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Intrauterine malnutrition disrupts leptin and ghrelin milk hormones, programming rats

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Abstract

Herein, we assessed milk hormones, the biochemical composition of milk, and its association with neonatal body weight gain and metabolic homeostasis in weaned rats whose mothers were undernourished in the last third of pregnancy. From the 14th day of pregnancy until delivery, undernourished mothers had their food restricted by 50% (FR50), whereas control mothers were fed *ad libitum*. The litter size was adjusted to eight pups, and rats were weaned at 22 days old. Milk and blood from mothers, as well as blood and tissues from pups, were collected for further analyses. At birth, FR50 pups were smaller than control pups, and they exhibited hyperphagia and rapid catch-up growth during the suckling period. On day 12, the milk from FR50 mothers had higher energy content, glucose, total cholesterol, triglycerides, and acylated ghrelin but lower leptin and corticosterone levels. Interestingly, FR50 mothers were hypoglycemic and hyperleptinemic at the end of the nursing period. Weaned FR50 pups had an obese phenotype and exhibited insulin resistance, which was associated with hyperglycemia and hypertriglyceridemia; they also had high blood levels of total cholesterol, leptin, and acylated ghrelin. In addition, the protein expression of growth hormone secretagogue receptor (GHSR) in the hypothalamus was increased by almost 4-fold in FR50 pups. In summary, maternal calorie restriction during the last third of pregnancy disrupts energy and metabolic hormones in milk, induces pup hyperleptinemia and hyperghrelinemia, and upregulates their hypothalamic GHSR, thus suggesting that the hypothalamic neuroendocrine circuitry may be working to address the early onset of obesity.

Key Words

- food restriction
- metabolic programming
- metabolic hormones
- milk composition
- obesity
- metabolic syndrome

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Introduction

In the early stages of development, individuals are in a critical process of plasticity that is sensitive to different types of stress, such as physiological perturbations and including inadequate maternal nutrition. This lack of adequate nutrition can impair the homeostasis of circulating nutrients and important metabolic hormones

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for fetuses and/or babies in their early development (Ross & Desai 2014, Badillo-Suarez *et al.* 2017). In fact, gestational undernutrition affects not only offspring in fetal stages but also infants in their first stages of life (Facchi *et al.* 2020).

For human conditions, especially in low-income countries, it is known that undernutrition does not occur only at specific stages of life development, such as certain portions of the pregnancy or lactation period, but rather throughout pregnancy and lactation in general (Victora *et al.* 2008, Uauy *et al.* 2011). However, evidence from the Dutch (Lumey 1992) and the Leningrad (Stanner *et al.* 1997) famine studies has shown that maternal undernutrition programs long-term metabolic dysfunction in the offspring and that some subtle characteristics of this metabolic impairment depend on the period when the maternal undernutrition occurred. Similarly, while the offspring from women exposed to food scarcity only during pregnancy exhibit an obese phenotype, the same did not occur when maternal exposure to calorie restriction occurred during the lactating period (Ross & Desai 2014).

For instance, through the use of experimental rodent models, our and other groups have studied the effects of maternal undernutrition during specific periods of offspring development (for example, throughout pregnancy and lactation (Fernandez-Twinn *et al.* 2005, Zambrano *et al.* 2005), during a portion of pregnancy and throughout lactation (Delahaye *et al.* 2010), only during pregnancy (Zambrano *et al.* 2005, Burdge *et al.* 2007), only for a portion of the pregnancy (de Oliveira *et al.* 2016, Venci *et al.* 2018), only during lactation (Zambrano *et al.* 2005, Fagundes *et al.* 2007), and only during a portion of lactation (de Oliveira *et al.* 2011, Mathias *et al.* 2020)). Generally, the offspring are affected differently depending on the period when maternal food scarcity occurs, but they all exhibit metabolic dysfunction.

In a previous study, we showed that maternal protein restriction in the last third of pregnancy programs the offspring's obese phenotype with failure of the endocrine pancreas to secrete insulin and with hypothalamic–pituitary–adrenal axis dysfunction in adulthood (de Oliveira *et al.* 2016). In addition, maternal calorie restriction that begins in the last third of pregnancy and remains until weaning is responsible for the malprogramming of neonatal rats, drastically reducing the leptin surge with an associated reduction in hypothalamic proopiomelanocortin (POMC) gene expression (Delahaye *et al.* 2008). Interestingly, when mothers were exposed to protein restriction throughout lactation, milk leptin concentration was increased by approximately 42%, but no change was found in milk leptin concentration when the same maternal nutritional stress was present throughout pregnancy or during pregnancy and lactation (Bautista *et al.* 2008). In the literature, studies report that maternal nutritional insults such as calorie restriction, obesogenic diet consumption, and maternal obesity impair milk composition (Pico *et al.* 2021); therefore, these insults can affect the offspring's energy and metabolism homeostasis as long-term consequences.

Metabolic hormones, especially in the first periods of development, are essential to address neuroendocrine signaling maturation and connections, and their adequate content in milk and consumption during breastfeeding is undoubtedly pivotal for the metabolic health of infants (Badillo-Suarez *et al.* 2017). In this regard, to the best of our knowledge, leptin is the most widely studied metabolic hormone in our field. Its function during critical periods of development has been well demonstrated by its direct action on the hypothalamus and its induction of neuroendocrine projection stability and maturation; thus, it is an important neurotrophic factor for neuronal ontogenetic plasticity (Bouret & Simerly 2007). As also demonstrated, rat pups from mothers with a 20% dietary calorie restriction throughout gestation were protected against disturbances in energy homeostasis and metabolism by the use of leptin as a supplement in the suckling phase (Konieczna *et al.* 2013, Szostaczuk *et al.* 2017). Beyond leptin, ghrelin has also been shown to have an important action on neuroendocrine maturation during the suckling phase (Steculorum *et al.* 2015); it acts on hypothalamic orexigenic and anorexigenic neuronal circuitry as one important factor directly affecting its plasticity (Serrenho *et al.* 2019).

Thus, considering the role of metabolic hormones in early stages of development for infant health, we hypothesized that changes in maternal energy balance, even in the last third of pregnancy, can affect the content of these key hormones in milk, which may be underlying the origin of the metabolic syndrome that is commonly observed in their offspring as a long-term consequence. Nonetheless, little is known about the effects of undernutrition only during pregnancy or only in a portion of pregnancy, such as the last third of pregnancy, on the metabolic hormones in milk and its circulating levels in mothers, as well as its effect on offspring in early stages of development. Therefore, to better understand this interplay, herein, we assessed milk composition, including metabolic hormones, and its association with body and metabolic homeostasis in weaned male rat pups whose mothers were undernourished during the last third of pregnancy.

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Materials and methods

Ethical approval

The experimental protocol was approved by the Animal Ethics Committee of the Federal University of Mato Grosso (CEUA/UFMT; process number 23108.724433/2017-16), which adheres to the Brazilian Federal Law number 11.794/2008. Our study complies with the animal ethics checklist as described by Grundy (2015).

Experimental design

All the rats were kept under controlled conditions including temperature (23 ± 2°C), humidity (55 ± 5%), and lighting (12 h light cycle, lights switched on 06:00 h to 18:00 h) throughout the experimental period with water and rodent chow *ad libitum* (Nuvital®, Curitiba, PR, Brazil).

Virgin, female, 70-day-old Wistar rats were mated with proven male breeders, and afterward, a vaginal smear washed with saline solution (NaCl, 0.9%, w/v) was collected to evaluate the presence of spermatozoa. The day when spermatozoa were present in a vaginal smear was designated as the day of conception. Only rats that were pregnant within 10 days were used for experimental procedures (de Oliveira *et al.* 2016). The control group dams (CONT, $n = 6$) were fed throughout gestation and lactation *ad libitum*, whereas the 50% food-restricted group dams (FR50, $n = 6$) were fed 50% of the daily food intake of control mothers from day 14 of pregnancy until delivery (Delahaye *et al.* 2008). Subsequently, the mother returned to being fed throughout lactation *ad libitum*.

At delivery, the male-to-female ratio, as well as the live birth percentage, was assessed.

At birth, the birthweight and naso-anal length of the rat offspring were recorded, and then the litter size was adjusted to eight pups per lactating mother. Only litters containing at least eight male pups were used in the study.

At the end of the lactation period, after weaning, all dams were fasted overnight and euthanized for blood collection. Blood samples were centrifuged (10 min at 1248 ***g***), and plasma was stored at –20 °C for subsequent biochemical analyses.

Milk sample collection

Milk collection was performed on the 12th day of lactation, when mothers were separated from their pups for 2 h. Subsequently, the dams were anesthetized (ketamine-xylazine, 75 mg plus 15 mg/kg bw, i.m.) and, to stimulate milk ejection, received an injection (2.5 IU/kg bw, i.p.) of synthetic oxytocin (Oxytocin®, Chemical Union, Embu, São Paulo, SP, Brazil). A volume of 500 µL of milk was collected in a sterile Pasteur pipette by manually massaging the nipple (Miranda *et al.* 2017). Milk samples were stored at –80 °C for subsequent biochemical analyses.

The milk energy content was quantified by the creamatocrit technique, as previously reported (Lucas *et al.* 1978, Xavier *et al.* 2019). For this, a volume of 75 µL of milk was analyzed by the creamatocrit technique, in which the milk percentage fat content and milk energy (kcal/L) were calculated.

Milk intake

The assessment of milk intake by rat offspring was adapted from a previous study (Bautista *et al.* 2019). On the 6th, 11th, and 16th days of lactation, rat offspring were fasted for 4 h by separating them from their respective mothers. After that, the rats were weighed and returned to their cages to suckle every 1 h.

The difference between the rat weight after and before suckling was considered to be the amount of milk ingested (per gram) by each one of the rats. The values of milk intake were related to the percentage of the rats' body weight and accounted for the body weight obtained after the suckling period as follows: milk intake = ((rat body weight$_{(\text{after suckling})}$) – (rat body weight$_{(\text{before suckling})}$)/ rat body weight$_{(\text{after suckling})}$ × 100).

Body weight gain from birth until weaning assessment

During the lactation period, the body weight gain of rat pups was recorded every 2 days, from birth until 22 days of age, when weaning was performed. At weaning, at the end of the light cycle (at 19:00 h), pups were fasted overnight and then euthanized at 07:00 h by decapitation for blood and tissue sample collection.

Fat pad assessment

At the completion of all experimental procedures, the weaned rat offspring ($n = 6$ litters) were euthanized for the removal of visceral white adipose tissue samples (mesenteric, retroperitoneal, and periepididymal fat pads), which were weighed to assess fat mass as representative measures of fat pad stores. Additionally, the naso-anal length of the rats was recorded to quantify their BMI with the Lee index = ((body weight$_{(g)}$)$^{1/3}$/naso-anal length$_{(cm)}$) × 1000, which has commonly been used as a predictor of the BMI in some rodent models (Bernardis & Patterson 1968).

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The interscapular brown adipose tissue (iBAT) in the region of the neck was removed and weighed. To dissect the hypothalamus, the whole brain of the rats was removed, and then all the hypothalamic masses located in the region above the pituitary and below the thalamus were removed and stored at –80 °C for further protein expression assessment by western blotting. From the ventral face, using as reference the pituitary stalk, hypothalamus was carefully dissected by performing two frontal cuts, as rostral-caudal bounders, one at the posterior border of the optic chiasma and the other just around in front of mammillary bodies. In addition, at the lateral borders, a bilateral cut just around of the fornix region was performed, and at the level of thalamus, a horizontal cut was made to remove the hypothalamus (Pozzo Miller & Aoki 1992, Papp *et al.* 2014).

Glucose–insulin homeostasis assessment

After blood collection, the pup blood was centrifuged (10 min at 1248 ***g***), and plasma samples were stored at –20 °C for subsequent blood glucose, triglycerides, total cholesterol, and total protein evaluation through spectrophotometry using commercial kits (Gold Analisa®, Belo Horizonte, MG, Brazil).

Regarding peripheral glucose–insulin homeostasis, the values of the TyG index were calculated to predict body insulin sensitivity, since the TyG index is widely used by clinicians to identify insulin resistance in apparently healthy individuals as follows: TyG index = Ln((fasting blood glucose$_{(mg/dL)}$) × (fasting triglycerides$_{(mg/dL)}$)/2) (Simental-Mendia *et al.* 2008).

Metabolic hormone level detection

The plasma and milk levels of corticosterone (catalog number ADI-900-097, Enzo® Life Sciences, Plymouth Meeting, PA, USA), leptin (catalog number ADI-900-015A, Enzo® Life Sciences, Plymouth Meeting, PA, USA), and acylated ghrelin (catalog number EZRGRA-90K, EMD Millipore® Corporation) were quantified by commercial ELISA kits following the manufacturer's recommendations.

The intra- and interassay coefficients of variation were 7.7% and 9.7%, respectively, for corticosterone, 5.9% and 7.2%, respectively, for leptin, and 0.8% and 3.1%, respectively, for acylated ghrelin. The hormone level detection limits were 74.5 pmol/L for corticosterone, 4.2 pmol/L for leptin, and 2.4 pmol/L for acylated ghrelin.

Western blot analyses

The protein content of growth hormone secretagogue receptor (GHS-R) expressed in the hypothalamus homogenate from weaned rat offspring ($n = 4$ litters) was determined by western blotting, as previously described (Miranda *et al.* 2022). The hypothalamus samples, previously frozen at –80 °C, were thawed and homogenized (twice, 10-s pulses using an Ultraturrax® disperser, IKA® T10 basic, Germany) in 600 mL of lysis buffer (mmol/L: HEPES, 50; $MgCl_2$, 1; EDTA, 10; Triton X, 1%; v/v) containing a protease inhibitor cocktail (5 µL/mL, Roche®). Then, the homogenate was centrifuged at 14,676 ***g*** for 5 min at 4 °C. After that, the supernatant was collected, and the total protein content in the homogenate was determined by a BCA kit for protein analysis (Thermo Scientific®) using a microplate reader (Bioclin Biolisa Reader®). The samples were treated with Laemmli buffer (4:1, v/v: glycerol, 30%; β-mercaptoethanol, 20%; sodium dodecyl sulfate (SDS), 8%; Tris-HCl, 0.25 mol/L at pH 6.8, 0.5%; deionized water, and bromophenol blue) and heated in a boiling water bath for 3 min (Laemmli 1970).

The total protein extract (40 µg) from the hypothalamus was separated by 10% SDS–PAGE at 50 V for 30 min followed by 100 V for 120 min. The hypothalamus homogenate from three of four rats in each experimental group was duplicated in electrophoresis wells. A standard molecular weight marker (Broad range protein, product code: V849A, Madison CA, USA) was loaded in parallel. Then, the proteins were transferred from the gel to a nitrocellulose membrane using a Trans-Blot® Electrophoretic Transfer Cell (Bio–Rad®) at 120 V for 90 min. Then, nonspecific binding was blocked with 5% skimmed powdered milk (Molico brand) in Tween–Tris-buffered saline (Tris-HCl, 1 mol/L; NaCl, 5 mol/L; Tween-20, 0.05%, v/v) at room temperature for 90 min under continuous shaking. Next, the membranes were incubated overnight at 4 °C with one of the primary antibodies of interest: rabbit anti-GHS-R (1:1000, product code: ab-85104, Abcam®) or mouse anti-β-actin (C4) (1:1000, product code: sc-47778, Santa Cruz Biotechnology®) polyclonal primary antibody. The same membrane in which GHS-R was detected was stripped to be reused for the detection of β-actin protein expression. After that, membranes were incubated for 90 min with goat anti-rabbit (1:10,000, product code: ab97069, Abcam®) or goat anti-mouse (1:10,000, product code: ab98808, Abcam®) IgG-HRP secondary polyclonal antibody. The antibodies were diluted in TBS 1× with 2% albumin.

The specific bands were detected with the chemiluminescent substrate, where immunoreactive proteins were visualized using a hyperfilm ECL™ kit (GE Healthcare). The bands were quantified by densitometry

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using ImageJ 1.4 software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) and normalized against the bands obtained for β-actin and then normalized again by the mean value of the expressed protein in the control group. Normalized values are reported as the relative percentage (%) of the values found in the control group.

Statistical analyses

The results are given as the mean ± S.D. and were subjected to the D'Agostino-Pearson and/or Shapiro–Wilk normality tests, and where the data were parametric, they were subjected to Student's t-test, in which $P < 0.05$ was considered statistically significant. When data did not assume a Gaussian distribution, the Mann–Whitney test was used to compare groups.

Tests were performed using GraphPad Prism version 8.0 for Windows (GraphPad Software Inc.).

Results

Maternal status during pregnancy and nursing period

As shown in Fig. 1, the body weight gain of mothers varied depending on the food offered. As expected, during the first two-thirds of pregnancy, the body weight gain (Fig. 1A and B), food intake (Fig. 1C and D), and water consumption (Fig. 1E and F) did not differ between groups ($P > 0.05$, $n = 6$; Fig. 1A). However, during the last third of pregnancy, when maternal food supply was restricted by 50%, the body weight gain was reduced by 34.3% in FR50 mothers ($P < 0.001$, $n = 6$; Fig. 1A and B), while the food intake was reduced by 44.8% in FR50 mothers in relation to CONT mothers ($P < 0.001$, $n = 6$; Fig. 1C and D).

After delivery and throughout lactation, the body weight gain of FR50 mothers remained 38% lower than that of CONT mothers ($P < 0.05$, $n = 6$; Fig. 1A and B); however, the food intake was observed to be 29.5% higher

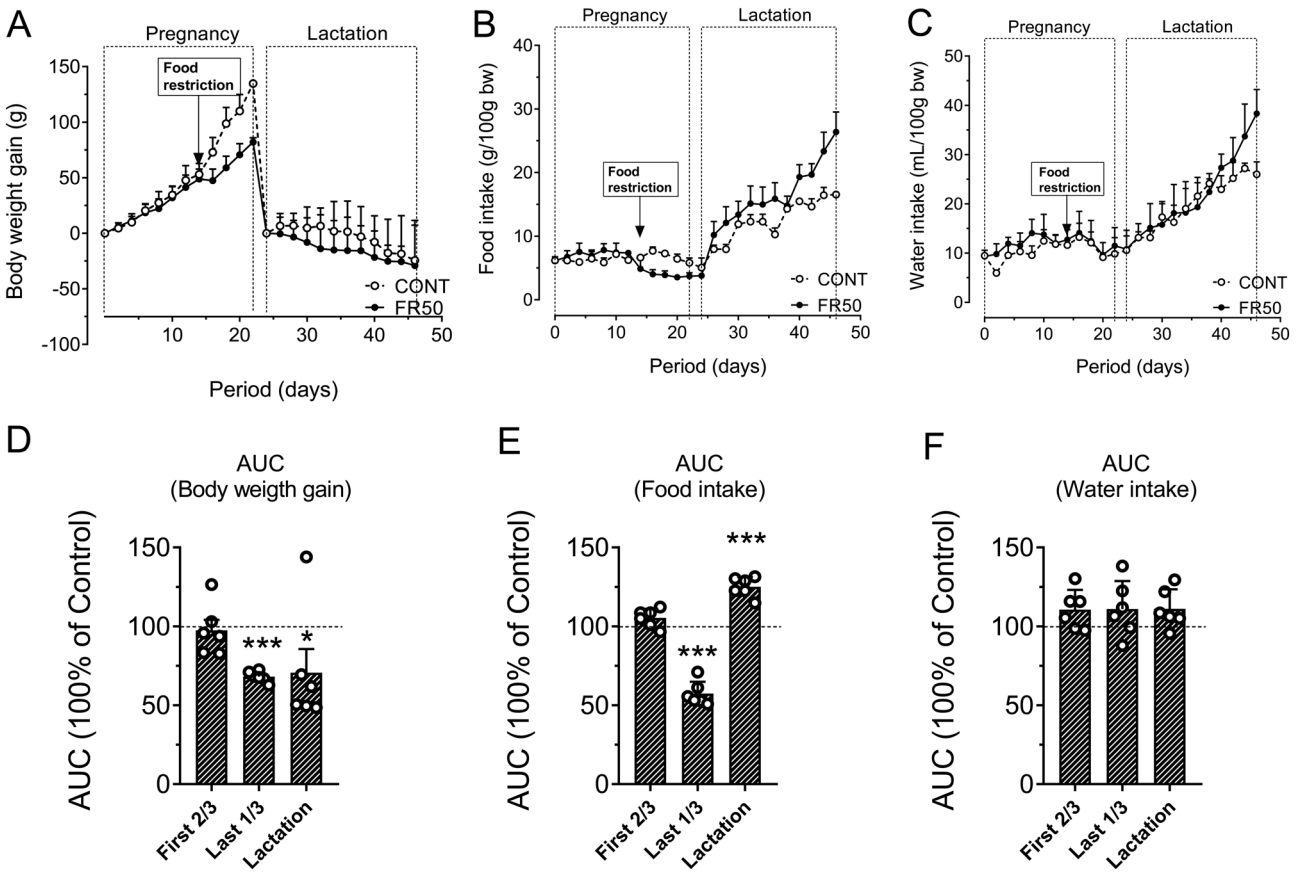

Figure 1
Body weight gain (A), food (C), and water intake (E) of mothers during the period of pregnancy and lactation. Data are presented as the mean ± S.D. of six different mothers for each experimental group. The statistical analyses between groups were obtained by Student's t-test, where $*P < 0.05$, $***P < 0.001$. Figures B, D, and F represent the area under the curve of Figures A, C, and E, respectively. The line in the hundred level on the Y-axis depicts 100% of the control values. CONT, control mothers; FR50, mothers who were under 50% food restriction during the last third of pregnancy.

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in FR50 mothers than in CONT mothers ($P < 0.001$, $n = 6$; Fig. 1C and D).

Regarding water consumption, there was no significant difference between groups throughout pregnancy and lactation ($P > 0.05$, $n = 6$; Fig. 1E and F).

The number of mothers excluded from the study because they did not deliver at least 8 male pups was 33.3% (3/9) for CONT mothers, with 4.33 ± 1.53 male rat pups per dam. In turn, the FR50 group had 40% (4/10) of dams delivering fewer than 8 rat pups (4.25 ± 1.71, $P = 0.97$ by the Mann–Whitney test). Additionally, the ratio of males to females live to dead birth was not affected by intrauterine food restriction ($P > 0.05$, Table 1).

Table 1 Effect of calorie restriction in the last third of pregnancy on the female–male ratio and live-birth percentage at birth.

Parameters	CONT	FR50
Ratio of male/female rats (at birth)	1.29 ± 0.65	1.28 ± 0.71
Live birth rats (%)	96.70 ± 5.31	94.44 ± 7.77

Data are presented as the mean ± S.D. of 9 CONT and 10 FR50 litters. The statistical analyses between groups were performed by Student's t-test ($P = 0.475$). All the mothers were considered for these parameter analyses.
CONT, control group; FR50, food restricted group.

Breast milk composition and maternal biochemical parameters

Table 2 shows the milk content obtained at the 12th lactation. In comparison with the CONT group, milk from FR50 mothers had increased levels of glucose (27.1%, $P < 0.01$), triglycerides (1.8%, $P < 0.001$), and total cholesterol (40.6%, $P < 0.05$), while the levels of total proteins were not changed ($P > 0.05$). The energy content of milk from FR50 mothers was 22.1% higher than that in the CONT group ($P < 0.05$, Table 2).

Regarding the metabolic hormones in milk, in comparison with the CONT group, leptin was reduced by 78.5% ($P < 0.001$, Table 1), and corticosterone was reduced by 14.2% ($P < 0.001$, Table 1) in milk from FR50 mothers. On the other hand, the values of acylated ghrelin were found to be increased by 70.2% in milk from FR50 mothers in relation to the CONT group ($P < 0.05$, Table 2).

At the end of the nursing period, in comparison with CONT, the FR50 mothers were hypoglycemic (23.4%, $P < 0.001$, Table 3); nonetheless, their triglycerides increased by 38.4% ($P < 0.01$, Table 3) without a significant difference occurring between the values of the TyG index. Likewise, there were no significant differences between groups regarding values of total cholesterol, HDL cholesterol, LDL cholesterol, and total proteins ($P > 0.05$, Table 3), while the levels of VLDL cholesterol were increased by 38.4% in FR50 mothers ($P < 0.01$, Table 3).

In comparison to that of CONT mothers, levels of blood leptin were increased by 2.5-fold in FR50 mothers ($P < 0.01$, Table 3), while corticosterone and acylated ghrelin were not changed.

Biometric assessment at birth and body weight gain and milk intake at the suckling phase

At birth, FR50 rats had body weight reductions of 18.3% ($P < 0.01$, $n = 6$ litters; Fig. 2A), as well as naso-anal length reductions of 11.7% ($P < 0.001$, $n = 6$ litters; Fig. 2B and C), without changes in the Lee index, in comparison with CONT rats (data not shown).

On the other hand, during the period of lactation, the body weight gain from the second week until weaning was higher in FR50 rats than in CONT rats ($P < 0.05$, $n = 6$ litters; Fig. 2D). As demonstrated in the area under the curve (AUC) for body weight gain, body weight increased by 10.4% in FR50 rats compared to that of CONT rats ($P < 0.05$, $n = 6$ litters; insert of Fig. 2D).

As shown in Fig. 2E, during the suckling phase, in comparison with that of CONT rats, the milk intake assessed in FR50 rats was 61.3% higher at the 6th ($P < 0.05$, $n = 6$ litters) and 58.1% higher at the 11th ($P < 0.05$, $n = 6$ litters) day of age. On the other hand, milk intake at 16 days of age did not differ between groups ($P > 0.05$, $n = 6$ litters).

Biometric assessment at weaning

At weaning, the body weight was 18.8% higher in FR50 rats than in CONT rats ($P < 0.001$, $n = 6$; Fig. 3A), and the Lee index increased by 4.0% ($P < 0.05$, $n = 6$; Fig. 3B) without changes in naso-anal length (data not shown). The brown adipose tissue was found to be more abundant by 86.2% ($P < 0.05$, $n = 6$; Fig. 3C) in FR50 rats than in CONT rats.

Regarding the main representative stores of white adipose tissue, weaned FR50 rats had mesenteric fat pads that were 37.5% larger ($P < 0.05$, $n = 6$; Fig. 3D), retroperitoneal fat pads that were 77.2% larger, and periepididymal fat pads that were 134.6% larger when compared to CONT rats ($P < 0.01$, $n = 6$; Fig. 3D, E and F).

Glucose–insulin homeostasis and metabolic hormone parameters

Weaned FR50 rat offspring were hyperglycemic (27.2%, $P < 0.01$, $n = 6$; Fig. 4A), hypertriglyceridemic (59.7%, $P < 0.05$, $n = 6$; Fig. 4B), and had a TyG index increase of 6.9% compared to that of CONT rat offspring ($P < 0.05$, $n = 6$; Fig. 4C).

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Table 2 Effect of calorie restriction during the last third of pregnancy on the milk composition on the 12th day of lactation.

Milk parameters	CONT	FR50
Glucose (mg/dL)	120.50 ± 7.38	153.10 ± 21.34**
Total proteins (g/dL)	23.80 ± 1.32	20.72 ± 3.76
Triglycerides (mg/dL)	955.30 ± 7.48	972.80 ± 4.35***
Total cholesterol (mg/dL)	235.40 ± 69.75	331.00 ± 71.12*
Energy content (kcal/L)	1558.0 ± 121.10	1902.00 ± 221.40**
Leptin (pg/mL)	7574.00 ± 2465.00	1627.00 ± 1033.00***
Corticosterone (ng/mL)	665.10 ± 10.53	570.80 ± 28.56***
Acylated ghrelin (pg/mL)	832.70 ± 173.60	1417.00 ± 459.80*

Data are presented as the mean ± S.D. of six mothers. Significant differences between groups were obtained by Student's t-test, where *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.
CONT, control group; FR50, food restricted group.

Total cholesterol was 51.3% higher in FR50 than in CONT rat offspring ($P < 0.01$, $n = 6$; Fig. 4D) without changes in total proteins ($P > 0.05$, $n = 6$; Fig. 4E) or corticosterone between groups ($P > 0.05$, $n = 4$; Fig. 4F).

In comparison with weaned CONT rat offspring, the FR50 rat offspring showed higher plasma levels of leptin (82.3%, $P < 0.05$, $n = 4$; Fig. 4G) and acylated ghrelin than the CONT group (81.7%, $P < 0.05$, $n = 4$; Fig. 4H).

Hypothalamus growth hormone secretagogue receptor expression

Weaned FR50 rat offspring exhibited a 4.1-fold higher expression of hypothalamic GHSR protein than that in CONT rat offspring ($P < 0.01$, $n = 4$ litters; Fig. 5).

Discussion

The present study demonstrates the role of metabolic hormones, which were changed in both the maternal environment (milk and circulating blood levels) and in the blood of rat pups as a result of maternal adverse conditions in the last third of pregnancy, as a critical factor contributing to programming in weaned rat pups, resulting in a metabolic syndrome phenotype being already present in infancy.

Regarding the FR50 mothers' phenotype during lactation, considering the physiological effect known to restore catch-up growth just after a short period of food restriction, which occurs in addition to hyperphagia (Griffin 2015), we did not observe physiological catch-up growth in FR50 mothers that were hyperphagic during lactation. This may be associated with high milk consumption by FR50 rat pups and the energy values of milk, which suggest a high physiological demand for mothers to offer their rat offspring an adequate nutritional content as a mechanism to guarantee offspring survival in a hostile environment (Gluckman & Hanson 2004).

Offspring whose dams were fed a poor protein diet only in the last third of pregnancy displayed dyslipidemia, hyperglycemia, hyperinsulinemia, hypercorticosteronemia, hyperleptinemia, and reduced levels of adiponectin and adrenocorticotropic hormone

Table 3 Effect of calorie restriction in the last third of pregnancy on the glucose homeostasis, lipid profile, and metabolic hormones of mothers just after nursing their offspring.

Blood parameters	CONT	FR50
Glucose (mg/dL)	129.50 ± 17.10	99.25 ± 16.54***
Triglycerides (mg/dL)	134.30 ± 53.54	185.90 ± 64.97**
TyG index	4.89 ± 0.22	4.87 ± 0.23
Total cholesterol (mg/dL)	106.90 ± 38.13	107.01 ± 27.82
HDL cholesterol (mg/dL)	43.94 ± 25.91	49.53 ± 21.37
LDL cholesterol (mg/dL)	27.55 ± 55.33	18.10 ± 38.76
VLDL cholesterol (mg/dL)	26.86 ± 10.71	37.18 ± 12.99**
Total proteins (g/dL)	7.89 ± 0.59	7.88 ± 0.27
Leptin (pg/mL)	14,512.00 ± 7544.00	36,957.00 ± 14,629.00**
Corticosterone (ng/mL)	591.50 ± 135.70	562.70 ± 117.00
Acylated ghrelin (pg/mL)	49.13 ± 13.56	54.68 ± 12.04

Data are presented as the mean ± S.D. of six mothers. Significant differences between groups were obtained by Student's t test, where **$P < 0.01$, ***$P < 0.001$.
CONT, control group; FR50, food restricted group.

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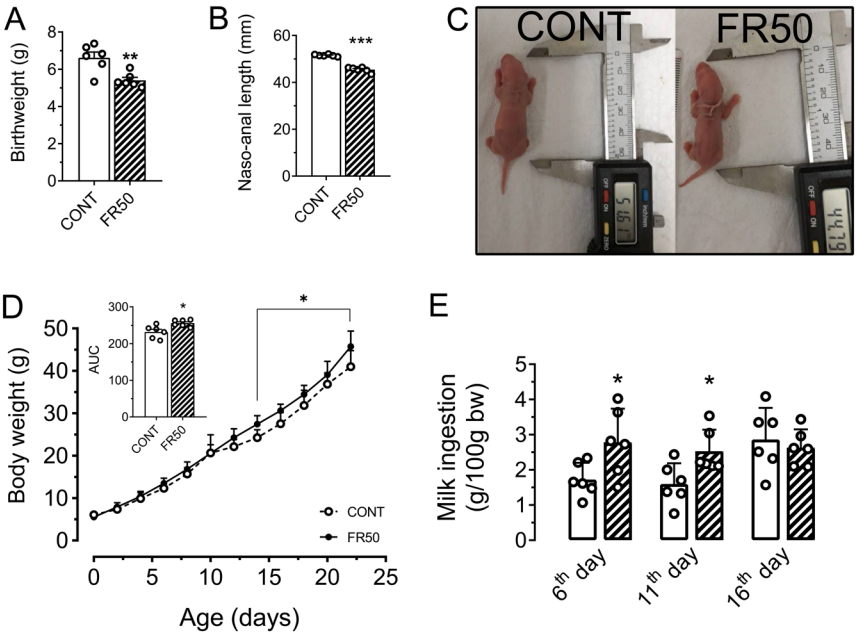

Figure 2
Body weight (A) and naso-anal length (B) at birth and body weight gain (D) and milk intake during lactation (E). Data are presented as the mean ± S.D. of six litters for each experimental group. The statistical analyses between groups were obtained by Student's t-test for Figures A, B, and D and by the Mann–Whitney test for Figure E, where $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Figure C depicts a photo illustration of the size of rats at birth, and the insert in Figure D represents the area under the curve of body weight gain during lactation. CONT, control group; FR50, food restricted group. A full color version of this figure is available at https://doi.org/10.1530/JOE-21-0427.

in adulthood; these changes were associated with insulin resistance, poor ability of the pancreas to secrete insulin, and an obese phenotype (de Oliveira *et al.* 2016). In addition, these rat pups were more sensitive to the effects of a high-fat diet, becoming obese at a high pace when challenged by an obesogenic diet (Venci *et al.* 2018).

Metabolic hormones, especially leptin and ghrelin, are critical not only to regulate physiological functions that occur in adulthood but also to regulate neuroendocrine connections as growth factors, promoting the stability and maturation of the several pathways involved in energy metabolism regulation (Bouret & Simerly 2007, Steculorum *et al.* 2015, Croizier *et al.* 2016). Given that in the present study, the milk consumed by FR50 litters had high levels of ghrelin, as well as a reduced amount of leptin and corticosterone, this altered hormone content is a pivotal factor contributing to inadequate signaling in hypothalamic neuronal connections in early development, which suggests that these hormones are involved in the programming of the FR50 rat offspring metabolic phenotype. Herein, we show that FR50 rat pups that were born smaller have a more prominent catch-up growth from the middle of the suckling period forward, were hyperphagic on milk intake, and stored more fat depots at weaning age. It brings to light

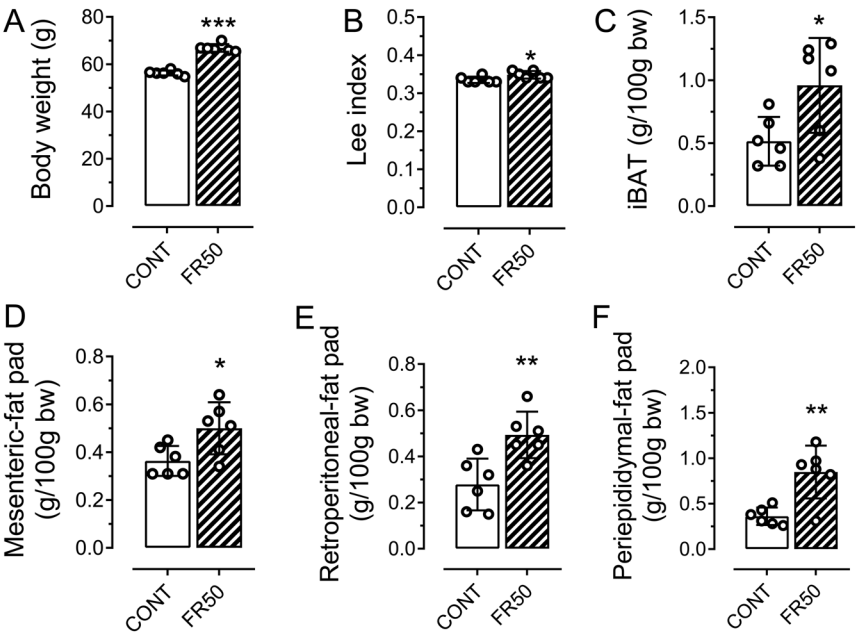

Figure 3
Biometric parameters from weaned rat offspring. Data are given as the mean ± S.D. obtained from six different litters. The statistical analyses between groups were obtained by Student's t-test, where $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. CONT, control group; FR50, food restricted group.

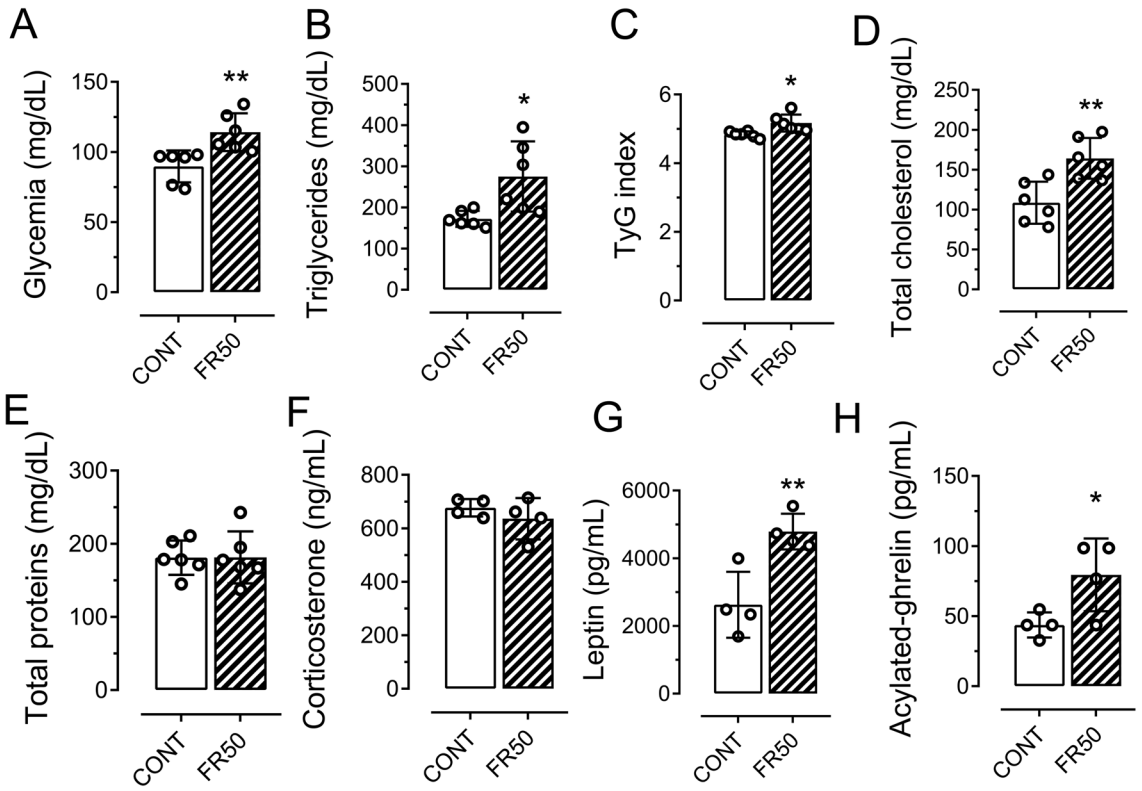

Figure 4
Glucose–insulin homeostasis and metabolic hormones in weaned rat offspring. Data are given as the mean ± s.d. obtained from at least four different litters. The statistical analyses between groups were obtained by Student's t-test, where $*P < 0.01$, $**P < 0.01$. CONT, control group; FR50, food restricted group. TyG, index for peripheral insulin sensibility inference obtained from glucose and triglycerides fasting values.

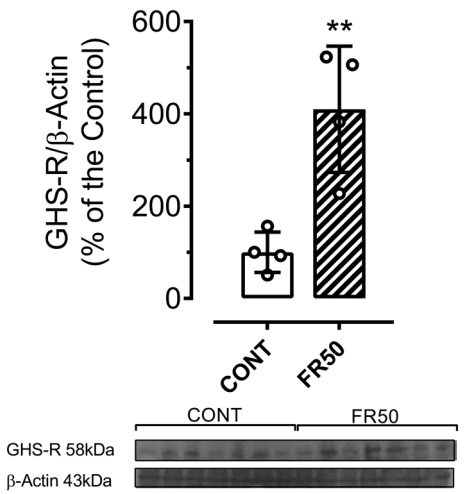

Figure 5
Protein expression of hypothalamic growth hormone secretagogue receptor (GHSR). Data are given as the mean ± s.d. of four different litters. The statistical analyses between groups were obtained by Student's t-test, where $**P < 0.01$. CONT, control group; FR50, food restricted group.

the knowledge about the thrifty phenotype hypothesis well postulated by doctors David J Baker and Charles N Hales (Hales & Barker 1992, Barker *et al.* 1993, Vaag *et al.* 2012), where a lower birthweight is a strong predictor of the early onset of metabolic disorders. In this regard, even after birth, plasticity in hypothalamic neuroendocrine pathways still occurs (Bouret & Simerly 2006), and it is a critical moment for the developmental programming of orexigenic/anorexigenic neuronal signaling and response, which may be modulated due to food scarcity (Delahaye *et al.* 2008, Breton *et al.* 2009), thus contributing to an excessive appetite, reduced satiety, and the development of obesity and associated comorbidities. In fact, children who show rapid weight gain are under a higher risk of adult diseases, regardless of their birth weight (Ong 2007). This highlights a link between the rapid catch-up group in early life and the risk for the development of metabolic diseases associated with energy control pathway modulation early in life (Ross & Desai 2014).

Herein, we show that milk obtained from FR50 mothers on the 12th day of lactation was richer in glucose, total cholesterol, and triglycerides and higher in energy,

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which is associated with milk hyperphagia by neonatal rat offspring and is strongly suggestive of obesity onset and metabolic disorder programming. In addition, milk from FR50 mothers displayed high levels of acylated ghrelin and reduced levels of leptin and corticosterone. As discussed recently, the adequate consumption of leptin during breastfeeding is crucial to protect neonates from the early programming of metabolic diseases (Palou *et al.* 2018). Similarly, changes in the levels of metabolic hormones other than leptin, such as ghrelin, corticosterone/cortisol, and insulin, among others supplied by milk, negatively impact the ideal maturation of neuronal connections and neuroendocrine signaling during the critical plasticity window (Badillo-Suarez *et al.* 2017, Palou *et al.* 2018, Pico *et al.* 2021), which may be one of the factors contributing to the obese phenotype and metabolic-associated disorders observed herein in the FR50 rat offspring. As previously shown in a similar rat model, male rats whose mothers underwent 50% food restriction from the last third of pregnancy until the end of the suckling phase displayed a reduced surge of leptin at postnatal day 7 and negative anorexigenic signaling as a result of reduced mRNA expression of hypothalamic POMC from postnatal day 14 until postnatal day 30 (Delahaye *et al.* 2008).

Interestingly, in our data, we found that weaned FR50 rat pups were hyperleptinemic, which when viewed together with the body of data as higher fat pad accumulation, hyperglycemia, hypertriglyceridemia, and, according to TyG index, insulin resistance, can indicate a central leptin signaling disorder in early life. Regarding leptin levels, in our study, and in contrast to the study by Delahaye, a 50% maternal food restriction was performed only in the last third of pregnancy, and pup leptinemia was assessed at 21 days old. This implies that undernutrition during the nursing period modulates metabolism to lower leptin levels. Corroborating this hypothesis, we had previously shown that weaned rat offspring from mothers fed a low-protein diet during the first two-thirds of lactation exhibited lower leptinemia and insulinemia and that the milk contained reduced insulin levels (Mathias *et al.* 2020).

The fact that we did not analyze thermogenesis markers in weaned rat offspring is one of the limitations of our work, as weaned FR50 rat pups had high iBAT. Even though it is not a precise marker, this can indicate high thermogenic activity that may be an adaptation fighting the physiological changes converging to the thrifty phenotype hypothesis in these rats. As recently shown by our group, adult rat offspring whose mothers were fed 50% less food during the two-thirds of lactation showed high iBAT mass and high protein expression for uncoupling protein 1 (UCP1) in iBAT (Miranda *et al.* 2022). Additionally, the gene expression of UCP1 was greatly increased in gonadal fat pads from weaned rat pups whose mothers were fed 50% less food during the last third of pregnancy until the end of the suckling period (Delahaye *et al.* 2010).

Corroborating the onset of obesity in the FR50 rat offspring, we can appoint ghrelin as an important contributing factor because formula-fed infants, compared to breastfed children, have high plasma levels of ghrelin, which has been correlated positively with greater infant weight gain in newborns (Badillo-Suarez *et al.* 2017). In this regard, to the best of our knowledge, we are the first to show high levels of acylated ghrelin in milk consumed by FR50 rat offspring and that weaned FR50 rat offspring displayed high plasma levels of acylated ghrelin, as well as high protein expression of GHS-R in the hypothalamus, which can contribute to the hyperphagia and rapid catch-up growth observed in FR50 rat pups that can be viewed as a short-term physiological effect. At the same time, persistent acylated ghrelin signaling (high blood levels and high GHS-R expression in the hypothalamus) may contribute to the input of metabolic memory, resulting in orexigenic neuronal plasticity, for example, increasing the hypothalamic density of neuronal projections and/or the number of dendritic spines and then strengthening the density of excitatory synapses (Cabral *et al.* 2020) that contributes to the maintenance of hunger signals due to stimulation in the hypothalamic agouti-related peptide (AgRP)-expressing neurons, which has a long-term effect (Lopez Soto *et al.* 2015).

As one well-reviewed possible mechanistic explanation, the excitatory synapses on hypothalamic AgRP-expressing neurons are increased by the action of ghrelin on hypothalamic glutamatergic neurons. Simultaneously, ghrelin promotes the activation of N-methyl-D-aspartate-type glutamate receptors, and associated with the hunger signal, pathways involving energy sensors such as AMP-activated protein kinase and sirtuin 1 or mammalian target of rapamycin and ribosomal protein S6 kinase 1 are activated, which drive state-dependent excitatory synaptic plasticity in AgRP-expressing neurons (Spanswick *et al.* 2012, Stoyanova & Lutz 2021). In turn, disruption in ghrelin physiological action during the neonatal period programs lifelong metabolic dysfunction; the role of ghrelin in this critical period is pivotal for normal maturation of axonal projections in the hypothalamus neural circuitry involved in metabolic regulation, such as that by influencing neuropeptide Y and POMC synaptic plasticity (Steculorum *et al.* 2015, Serrenho *et al.* 2019).

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Considering adversities, like undernutrition as one physiological stress factor, in the present study, we interestingly did not find alterations in corticosterone levels in the weaned FR50 rat pups, while it was reduced in milk ingested by FR50 rat pups. High levels and/or local action of glucocorticoids in early life (cortisol (Salvante *et al.* 2017, Niwa *et al.* 2020) in humans and corticosterone (Somm *et al.* 2012) in rodents have been shown to be involved in the programming of, among other diseases, metabolic malfunction later in life (Moisiadis & Matthews 2014, Chen *et al.* 2019).

Therefore, it is important to bear in mind that the consumption and provision of inappropriate foods, such as high-sugar and high-fat diets that exceed the ideal nutritional guidelines, contribute to the fast increase in metabolic diseases worldwide. Additionally, inadequate food intake falling below the ideal nutritional guidelines is also a problem that affects people around the world, especially with the current COVID-19 pandemic that has been amplifying this nutritional adversity (Dzinamarira *et al.* 2020, Perez-Escamilla *et al.* 2020, Paslakis *et al.* 2021). In Brazil, for example, the discrepancy between the different social realities coexisting in the country has been amplified by the effects of COVID-19, which calls for appropriate social policies to mitigate this issue (Alpino *et al.* 2020, Ribeiro-Silva *et al.* 2020, Carvalho *et al.* 2021, Rodrigues *et al.* 2021). The COVID-19 pandemic has negative implications and aggravates the food and nutritional security of less favored individuals, which in fact could have long-term implications on the metabolism of individual exposed to food scarcity in critical stages. The fact that adequate health care for pregnant and lactating mothers is not comprehensive and equal for all people (Uauy *et al.* 2011) reinforces the need for policies that provide adequate maternal nutrition for an optimal milk nutrient and hormone composition, which in addition to promoting breastfeeding, will also mitigate the onset of metabolic diseases, which could bring health, educational, and economic benefits.

In summary, rat offspring from undernourished dams under dietary restriction during the last third of pregnancy had a small birthweight associated with prominent neonatal catch-up growth and an obese phenotype at weaning, which seems to be associated with changes in the energy content of milk and in the supply of metabolic hormones, leptin, and ghrelin.

In addition, the GHS-R protein expression by the hypothalamus and the increased blood levels of leptin and ghrelin are suggestive of influencing neuroendocrine circuitry development in FR50 rat offspring.

Declaration of interest

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

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