present changes in the plasma levels of sexual hormones.[38]

Thus, the present work shows that the offspring of dams with lactational undernutrition induced by the litter size expansion, a preconception maternal undernutrition model, causes metabolic changes, mainly in male offspring, without affecting reproduction in offspring of both sexes, as the weight of reproductive organs, puberty onset, sexual hormones, and estrous cyclicity. These data, along with data shown by Kwong et al.,[39] demonstrated that maternal undernutrition during the preconception phase can generate long-term changes in their offspring, such as in their postnatal growth. Altogether with data by Ferreira et al.,[38] the current results suggest that the metabolic status of the mothers may be responsible for metabolic and/or reproductive changes in the offspring, where males are generally more likely to present metabolic alterations than females, since females seem to have a greater capacity to maintain their homeostasis in response to metabolic challenges than males.

In conclusion, the present work showed that in the maternal undernutrition preconception model, where these mothers had

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Table 1. Weight of testis (g/100g), preputial separation (postnatal day: PND), and plasma testosterone concentration (ng/ml) of male offspring from NL (oNL) and LL (oLL) female rats

Groups	Male oNL	Male oLL
Weight of testes (g/100g)	0.41 + 0.07[18]	0.43 + 0.04[15]
Preputial separation (PND)	45.1 + 0.54[20]	45.0 + 0.31[20]
Testosterone (ng/ml)	1.78 + 0.13[9]	2.02 + 0.32[10]

Data were analyzed by the Student's *t*-test, and expressed as mean ± SEM. The number of animals in each group is described above the data.

Table 2. Weight of ovaries and uterus (g/100g), vaginal opening (postnatal day: PND), first estrus (PND), cycle regularization (PND), cycle duration (days), proestrus coefficient (%), estrus coefficient (%), diestrus coefficient (I and II) (%), and plasma estradiol concentration (pg/ml) of female offspring from NL (oNL) and LL (oLL) female rats

Groups	Female oNL	Female oLL
Weight of ovaries (g/100g)	0.02 + 0.00[20]	0.02 + 0.00[18]
Weight of uterus (g/100g)	0.21 + 0.009[20]	0.21 + 0.008[20]
Vaginal opening (PND)	32.1 + 0.34[20]	32.1 + 0.22[20]
First estrus (PND)	34.5 + 0.65[20]	33.8 + 0.39[20]
Onset of cyclicity (PND)	48.7 + 0.58[20]	49.4 + 0.48[20]
Cycle duration (days)	4.41 + 0.18[20]	4.24 + 0.14[20]
Proestrus coefficient (%)	22.0 + 0.77[17]	23.2 + 0.67[18]
Estrus coefficient (%)	23.4 + 0.76[20]	24.3 + 0.77[20]
Diestrus coefficient I and II (%)	53.1 + 1.06[20]	51.9 + 0.80[20]
Estradiol (pg/ml)	41.1 + 4.05[9]	40.7 + 1.94[10]

Data were analyzed by the Student's *t*-test, and expressed as mean ± SEM. The number of animals in each group is described above the data.

access to an adequate nutritional diet after lactation, maternal undernutrition induced by litter size expansion causes metabolic programming in their offspring. Such changes had more repercussions on the male offspring, without reproductive effects on the offspring of both sexes. This sexual dimorphism reinforces the hypothesis that females have a greater capacity to maintain their homeostasis in response to metabolic challenges.

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Competing interests. None.

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