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WILLIAN DO NASCIMENTO DE SOUZA RODRIGUES

A low-protein diet during breastfeeding alters metabolism and physiological functions in the hypothalamus–pituitary–testle axis of offspring male *Wistar* rats during peripuberty

Maringá

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Tese apresentada ao programa de Pós-Graduação em Ciências Biológicas (área de concentração – Biologia Celular e Molecular), da Universidade Estadual de Maringá para a obtenção do grau de Doutor em Ciências Biológicas.

Orientador: Paulo Cezar de Freitas Mathias

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Aprovado em: ...../...../.....

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BIOGRAFIA

Willian do Nascimento de Souza Rodrigues nasceu em Sarandí, Paraná. Graduou-se em Ciências Biológicas (Licenciatura Plena) pela Universidade Estadual de Maringá (UEM) entre 2012 e 2016 e concluiu o Mestrado em Ciências Fisiológicas na mesma instituição (2017--2019). Atualmente, é doutorando no Programa de Pós-Graduação em Ciências Biológicas, com área de concentração em Biologia Celular e Molecular. Durante sua trajetória acadêmica, participou de três projetos de iniciação científica (PIBIC-UEM) e de uma iniciação à docência (PIBID-UEM). Também esteve envolvido na organização de palestras e eventos acadêmicos, escreveu capítulo de livro e apresenta seis artigos científicos. Recently, teve os resultados da dissertação de mestrado publicados em revista indexada, o artigo original é intitulado "Adrenalectomy Improves MSG-Induced Obesity in Rats by Increasing UCP-1 Levels in Interscapular Brown Adipose Tissue", a presente tese visa gerar dois artigos científicos.

BIOGRAPHY

Willian de Souza Rodrigues was born in Sarandí, Paraná, Brazil. He earned a Bachelor's degree in Biological Sciences from the State University of Maringá (UEM) from 2012--2016 and a Master's degree in Physiological Sciences at the same institution from 2017--2019. He is currently pursuing a PhD in Biological Sciences, a concentration area in Cellular and Molecular Biology. He took part in three scientific projects and one teaching project. He has also helped organize lectures and academic events, write a book chapter, and publish six scientific articles. Recently, the results of his Master's thesis were published in a journal. The original article is titled "Adrenalectomy Improves MSG-Induced Obesity in Rats by Increasing UCP-1 Levels in Interscapular Brown Adipose Tissue." This current thesis aims to wage two scientific articles.

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APRESENTAÇÃO

Esta tese é composta por dois artigos originais.

RODRIGUES *et al*. 2024. **Poor protein nutrition during breastfeeding alters ontogenetic development in male offspring by influencing hypothalamic DNA methyltransferases and liver functions from puberty onward.** JOURNAL OF ENDOCRINOLOGY. 2024. **(**Qualis A1- IF: 3.4)**.**

RODRIGUES *et al*. 2024. **A low-protein diet during breastfeeding delays puberty by altering reproductive physiology in underweight male rats in the peripubertal window.** Life Sciences**. 2024 (**Qualis A1- IF: 5.2)**.**

RESUMO GERAL

INTRODUÇÃO: O período pós-natal é um momento vulnerável para o desenvolvimento ontogenético, durante o qual os neonatos passam da dependência placentária para a amamentação. Esse processo fornece nutrientes essenciais para o crescimento e maturação dos órgãos. A desnutrição materna, particularmente durante a lactação, tem o potencial de alterar o fenótipo da prole, resultando em mudanças notáveis durante a peripuberdade, fase de maturação sexual que diferencia os sexos biológicos. Além disso, essa fase é considerada a última janela de vulnerabilidade an insultos metabólicos, especialmente no hypotálamo, que atua como ponte entre os sistemas nervoso e endócrino (neuroendócrino) e é suscetível à programação materna. Este estudo elucida o impacto da restrição proteica materna durante os dois terços iniciais da lactação em descendentes com fenótipo magro, com ênfase especial na regulação de genes envolvidos no eixo hypotálamo-hipófise-testicular (HPT), esteroidogênese, espermatogênese, metabolismo intermediário, padrões de metilação hypotalâmica durante a peripuberdade até a vida adulta e no acúmulo de gordura hepática, danos e proteção.

MATERIAIS E MÉTODOS: O estudo foi conduzido de acordo com as diretrizes ARRIVE e recebeu aprovação ética do Comitê de Ética (protocolo 8620070222/2022). Foram utilizados ratos Wistar machos e fêmeas, com idades de 80 e 70 dias, respectivamente. Após o acasalamento, as fêmeas prenhes foram aleatoriamente distribuídas em dois grupos alimentares: o grupo controle (NP), que recebeu uma dieta normal de proteínas (23%), e o grupo de baixa proteína (LP), que recebeu uma dieta com 4% de proteínas durante os primeiros 14 dias de lactação, seguida por uma dieta normal na última semana. A prole foi desmamada no dia pós-natal (PND) 21 e monitorada até o PND90, com medidas de peso corporal e marcadores metabólicos em intervalos (PN14, PN35, PN45, PN55 e PN90). Foram realizadas análises biométricas e bioquímicas, incluindo avaliações do tempo de puberdade, peso na puberdade, testes de tolerância à glicose e sensibilidade à insulina. As concentrações séricas de hormônios relacionados ao equilíbrio energético e à função reprodutiva, incluindo insulina, testosterona e corticosterona, foram mensuradas. O eixo hypotálamo-hipófise-testicular (HPT) foi investigado por meio da expressão de peptídeos estimuladores e inibidores do gene Gnrh1 e genes relacionados à função testicular, incluindo aqueles envolvidos na biossíntese de esteroides e espermatogênese. Amostras de hypotálamo foram utilizadas para avaliar o mRNA das metilases de DNA envolvidas na programação epigenética.

RESULTADOS E DISCUSSÃO: A restrição proteica materna durante a lactação resultou em atraso no tempo de puberdade e na redução do peso da prole no início da puberdade. A regulação do eixo HPT foi alterada, com aumento da kisspeptina (mRNA de Kiss1) no dia pós-natal (PND) 45. No entanto, a transcrição do hormônio liberador de gonadotrofina 1 (Gnrh1) permaneceu inalterada, indicando que, embora o eixo tenha sido modulado, ele continuou funcional. A atividade da aromatase foi observada com alterações durante o período peripuberal, resultando em impactos nos níveis de testosterona. A dieta LP afetou o crescimento corporal, o ganho de peso dos tecidos periféricos e a expressão de metilases de DNA durante a peripuberdade. A sensibilidade à insulina apresentou melhora próxima ao início da puberdade, acompanhada de alterações no metabolismo de lipídios e glicose. Embora o conteúdo de lipídios hepáticos estivesse elevado durante an infância, retornou aos níveis normais na peripuberdade. A elevação de marcadores de estresse hepático indicou a presença de estresse oxidativo na prole LP, embora o aumento concomitante da capacidade antioxidante sugira uma

resposta compensatória. Observou-se comprometimento da espermatogênese, acompanhado por alterações na expressão de genes envolvidos nesse processo, incluindo ***Kit*** (espermatogônias diferenciadas), ***Rhcg*** (espermatócitos) e ***Lrrc34*** (espermátides). Além disso, genes associados às células de Sertoli, como ***Lgals1,*** também foram afetados, indicando que a desnutrição materna pode comprometer o ambiente intratesticular, afetando tanto a síntese de esteroides quanto a diferenciação das células espermáticas.

CONCLUSÃO: Os resultados revelaram o impacto da desnutrição materna durante o período pós-natal no metabolismo e nas funções reprodutivas da prole. Os animais LP apresentaram atraso no início da puberdade, no crescimento corporal, baixa adiposidade e níveis alterados de insulina, corticosterona e testosterona em diferentes estágios da ontogenia. A atividade da aromatase testicular, essencial para a função reprodutiva, sofreu alterações ao longo do tempo. Além disso, os transcritos que codificam genes envolvidos na espermatogênese foram alterados. Apesar da baixa adiposidade persistir até a vida adulta, esses descendentes apresentaram aumento da sensibilidade à insulina durante a peripuberdade, acompanhada pelo acúmulo de gordura visceral. O fígado, embora produza níveis elevados de espécies reativas, também demonstrou maior produção de antioxidantes, indicando um sistema redox eficaz. Essas mudanças, resultantes da desnutrição durante a lactação, reprogramaram o metabolismo hepático, afetando sua função ao longo do tempo, potencialmente devido à hiperfagia e à alteração na composição corporal.

PALAVRAS-CHAVE: Desnutrição materna na amamentação; fenótipo magro; peripuberdade; eixo hypotálamo-hipófise-testicular (HPT); aromatase; esteroidogênese e espermatogênese; metabolismo hepático.

ABSTRACT

INTRODUCTION: The postnatal period is a vulnerable time for ontogenetic development, during which neonates undergo a transition from placental dependence to breastfeeding. This provides essential nutrients for growth and organ maturation. Maternal malnutrition, particularly during lactation, has the potential to alter the phenotype of offspring, resulting in notable changes during peripuberty, a phase of sexual maturation that distinguishes biological sexes. Furthermore, this phase is regarded as the final window of vulnerability to metabolic insults, particularly in the hypothalamus, which serves as a bridge between the nervous and endocrine (neuroendocrine) systems and is susceptible to maternal programming. This study elucidates the impact of maternal protein restriction during the initial two-thirds of lactation on offspring with a lean phenotype, with a particular emphasis on the regulation of genes involved in the hypothalamic–pituitary–testicular (HPT) axis, steroidogenesis, spermatogenesis, intermediate metabolism, hypothalamic methylation patterns during peripuberty until adulthood, and liver fat accumulation, damage, and protection.

MATERIALS AND METHODS: This study was conducted in accordance with the ARRIVE guidelines and received ethical approval from the relevant Ethics Committee (protocol number 8620070222/2022). The study employed male and female Wistar rats aged 80 and 70 days, respectively. Following mating, pregnant females were randomly assigned to one of two dietary groups: the control group (NP), which received a normal protein diet (23%), or the low-protein group (LP), which received a 4% protein diet during the first 14 days of lactation, followed by a normal diet during the last week. The offspring were weaned on postnatal day (PND) 21 and monitored until PND90, with body weight and metabolic markers measured at intervals (PN14, PN35, PN45, PN55, and PN90). Biometric and biochemical analyses were conducted, including assessments of pubertal timing, weight at puberty, glucose tolerance tests, and insulin sensitivity. The serum concentrations of hormones that are linked to energy balance and reproductive function, including insulin, testosterone, and corticosterone, were measured. The hypothalamic–pituitary–testicular (HPT) axis was investigated through the expression of stimulatory and inhibitory peptides of the *Gnrh1* gene and genes related to testicular function, including those involved in steroid biosynthesis and spermatogenesis. Hypothalamic samples were utilized to assess the mRNA levels of DNA methylases involved in epigenetic programming.

RESULTS AND DISCUSSION: Maternal protein restriction during lactation results in delayed pubertal timing and a reduction in offspring weight at the onset of puberty. The regulation of the hypothalamic–pituitary–thyroid (HPT) axis was altered, with increased kisspeptin (*Kiss1 mRNA*) levels on postnatal day (PND) 45. However, gonadotropin-releasing hormone 1 (*Gnrh1*) transcription remained unchanged, indicating that although the axis was modulated, it remained functional. Aromatase activity was observed to undergo alterations during the peripubertal period, resulting in a corresponding effect on testosterone levels. The LP diet affected body growth, peripheral tissue weight gain, and DNA methylase expression during the peripubertal period. Insulin sensitivity improved in proximity to the onset of puberty, accompanied by alterations in lipid and glucose metabolism. Although the liver lipid content was elevated during infancy, it returned to normal levels during peripuberty. The elevation of hepatic stress markers indicated the presence of oxidative stress in LP offspring, yet the concomitant increase in antioxidant capacity suggested a compensatory response. Impairment of spermatogenesis was observed, accompanied by altered expression of genes involved in this process, including *Kit* (differentiated spermatogonia), *Rhcg* (spermatocytes), and *Lrrc34* (spermatids).

Additionally, genes associated with Sertoli cells, such as *Lgals1*, were found to be affected, indicating that maternal malnutrition can compromise the intratesticular environment, affecting both steroid synthesis and sperm cell differentiation.
CONCLUSION: The results revealed the impact of maternal malnutrition during the postnatal period on offspring metabolism and reproductive functions. LP animals exhibit delayed timing of puberty onset and body growth; low adiposity; and altered levels of insulin, corticosterone, and testosterone at different stages of ontogeny. The activity of testicular aromatase, which is crucial for reproductive function, was observed to undergo alterations over time. Moreover, the transcripts encoding the genes involved in spermatogenesis are altered. Despite low adiposity persisting into adulthood, these offspring exhibited increased insulin sensitivity during peripuberty, accompanied by visceral fat accumulation. The liver, while producing elevated levels of reactive species, also demonstrated increased antioxidant production, indicating an effective redox system. These changes, resulting from malnutrition during lactation, reprogram hepatic metabolism, affecting its function over time, potentially through hyperphagia and altered body composition.
KEYWORDS: Maternal malnutrition during breastfeeding; lean phenotype; peripuberty; hypothalamic–pituitary–testicular (HPT) axis; aromatase; steroidogenesis and spermatogenesis; hepatic metabolism.

Poor protein nutrition during breastfeeding alters ontogenetic development in male offspring by influencing hypothalamic DNA methyltransferases and liver functions from puberty onward

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Short title: Impact of poor protein nutrition on male offspring.

ABSTRACT

During vulnerable developmental windows, such as gestation, lactation, and puberty, maternal dietary insults can alter metabolic signals and impact offspring health. This study investigated whether maternal caloric–protein restriction (LP) during the first two-thirds of lactation affects the offspring phenotype throughout ontogenetic development, with a focus on intermediary metabolism, insulin sensitivity, and hypothalamic DNA methyltransferase expression. Additionally, hepatic tissue was analyzed for hepatic fat accumulation, oxidative stress, and enzymatic activity, and body mass gain and metabolic changes were monitored. Female Wistar rats were divided into two dietary groups: those fed a normal protein diet (NP, 20.5%) and those fed a low-protein diet (LP, 4%) during the first two-thirds of lactation. In male offspring, body weight, food intake, glucose tolerance, insulin sensitivity, biochemical assays, and biometric measurements were evaluated on postnatal days (PNs) 35, 45, 55, and 90. Liver samples were analyzed for oxidative stress, lipid content (Folch method), and enzymatic activity (AST, ALT). DNA methyltransferase gene expression in the hypothalamus was assessed by RT–qPCR. LP offspring exhibited hypoinsulinemia only during infancy and adulthood. After weaning, all these rodents exhibited hyperphagia, delayed body weight gain, and transient hepatic fat accumulation. Activation of the antioxidant system, particularly through catalase, helps prevent persistent liver fat accumulation and reduces oxidative damage. The reduction in DNA methyltransferases during peripuberty suggested the presence of epigenetic alterations. Despite changes in intermediary metabolism, the LP phenotype adapts to postweaning stress, maintaining metabolic control and improving insulin sensitivity into adulthood.

Keywords: DNA methyltransferases, glucose tolerance, hepatic metabolism, oxidative stress, low adiposity, hyperphagia, insulin sensitivity.

Introduction

Poor nutrition in the womb and in early postnatal life can increase the risk of metabolic syndrome, impacting an offspring's long-term health later in life (Ravelli *et al.* 1976; Forsdahl 1977; Yan *et al.* 2020). Despite globalization, undernutrition remains a global health concern; in developing countries, it is worsened by social isolation (Workie *et al.* 2020), civil wars (Osendarp *et al.* 2022), seasonality (Marshak *et al.* 2021) and infectious diseases (Narayan *et al.* 2019). In *Homo sapiens*, the first 1,000 days of life are crucial for early postnatal development, particularly during the breastfeeding phase (Lisboa *et al.* 2021). Early malnutrition manifests in infancy as low height and body weight, indicating both chronic and acute undernutrition, as reported in South Asia (Benjamin-Chung *et al.* 2023). Reduced growth specifically reflects chronic undernourishment, whereas low body weight is indicative of acute malnutrition. In 2022, nearly 150 million children worldwide did not receive sufficient calories for normal growth, with more than 45 million exhibiting signs of wasting due to malnutrition. Both conditions can impair cognitive development, with stunting estimated to cause more than 250,000 deaths annually and wasting resulting in more than one million deaths (Benjamin-Chung *et al.* 2023; Mertens *et al.* 2023a, b). Childhood malnutrition has the potential to cause lifelong metabolic problems. For example, severe protein malnutrition can result in kwashiorkor disease, reducing growth and impairing development in infancy. It is characterized by hypoalbuminemia and hypercortisolism associated with edema, systemic inflammation, and low beta-cell function, which contribute to dysfunction in intermediary metabolism (Bhutta *et al.* 2017; Dipasquale *et al.* 2020).

Maternal nutritional restriction during periods of phenotypic plasticity in her progeny, such as early postnatal life, can lead to slowed growth in the offspring,

increasing the risk of metabolic disorders (Passos *et al.* 2000; Martins *et al.* 2018a; Vargas *et al.* 2023). This adaptive response is aimed at producing a more energy-efficient phenotype (Vaag *et al.* 2012a; Ribeiro *et al.* 2015), but the medium-term effects of these early nutritional deficiencies, from peripubertal to adult life in male offspring, remain unclear. Maternal protein restriction during lactation mimics undernutrition, causing lower body gain, low adiposity and high insulin sensitivity in developing offspring (Fagundes *et al.* 2009a; de Oliveira *et al.* 2013a; Martins *et al.* 2018a; VARGAS *et al.* 2024). Moreover, maternal malnutrition results in later hyperphagic offspring (Lesage *et al.* 2006a; Bouret 2012) and affects maternal behavior, milk composition (Martins *et al.* 2023a) and milk energy (Passos *et al.* 2000). A lean maternal phenotype can cause metabolic syndrome in offspring, particularly if they are exposed to a high-calorie diet incompatible with their early development (Vargas *et al.* 2023).

The peripubertal phase is distinguished by notable phenotypic plasticity and the synthesis of sex hormones that are crucial for the development of sexual dimorphism (Korenbrot *et al.* 1977a; Stoker *et al.* 2000; Soliman *et al.* 2014; Abreu & Kaiser 2016). In rats, this phase occurs between postnatal days 35 and 55; however, the precise timing may be influenced by nutritional changes (Bell 2018; Sominsky *et al.* 2018). Epigenetic modifications during development and growth can affect the hypothalamus, affecting pre- and posttranscriptional regulation of messenger RNAs and thereby altering gene expression. (Wadhwa *et al.* 2009; Zheng *et al.* 2014; Yang *et al.* 2016; Briollais *et al.* 2021). These enzymes add methyl groups to DNA and specialize in maintaining and introducing DNA methylation; these enzymes are called *Dnmt1, Dnmt3a, and Dnmt3b* (DNA methyltransferases) (Wadhwa *et al.* 2009; Allison *et al.* 2021; Briollais *et al.* 2021). Paternal high-fat diets cause POMC gene hypermethylation at weaning, increasing the

risk of obesity in offspring (Haberman *et al.* 2024). Thus, maternal epigenetic markers passed down during pregnancy or lactation influence the offspring phenotype. However, there is limited research on how maternal nutrition during breastfeeding influences hypothalamus methylation, particularly during the peripubertal transition.

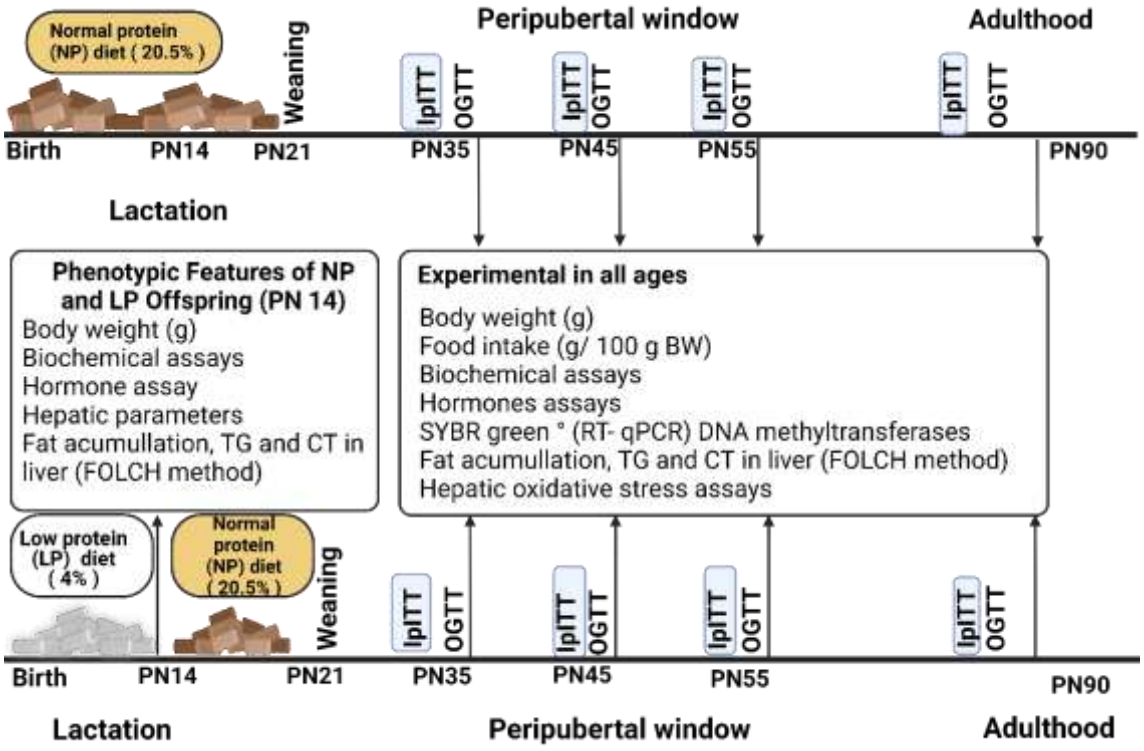
The liver is an indispensable nutrient sensor that facilitates the uptake, processing, and storage of energy substrates, including monosaccharides, amino acids, and fatty acids (Sharabi *et al.* 2019a). Moreover, they participate in the oxidation and biosynthesis of energy reserves, processes that invariably involve the generation of reactive oxygen species (ROS) (Scoditti *et al.* 2024; VARGAS *et al.* 2024). In liver metabolism, both are responsive to insulin and corticosterone, which affect carbohydrate and lipid metabolism and contribute to both physiologic and pathophysiologic conditions (Sharabi *et al.* 2019a, b; Scoditti *et al.* 2024). Perinatal nutrient restriction can predispose offspring to the development of nonalcoholic fatty liver disease (NAFLD)(Ravelli *et al.* 1976; Li *et al.* 2015; Chen *et al.* 2016; Du *et al.* 2020), highlighting the relevance of nutrition during early life. Maternal caloric restriction in rats can impact hepatic autophagy and redox status in both weaning and aged male offspring (Devarajan *et al.* 2019), revealing the complex links among nutrition, antioxidant capacity and ROS. However, the effects of maternal undernutrition on intermediary metabolism and liver functions, as well as their consequences for early development, i.e., peripubertal until adult life, have been poorly studied.

Alterations in intermediary metabolism and liver function are found in LP offspring (Passos *et al.* 2000, 2002; Bertasso *et al.* 2022; Vargas *et al.* 2023). We hypothesized that caloric-protein restriction during the initial two-thirds of lactation may impair the intermediary metabolism, insulin sensitivity, and DNA methyltransferases in

whole hypothalamic tissue of male offspring from the peripuberty window until adulthood.

Materials and methods

Experimental Setup



Frame 1: Experimental design of the study. Created with https://www.biorender.com/

Research Ethics Approval

The handling of the animals and all the experimental procedures followed the ARRIVE Guidelines 2.0 for animal experiments and the guidelines of the National Council for the Control of Animal Experimentation (CONCEA). The study was approved by the Ethics Committee on Animal Use of the State University of Maringá (CEUA-UEM, protocol n° 8620070222/2022).

Murinometric model and nutritional intervention during breastfeeding

Female Wistar rats (n= 6 dams/group) aged 70 days were mated and kept under controlled conditions with a 12-h light–12-h dark cycle and temperature, with access to standard chow and water. After one week of adaptation, the rats were mated at a ratio of three females (n= 60 female virgins) to each male (n=20 male virgins). Pregnant females were randomly housed in individual cages until they gave birth. The litter size was adjusted to eight pups, and the dams were randomly divided into two groups. During the first 14 days of lactation, the first group was supplied with a normal protein diet (20.5% protein), while the second group was supplied with a low-protein diet (4% protein). On day 15 of lactation, both dam groups were fed a standard diet until weaning. After lactation, the offspring of the NP and LP groups were separated, and only male rats were housed (4 rats/cage) and fed a standard diet, conformed shown in **frame 1**.

Body Weight and Offspring Food Intake during Maternal and Offspring Evolution

The maternal and offspring body weights of the NP and LP groups within the PN 90 cohort were assessed at four time points throughout the lactation period: PN1, PN7, PN14, and PN21. Furthermore, the body weight and food intake of each experimental group (n = 4 to 6 litters per group) were measured every two days from weaning until PN31, PN41, PN51, and PN85. The food intake values were determined by calculating the difference between the total amount of food provided initially (D initial) and the amount of food remaining (D final), which was divided by the number of days and the number of rats per cage (de Oliveira *et al.* 2013b). The grams of food consumed by each rat were calculated via the following formula: (D Initial – D Final)/(2 × 4). This value was then divided by the average body weight of the four rats in each cage to obtain the relative food intake. Body weight (BW) was evaluated throughout the experimental period for all groups. The area under the curve (AUC) was subsequently calculated for both body weight and food intake assessments.

Intraperitoneal insulin tolerance test (ipITT) and glucose disappearance test (kITT)

After a six-hour fast, other batches of rat offspring (n= 2 animals/four litters per group) from the NP90 and LP90 groups received an intraperitoneal injection of insulin (1 U/kg body weight, i.p.). Blood samples were collected via a small cut at the tail, and blood glucose levels were measured via a glucometer (FreeStyle Optimum H, Abbott Laboratories) at time zero (before insulin injection) and at 15, 30, 45, and 60 minutes postinjection (Akinmokun *et al.* 1992). The kITT was determined via the formula $0.693/(t^{1/2})$, where $t^{1/2}$ is the slope angle of the linear decline in plasma glucose concentrations during the ITT, as previously described. The result is expressed as kITT (%/min) (Lundbaek 1962)

Oral glucose tolerance test (oGTT)

After an overnight fast (20:00–08:00), the NP90 and LP90 groups were given a glucose gavage (2.0 g/kg BW) on PN35, PN45, PN55, and PN90 (n=16 animals from four litters). Blood samples were collected immediately before the glucose challenge (time zero, fasting glucose) and at 15, 30, 45, 60, and 120 min after gavage. All the samples were collected from the tail vein, and glucose was measured via a digital glucometer (Freestyle Optimum H, Abbott) via an adapted methodology (VARGAS *et al.* 2024).

Experimental procedures in offspring

On postnatal day (PN) 14, after nutritional insult, a group of male pups (n = 24 rats from 6 litters per NP and LP group) were fasted for 6 hours, weighed, and then euthanized via decapitation via a rodent guillotine for blood sample collection as well as organ and tissue harvesting. On days PN35, PN45, PN55, and PN90, the animals (n = 16--20 from all groups) were fasted for 12 h (20:00--08:00 h), anesthetized with thiopental

sodium (45 mg/kg BW, i.p., Thiopentax®, Cristália, Itapira, São Paulo, Brazil), and euthanized via decapitation via guillotine. Blood (plasma and serum), hypothalamus, liver, visceral adipose tissue (mesenteric, retroperitoneal, and periepididymal) and interscapular adipose tissue were then collected. The absolute and relative weights of the liver and all adipose tissue were calculated (g) and (g/100 g BW), respectively. The relative weight gain difference at each analyzed age was measured for hepatic tissue, visceral fat, and interscapular brown adipose tissue. This value was calculated by subtracting the weight of the tissue at each age from the weight at the previous euthanasia point. The difference in mass tissue gain (MTG) between the older and younger ages of each animal was calculated via the following formula: (MTG of the subsequent age) = (final MTG – initial MTG).

Biochemical Assays

Sample collection was conducted at PN14 after a six-hour fasting period (from 08:00 to 14:00). Six litters were utilized for each group, with a total of 8–24 animals per litter. The samples were subjected to centrifugation at 2400 ×g for 20 minutes at 4°C. The serum (devoid of anticoagulant) was promptly pipetted and stored at -20°C for subsequent analysis of glycemia, total cholesterol (TC), triglyceride (TG), total protein, albumin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels via enzymatic-colorimetric methods with a commercial kit (Gold Analisa®, Belo Horizonte/MG, Brazil). The ketone levels were determined by measuring β-hydroxybutyrate levels via FreeStyle Optium Ketone test strips and a meter (FreeStyle Optium H). During the peripubertal period (PN35, PN45, PN55) and early adulthood (PN90), serum and plasma samples were also collected from six litters per group after 12 hours of fasting (from 20:00 to 08:00) and stored at -20°C for analysis of the aforementioned markers. The TyG index was used as a predictor of insulin resistance (IR)

and cardiometabolic syndrome and was calculated with the following formula: TyG index = Ln [fasting triglycerides (mg/dL) × fasting glucose (mg/dL)/2] (Araújo *et al.* 2022). The albumin/globulin (A/G) ratio was calculated via the following formula: A/G ratio = albumin/(total protein - albumin) (Tsai *et al.* 2020).

Insulin and corticosterone assays during the ontogenetic development of male offspring

A batch of rats (*n*= 4–10 animals per group, from five different groups) were centrifuged at 2400×g for 20 min (4 °C), and the plasma was collected between 08:00 a.m. and 09:00 a.m. and stored at −20 °C until the assays were performed. The insulin and corticosterone concentrations were quantified via the Este ELISA, which is based on the principles of sandwich ELISA and competitive ELISA (Elabscience®, Houston, Texas, USA). The procedures were conducted in accordance with the manufacturer's instructions. The intra-assay and interassay coefficients of variation were 5.29 and 4.68 and 5.43 and 6.00, respectively.

The Folch method to quantify fat pad accumulation and hepatic cholesterol and triglyceride levels

Liver batches from rats at PN 14, PN35, PN45, PN55, and PN90 were used to quantify hepatic cholesterol and triglyceride levels. A 250 mg sample of liver tissue (from the left lobe) was collected from each animal (n = 8–10 rats from five litters per group). The tissue was homogenized with 10 ml of a chloroform solution (2:1, v/v) to extract the lipids (Vargas *et al.* 2023). The total lipid content was then measured via the Folch method. The extract was subsequently evaporated and diluted with isopropanol. On the following day, the total fat content, cholesterol content, and triglyceride content were quantified via a commercial kit in accordance with the manufacturer's instructions (GoldAnalisa, Belo Horizonte, MG, Brazil) (FOLCH *et al.* 1957).

Hepatic oxidative stress assays

A portion of hepatic tissue from a cohort of rats (n = 8 rats/group) was collected by exposing the peritoneal cavity. The liver was then removed, freeze-clamped, and immediately stored in liquid nitrogen. The samples were then separately homogenized in van Potter–Elvehjem in a cold solution of 0.1 M potassium phosphate (pH 7.4). The remaining homogenate was subsequently subjected to centrifugation at 11,000 × g for 15 minutes to separate it into soluble fractions. The activity of antioxidant enzymes was subsequently analyzed.

Lipid peroxides: The concentrations of products of lipid peroxidation were estimated via the TBARS assay (thiobarbituric acid-reactive substances). The amount of TBARS was calculated from the standard curve prepared with 1,1',3,3'-tetraethoxypropane, and the values are expressed as ηmol/mg protein (Buege & Aust 1978).

Protein carbonyl groups**:** The levels of protein carbonyl groups were quantified spectrophotometrically via 2,4-dinitrophenylhydrazine ($\varepsilon 370 = 22 \times 103\ M^{-1}\ cm^{-1}$) and expressed as ηmol/mg protein (Levine *et al.* 1990)

Antioxidant enzymes activity: Catalase activity was measured by detecting the change in absorbance at 240 nm using H_2O_2 as a substrate and expressed as μmol/(min × mg protein) (Tegge 1985).

The activity of superoxide dismutase (SOD): was determined by measuring its capacity to inhibit pyrogallol antioxidation in an alkaline medium. The latter was quantified at 420 nm. One unit of SOD (U) was defined as the quantity of enzyme required to inhibit 50% of the reaction, and the results were expressed as U of superoxide dismutase per mg of protein (MARKLUND & MARKLUND 1974).

Assay for reduced glutathione (GSH): The assay for reduced glutathione was conducted to determine the levels of GSH present in the total homogenate. The concentration of GSH was determined spectrofluorimetrically (with excitation at 350 nm and emission at 420 nm) via an o-phthalaldehyde (OPT) assay, as previously described by Hissin and Hilf (1976) (Hissin & Hilf 1976). The fluorescence was calculated as a measure of GSH. Standard curves were prepared with GSH, and the contents are expressed as ηmol/mg protein.

Reactive oxygen species (ROS): Levels were quantified in the homogenate supernatant via spectrofluorimetry with 2',7'-dichlorofluorescein diacetate (DCFH-DA) (de Almeida Gonçalves *et al.* 2018)The assay quantifies the oxidation of DCFH-DA to the fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS. Following the termination of the reaction by the addition of ice, the formation of DCF was quantified via a spectrofluorimeter (RF-5301, Shimadzu) with excitation and emission wavelengths of 504 and 529 nm, respectively. The results are expressed as ηmol/mg protein, employing a standard curve with oxidized DCF.

Protein content: The total protein content was determined in both the homogenate and the supernatant via the Folin phenol reagent (LOWRY *et al.* 1951).

RNA purification and gene expression via reverse transcription followed by real-time quantitative PCR (RT–qPCR)

The whole hypothalamus and testes were pulverized in liquid nitrogen via gral and pistil, transferred to 1.5 ml microtubes and stored at 80°C. Total RNA was extracted via the guanidine-phenol–chloroform method (CHOMZYNSKI 1987) via the TRIzol

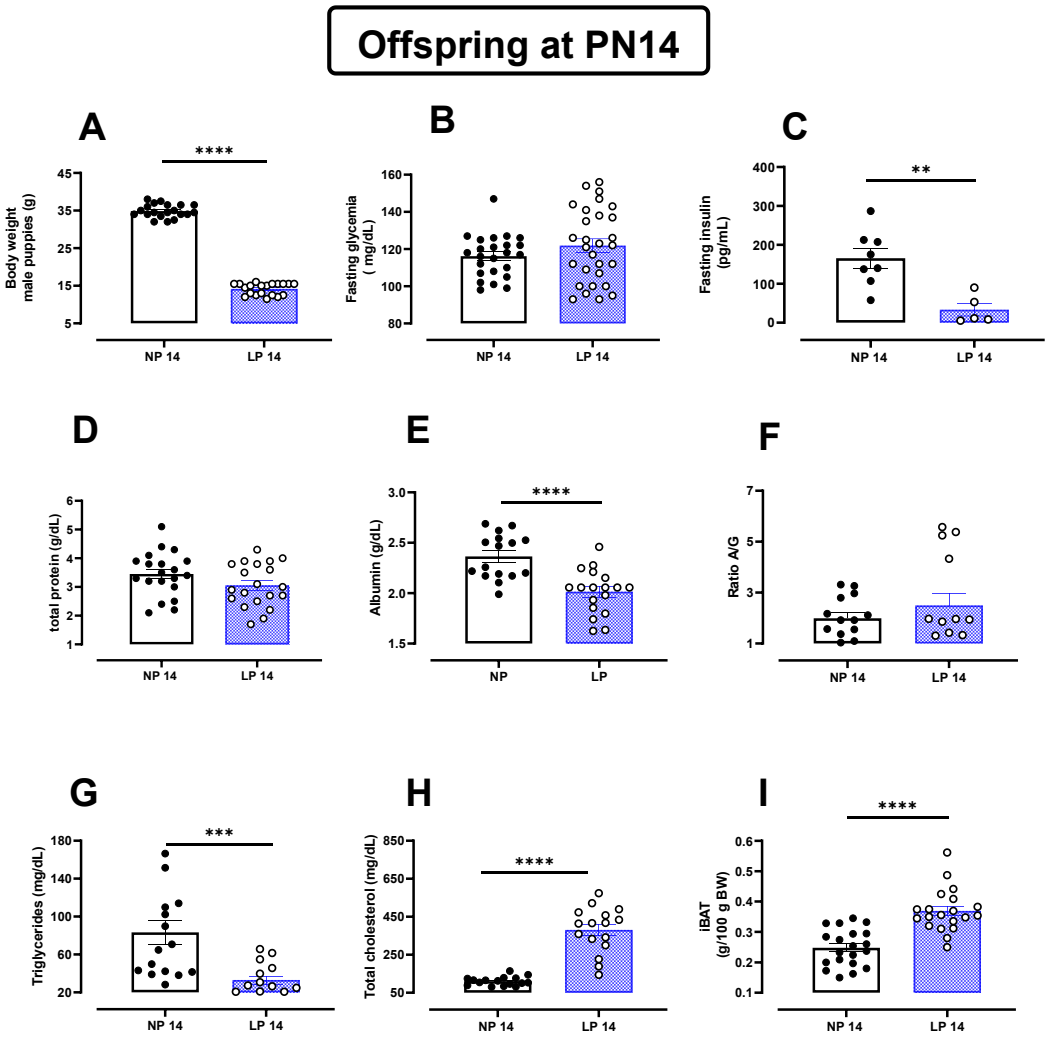
reagent (Life Technologies, Carlsbad, USA) according to the manufacturer´s instructions. The total RNA concentration was estimated by the optical density (OD) of the solution via nanospectrometry (KASVI model k23-002). The absorbance was measured at 260 nm, and the degree of purity was determined by the A260/280 nm ratio. RNA integrity was analyzed by electrophoresis on a 1.2% agarose gel in TBE buffer. Two-half micrograms of total RNA was reverse transcribed with a GoScript reverse transcription system kit (Promega, Madison, USA). Real-time PCR from the product of reverse transcription (RT–qPCR) was performed via the PowerUp SYBR Green Master Mix Kit (Life Technologies, Carlsbad, CA, USA), and the amplification conditions for these genes were performed with the resources Applied Biosystems StepOnePlusTM real-time PCR system (Applied Biosystems, Singapore) according to the manufacturer´s instructions. The cycle sequences were as follows: 50°C/2 min, 95°C/2 min, and 40 cycles of 95°C/15 s and 60°C/30 s. At the end of the reaction, a dissociation curve was generated to confirm the specificity of the reaction. The average values of the cycle threshold (Ct) were automatically determined by StepOneTM. Software v2.3 (Applied Biosystems). Quantification was performed via the $2^{-\Delta\Delta Ct}$ method via relative quantification analysis, as previously described (Livak & Schmittgen 2001a)The ribosomal protein L19 (*Rpl19*) gene was utilized as a reference gene. Samples from all ages were analyzed (n = 8 animals; six litters per group). The primer sequences and GenBank accession numbers of the genes for each tissue are shown in **Table 1**.

Statistical analysis

The results are presented as the mean ± standard error of the mean (SEM). The D'Agostino–Pearson test was employed to assess the Gaussian distribution of the dataset, with normality being the primary focus of this assessment. Two-way analysis of variance (ANOVA) was subsequently conducted to determine the influence of maternal diet (D),

offspring age (A), and their interaction. When significant differences were identified, the Bonferroni post hoc correction was employed. Student's t test was used for comparisons between two experimental groups (NP and LP). The results were deemed statistically significant when $p < 0.05$. All the statistical analyses were conducted via GraphPad Prism version 8.0 for Windows (GraphPad Software, Inc., San Diego, CA, USA).

Results



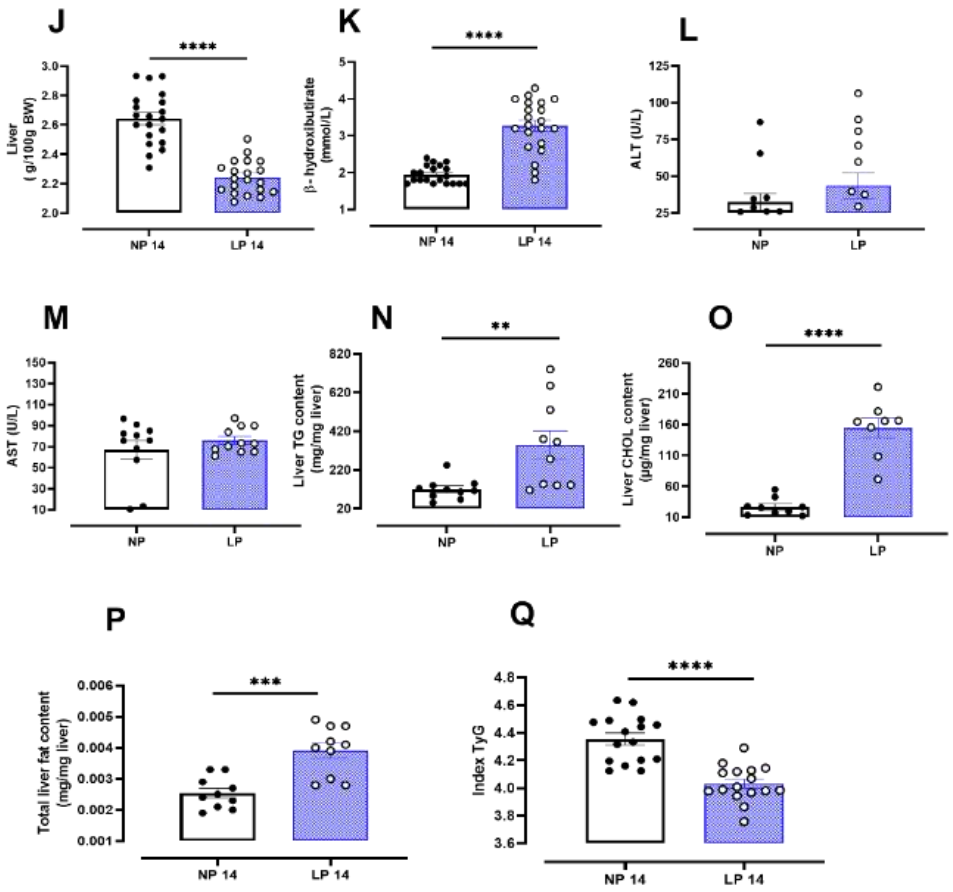


Figure 1: Features of lean-phenotype offspring compared with normal-phenotype offspring after maternal exposure to protein-caloric restriction with respect to biometric, biochemical, and hepatic metabolic functions in the offspring at PN 14. **(A)** Final body weight **(B)** Fasting glycemia **(C)** Insulin **(D)** Total protein **(E)** Albumin **(F)** AG ratio **(G)** Triglycerides **(H)** Total cholesterol **(I)** iBAT **(J)** Liver **(K)** β-Hydroxybutyrate **(L)** ALT **(M)** AST **(N)** Liver TG content **(O)** Liver CHOL content **(P)** Total liver fat content **(Q)** TyG index. The data are presented as the means ± S.E.M.ss of 5–20 male offspring from five to six different litters per group. * $P < 0.05$; Student's t test. NP: maternal normal protein diet; LP: maternal low protein diet.

After maternal exposure to a protein-supplemented diet, the body weights of the offspring were significantly lower (**NP:** 34.88 ± 0.39 vs. **LP:** 14.15 ± 0.34, $p < 0.05$), fasting glycemia (**NP:** 116.2 ± 2.31 vs. **LP:** 121.9 ± 3.66, ns) and total protein (**NP:** 3.45 ± 0.17 vs. **LP:** 3. 06 ± 0.16, ns) remained unchanged, whereas the insulin concentration was reduced (**NP:** 165.7 ± 25.10 vs. **LP:** 35.69 ± 12.80, $p < 0.05$), as was the albumin concentration (**NP:** 2.36 ± 0.05 vs. **LP:** 2.00 ± 0.05, $p < 0.05$), with no difference in the albumin/globulin ratio (**NP:** 1.99 ± 0.22 vs. **LP:** 2.59 ± 0.47, ns). Triglyceridemia decreased (**NP:** 83.32 ± 12.41 vs. **LP:** 33.08 ± 4.22, $p < 0.05$), whereas

hypercholesterolemia increased (**NP:** 110.8 ± 5.82 vs. **LP:** 380.8 ± 30.08, $p < 0.05$), as did subcutaneous brown adipose tissue mass (iBAT) (**NP:** 0.24 ± 0.01 vs. **LP:** 0.36 ± 0.01, $p < 0.05$). The offspring of LP dams with a malnourished phenotype presented altered hepatic regulation during infancy, with decreased liver mass (**NP:** 2.30 ± 0.01 vs. **LP:** 2.07 ± 0.02, $p < 0.05$), increased β-hydroxybutyrate (**NP:** 1. 94 ± 0.05 vs. **LP:** 3.26 ± 0.15, $p < 0.05$), whereas ALT (**NP:** 32.50 ± 5.83 vs. **LP:** 43.58 ± 8.58 ns) and AST (**NP:** 66.88 ± 8.80 vs. **LP:** 75.98 ± 3.68 ns) remained similar between the phenotypes. Lean offspring of nutritionally deprived dams during lactation developed hepatic steatosis in infancy, with a significant increase in triglycerides (**NP:** 120.1 ± 16.92 vs. **LP:** 349. 7 ±, $p < 0.05$), total cholesterol (**NP:** 26.63 ± 4.67 vs. **LP:** 154.5 ± 16.11, $p < 0.05$) and total fat accumulation in the liver (**NP:** 2.54 ± 0.15 mg vs. **LP:** 3.96 ± 0.25 mg, $p < 0.05$). These animals presented a decrease in the TyG index, indicating greater systemic insulin sensitivity (**NP:** 4.35 ± 0.04 vs. **LP:** 4.01 ± 0.03, $p < 0.05$).

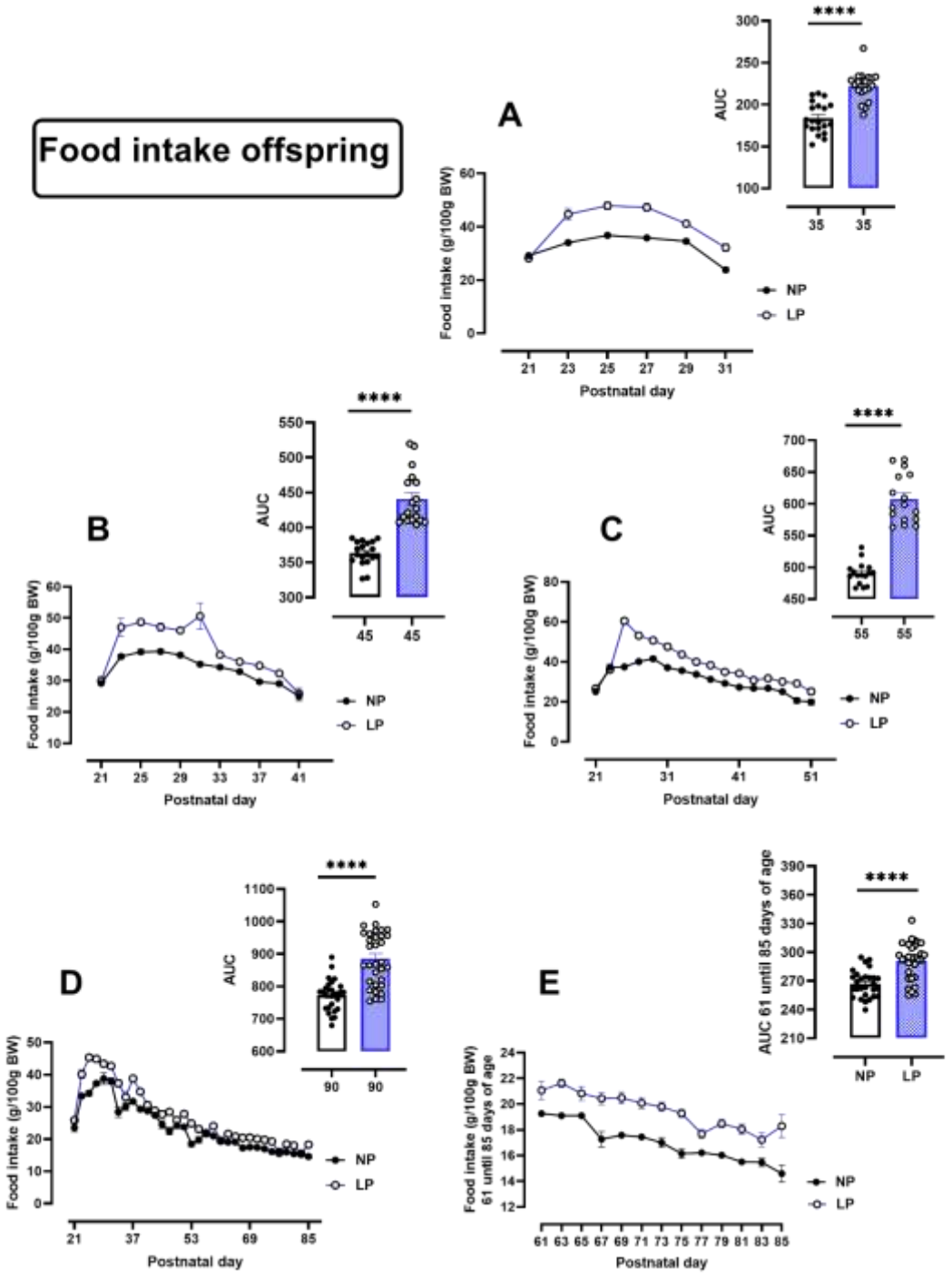


Figure 2: Food intake (FI) during ontogenetic development. FI **(A)** BW PN21 until PN31 **(B)** BW PN21 until PN 41 **(C)** BW PN21 until PN 51 **(D)** BW PN21 until PN 85 **(E)** PN 61 until PN 85. Data represent the mean ± SEM of 20–24 male offspring from four to six different litters showing food intake measurements during the prepubertal, pubertal, and postpubertal stages following maternal exposure to a low-protein (LP) diet. The graph next to each figure shows the AUC of the FI. *P<0.05 by Student's t test. NP: maternal normal protein diet; LP: maternal low protein diet.

As expected, the offspring of protein-restricted dams presented a hyperphagic phenotype that persisted into adulthood. Significant differences were detected at several developmental stages, including PN35 (Figure 2 A; **NP:** 184 ± 4.15 vs. **LP:** 222.3 ± 3.86, $p < 0.05$), PN45 (Figure 2 B, **NP:** 363.3 ± 7. 16 vs. **LP:** 440.7 ± 8.98, $p < 0.05$), PN55 (Figure 2 C; **NP:** 490.9 ± 4.39 vs. **LP:** 607.8 ± 9.57, $p < 0.05$), and PN90 (Figure 2 D; **NP:** 773.4 ± 9.03 vs. **LP:** 890.7 ± 10.22, $p < 0.05$). From PN61 to PN85, food intake also increased (Figure 2 E; **NP:** 266.8 ± 2.65 vs. **LP:** 290.8 ± 3.58, $p < 0.05$).

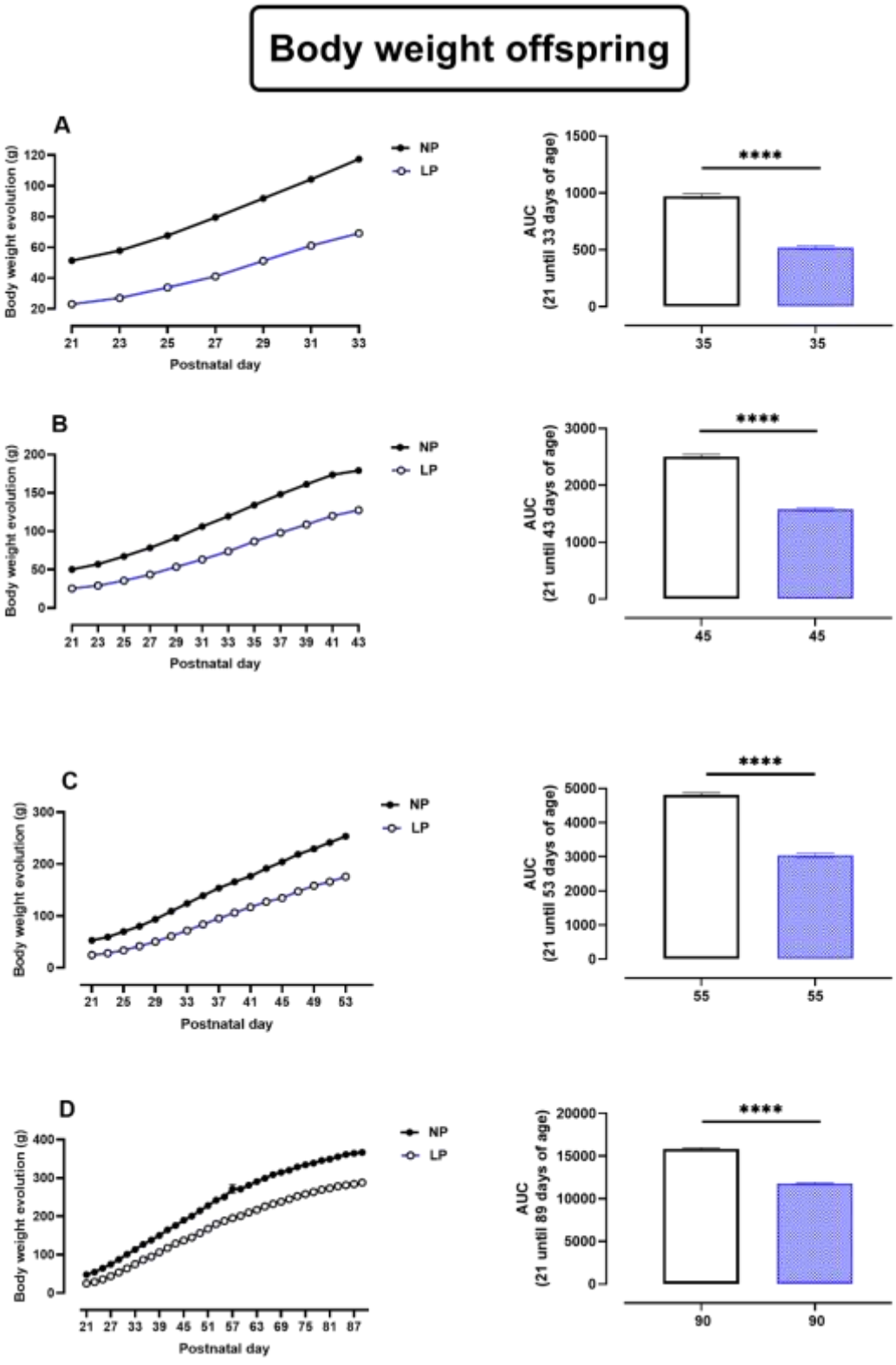


Figure 3: Progression of body weight (BW) during ontogenetic development. BW **(A)** BW PN21 to PN33 **(B)** BW PN21 to PN 43 **(C)** BW PN21 to PN 53 **(D)** BW PN21 to PN 87. The data are expressed as the means ± SEMs. of 20–24 rats from four to six different

litters. The graph next to each figure shows the AUC of BW. *P<0.05 according to Student's t test. NP: maternal normal protein diet; LP: maternal low protein diet.

Compared with the offspring of control dams, the offspring of dams fed the LP diet presented lower body weight gain from the prepubertal stage through young adulthood. Significant differences were detected at several developmental stages, including PN35 (Figure 3 A; **NP:** 971.6 ± 20.32 vs. **LP:** 813.2 ± 15.74, $p < 0.05$), PN45 (Figure 3 B; **NP:** 2504 ± 38. 89 vs. **LP:** 1579 ± 27.13, $p < 0.05$), PN55 (Figure 3 C; **NP:** 4819 ± 48.92 vs. **LP:** 3045 ± 64.14, $p < 0.05$), and PN90 (Figure 3 D; **NP:** 15838 ± 143.1 vs. **LP:** 11787 ± 137.1, $p < 0.05$).

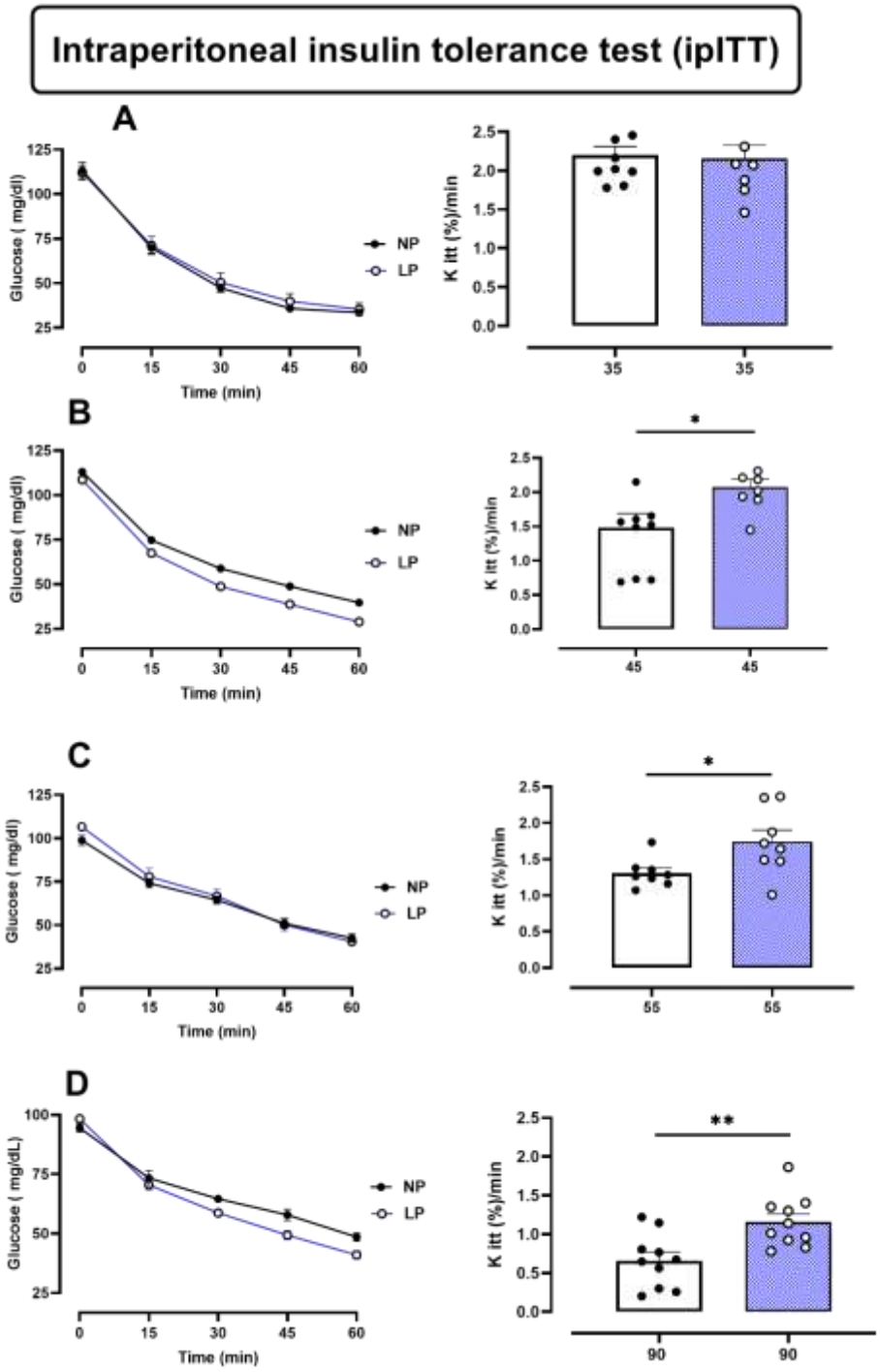


Figure 4: Glucose homeostasis during the insulin tolerance test (Kitt) was assessed at the prepubertal, pubertal, and postpubertal stages. Panels show Kitt values at **(A)** PN33, **(B)** PN43, **(C)** PN53, and **(D)** PN87. The data are expressed as the means ± S.E.M.s and are based on measurements from 8 rats per group (two animals from four different litters). *P < 0.05, ns: not significantly different according to Student's t test. NP: maternal normal protein diet; LP: maternal low protein diet.

During Kitt, as demonstrated by the AUC, LP animals presented altered glucose uptake throughout ontogenetic development (Figure 5). LP and NP animals showed no

differences at PN35 (Figure 4A; **NP:** 2.20 ± 0.11 vs. **LP:** 2.16 ± 0.17, ns). At the following ages, at PN45 (Figure 4B; **NP:** 1.48 ± 0.20 vs**. LP:** 2.10 ± 0.12, $p < 0.05$), PN55 (Figure 4C; **NP:** 1.31 ± 0.07 vs. **LP:** 1.85 ± 0.15, $p < 0.05$), and PN90 (Figure 4D; **NP:** 0.66 ± 0.10 vs. **LP:** 1.15 ± 1.0, $p < 0.05$), LP offspring presented increased glucose uptake.

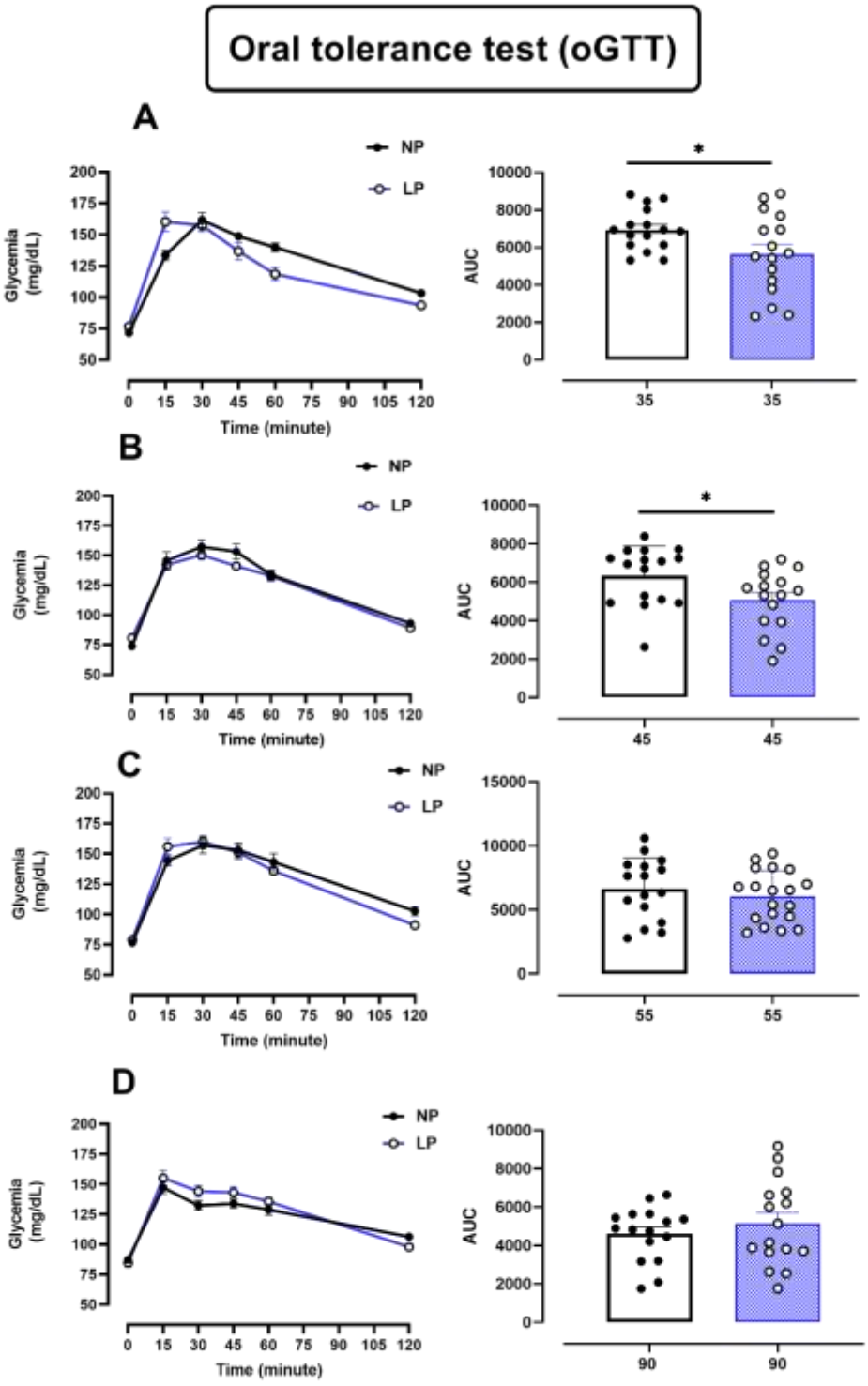
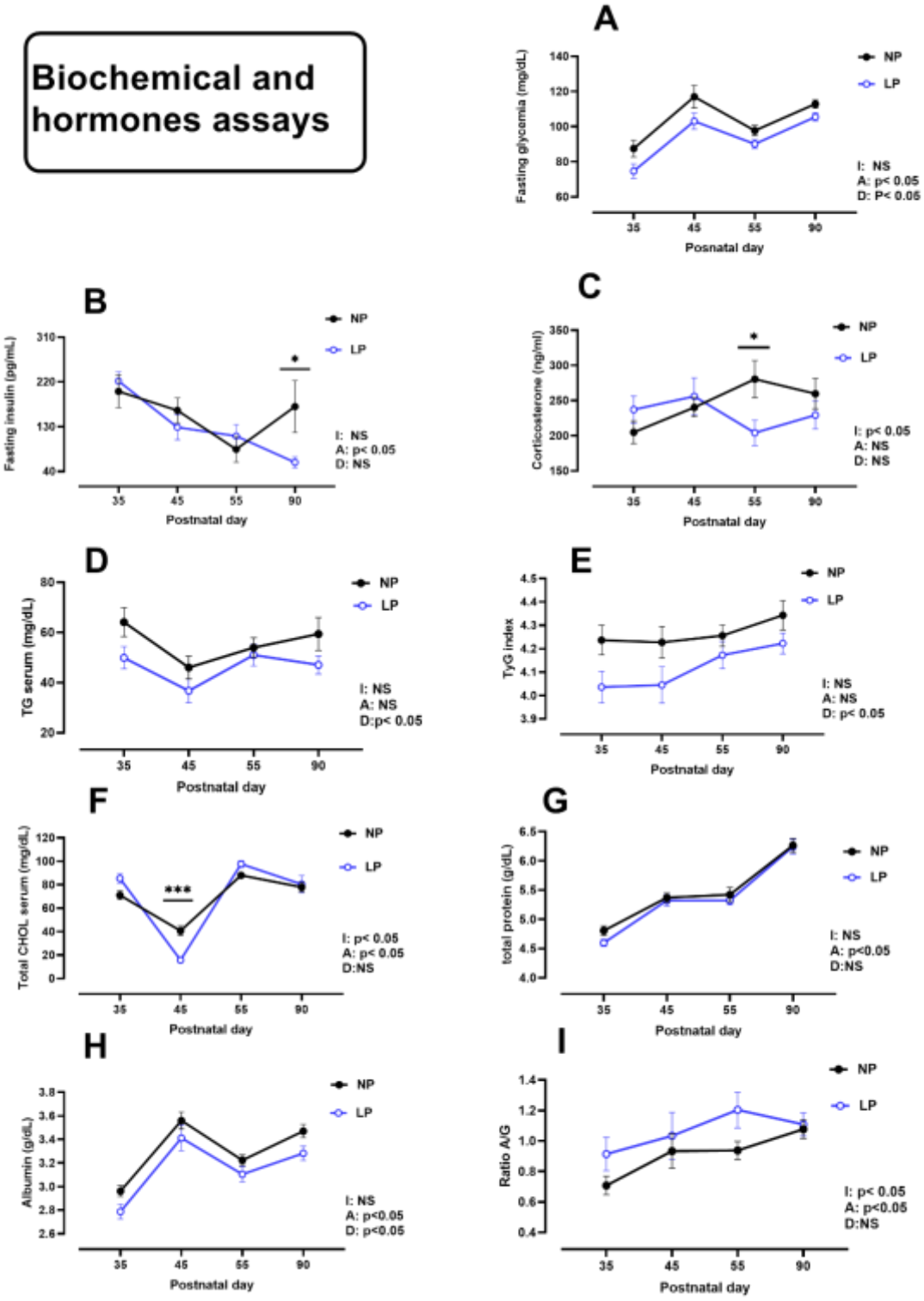


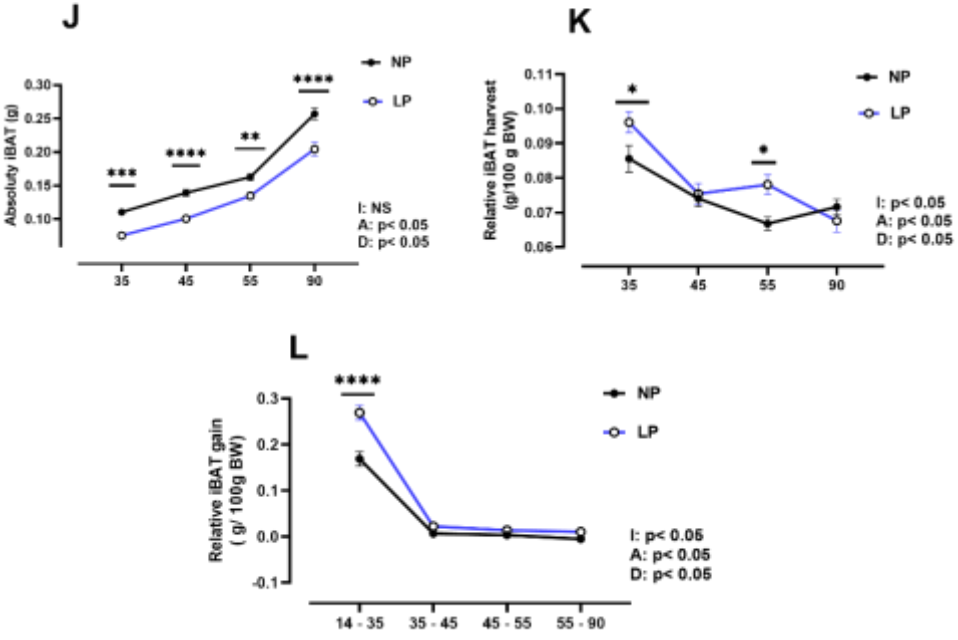
Figure 5: Glycemia curve during the oGTT, assessed throughout pubertal development into adulthood. oGTT results are shown for **(A)** PN35, **(B)** PN45, **(C)** PN55, and **(D)** PN90. The symbols represent the means ± S.E.M.ss of 16 rats from four to six different litters per experimental group. The graph next to each figure shows the AUC of the increase in glycemia during the oGTT. *P < 0.05 (Student's t test). NP: maternal normal protein diet; LP: maternal low protein diet.

During the oGTT, as shown by the AUC, LP animals presented altered glucose tolerance throughout ontogenetic development (Figure 5). Increased glucose tolerance was observed in LP animals at PN35 (Figure 5A; **NP:** 6939 ± 275.7 vs. **LP:** 5638 ± 214.7, $p < 0.05$) and PN45 (Figure 5B; **NP:** 6345 ± 385.5 vs. **LP:** 5073 ± 397.3, $p < 0.05$). At later ages, glucose tolerance remained unchanged (Figure 5C; **NP:** 6644 ± 603.6 vs. **LP:** 6038 ± 461.4 ns) (Figure 5D; **NP:** 4612 ± 355.0 vs. **LP:** 5155 ± 558.1 ns).

Biochemical and hormones assays



Interscapular adipose tissue Harvest



Visceral adipose tissue (VAT) in the offspring

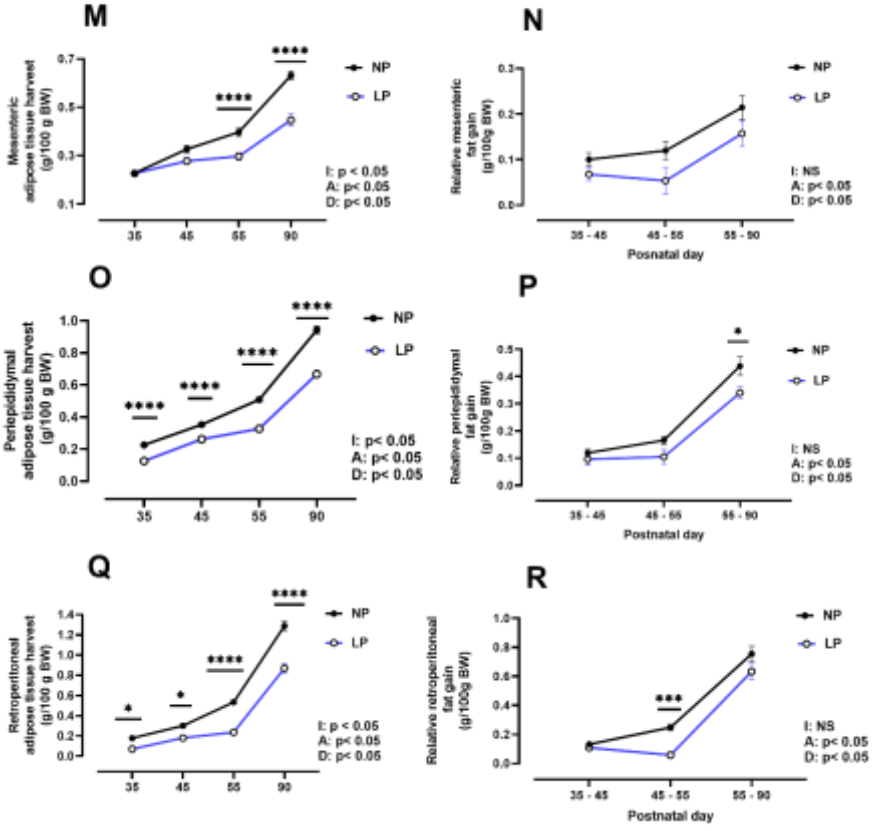


Figure 6: Effects of maternal protein restriction on plasma and serum markers, interscapular adipose tissue, and visceral adiposity in male offspring from puberty to maturity. **(A)** Fasting glycemia **(B)** Insulin **(C)** Corticosterone **(D)** Plasma TG **(E)** TyG index **(F)** Plasma CHOL **(G)** Serum total protein **(H)** Serum albumin **(I)** AG ratio **(J)** Absoluty iBAT **(K)** Relative iBAT harvest **(L)** Relative iBAT gain mass **(M)** Relative mesenteric harvest **(N)** Relative mesenteric gain mass **(O)** Relative periepididymal harvest **(P)** Relative periepididymal gain mass **(Q)** Relative retroperitoneal harvest **(R)** Relative retroperitoneal gain mass. The data are expressed as the means ± SEMs. Differences were assessed via two-way ANOVA, followed by the Bonferroni post hoc correction. The factors considered included D (LP diet), A (age), and I (interaction between the LP diet and age). Statistical significance was defined as $p < 0.05$, with "NS" indicating no significant difference. **NP:** offspring of dams fed an NP diet (n: 8--20); **LP:** offspring of dams fed an LP diet (n: 8--20).

A maternal low-protein (LP) diet during breastfeeding promoted a lean phenotype, along with changes in intermediate metabolism and body composition, from peripuberty to adulthood (Figure 6). Two-way ANOVA revealed a significant effect of diet (Figure 6 B; $pD < 0.05$) and age (Figure 6 B; $pA < 0.05$) on the fasting glucose concentration, with no significant interaction between the factors. However, post hoc analysis did not reveal any significant differences between groups at any age (Figure 6A). From peripuberty to adulthood, age significantly affected the insulin concentration ($pA< 0.05$, two-way ANOVA), whereas no effect was detected for diet or interaction between factors. LP offspring had lower fasting insulinemia than did NP rats at 90 days of age (Figure 6B; NP:180.54 ± 51.8 vs. LP: 58.36 ±10.5; $p < 0.05$; Bonferroni post hoc correction). Neither age nor diet affected the corticosterone concentration, but there was a significant interaction effect between these factors (Figure 6 C; $pI < 0.05$). Interestingly, LP offspring showed a decrease in corticosterone levels only at PN55 (Figure 6C; NP: 280.48 ± 26.41 vs. LP: 204.14 ± 17.91 $p< 0.05$, Bonferroni post hoc correction), with no differences observed at other ages.

Two-way ANOVA revealed a significant effect of diet (Figure 6D; $pD < 0.05$); however, the time factor and the interaction between factors did not significantly change.

Post hoc analysis revealed no significant differences between the groups studied. The TyG index also showed a significant effect of diet (Figure 6E; $pD < 0.05$), but neither age nor the interaction between age and diet significantly differed according to two-way ANOVA, and comparisons between the NP and LP groups throughout development did not reveal significant differences. There was a significant interaction effect between diet and age on the total serum cholesterol concentration (Figure 6F; $pI < 0.05$, two-way ANOVA). Cholesterol levels were lower in LP animals than in NP animals at 45 days of age (Figure 6F; NP: 42.75 ± 4.28 vs. LP: 15.76 ± 1.26; $p < 0.05$, Bonferroni post hoc correction).

According to two-way ANOVA, age had a significant effect on total protein levels (Figure 6G; $pA < 0.05$), whereas no significant differences were observed for the other factors. Serum protein concentrations remained unchanged when evaluated by multiple comparisons (Bonferroni post hoc correction) (Figure 6G). In contrast, both age (Figure 6H; $pA < 0.05$) and diet (Figure 6H; $pD < 0.05$) affected the serum ALB concentration, with no interaction between the factors. However, the albumin levels were not significantly different from those of the NP offspring according to the Bonferroni post hoc correction. There was a significant interaction effect between diet and age on the serum A/G ratio (Figure 6I; $pI < 0.05$, two-way ANOVA). However, Bonferroni post hoc analysis revealed no significant differences between the groups analyzed.

The absolute mass of brown adipose tissue was affected by age (Figure 6J; $pA < 0.05$) and diet (Figure 6J; $pD < 0.05$). However, there was no significant difference between the factors according to two-way ANOVA (Figure J). During the transition from peripuberty to young adulthood, the mass of iBAT was reduced at the following time points: PN 35 (NP: 0.010 ± 0.005 vs. LP: 0.076 ± 0.003, $p < 0.05$), PN 45 (NP: 0.139 ±

0.005 vs. LP: 0.101 ± 0.004, $p < 0.05$), PN 55 (NP: 0.163 ± 0.005 vs. LP: 0.135 ± 0.005, $p < 0.05$), and PN 90 (NP: 0.257 ± 0.009 vs. LP: 0.205 ± 0.009, $p < 0.05$). The relative weight and weight gain of iBAT during peripubertal development showed distinct responses that were significantly influenced by the interaction between diet and age (Figure 6K and L, $pI < 0.05$, two-way ANOVA), especially at the beginning and end of this developmental period. In a post hoc Bonferroni comparison between groups, LP animals had greater relative weights of iBAT at the beginning of the peripubertal period (PN 35: NP: 0.084 ± 0.004 vs. LP: 0.096 ± 0.003, $p < 0.05$) and at the end of the peripubertal period (PN 55: NP: 0.066 ± 0.002 vs. LP: 0.078 ± 0.003, $p < 0.05$). The weight gain of iBAT was greater in LP offspring only from PN 14 to PN 35 (NP: 0.169 ± 0.016 vs. LP: 0.269 ± 0.016, $p < 0.05$). In both analyses, no differences were observed at the other ages studied.

Age and maternal malnutrition had strong effects on mesenteric visceral fat (Figure 6M; $pI < 0.05$, two-way ANOVA). Starting at PN 45, LP rats had lower amounts of visceral fat than did NP rats (PN 45: NP: 0.327 ± 0.009 vs. LP: 0.298 ± 0.013, $p < 0.05$; PN 55: NP: 0.398 ± 0.015 vs. LP: 0.298 ± 0.015, $p < 0.05$), with this difference persisting into early adulthood at PN 90 (NP: 0.632 ± 0.018 vs. LP: 0.448 ± 0.025, $p < 0.05$). However, mesenteric fat gain was affected only by age (Figure N, $p < 0.05$) and the LP diet (Figure N, $p < 0.05$), with no significant interaction according to two-way ANOVA. No differences between the phenotypes were found via the post hoc Bonferroni correction.

These interaction effects also persisted in other visceral fat depots, such as periepididymal (Figure 6O; $pI < 0.05$, two-way ANOVA) and retroperitoneal fat (Figure 6Q; $pI < 0.05$, two-way ANOVA), in these lean-phenotype LP offspring, who had lower

visceral fat depots. LP offspring presented lower amounts of periepididymal fat during ontogenetic development than control offspring did (post hoc Bonferroni correction), with significant differences observed at PN 35 (NP: 0.226 ± 0. 011 vs. LP: 0.127 ±, $p < 0.05$), PN 45 (NP: 0.353 ± vs. LP: 0.262 ±, $p < 0.05$), PN 55 (NP: 0.509 ± 0.012 vs. LP: 0.328 ± 0.007, $p < 0.05$), and PN 90 (NP: 0.943 ± 0.024 vs. LP: 0.668 ± 0.017, $p < 0.05$). The same trend was observed for retroperitoneal fat, which decreased from the onset of peripuberty to adulthood, with differences at PN 35 (NP: 0.179 ± 0.010 vs. LP: 0.071 ± 0.005, $p < 0. 05$), PN 45 (NP: 0.301 ± 0.017 vs. LP: 0.181 ± 0.014, $p < 0.05$), PN 55 (NP: 0.534 ± 0.021 vs. LP: 0.237 ± 0.018, $p < 0.05$), and PN 90 (NP: 1.290 ± 0.047 vs. LP: 0.871 ± 0.048, $p < 0.05$).

With respect to the weight gain of periepididymal and retroperitoneal fat, both age and diet affected LP animals (Figures 6P and 6R, respectively; $pI < 0.05$, two-way ANOVA). However, while gonadal fat stores were lower in LP animals only during the transition to the postpubertal period, from PN 55 to PN 90 (Figure 6P; NP: 0.438 ± 0.035 vs. LP: 0.340 ± 0.020, $p < 0.05$), retroperitoneal fat was reduced only between PN 45 and PN 55 (Figure 6R; NP: 0.249 ± 0.020 vs. LP: 0.058 ± 0.020, $p < 0.05$). The gain in periepididymal and retroperitoneal fat did not differ between the NP and LP groups at other ages, indicating that both phenotypes are capable of accumulating adiposity, but this depends on the developmental phase they are in.

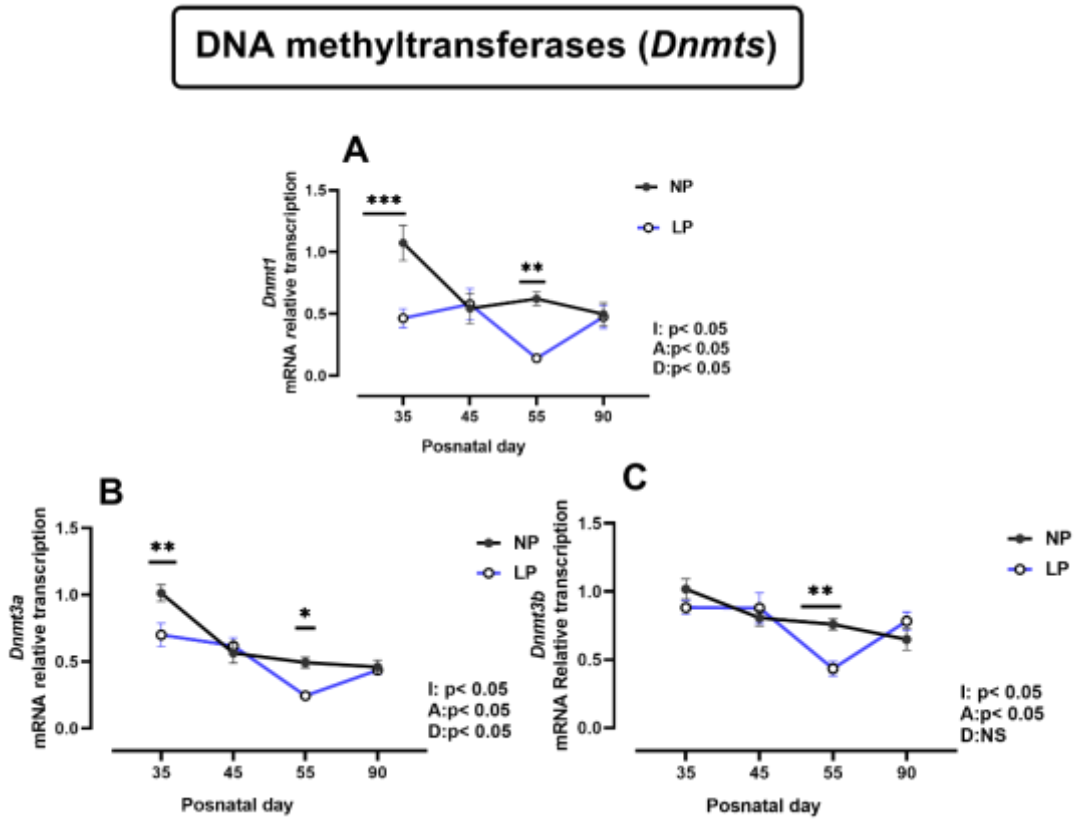


Figure 7: DNA methyltransferase (*Dnmt*) mRNA was quantified in the hypothalamus of male rat offspring from the onset of peripuberty to adulthood. Changes in the mRNA transcription levels of *Dnmt1* **(A)**, *Dnmt3a* **(B)**, and *Dnmt3b* **(C)**. This study highlights the regulation of DNA methylation enzymes during ontogenetic development. The data are expressed as the means ± SEMs. Differences were evaluated by two-way ANOVA followed by the Bonferroni post hoc correction, with significance set at $p < 0.05$. The factors considered included D (LP diet), A (age), and I (interaction between diet and age). **NP:** offspring of dams fed an NP diet (n: 7--8); **LP:** offspring of dams fed an LP diet (n: 6--8).

Protein malnutrition during lactation changed DNA methyltransferase expression in LP offspring throughout the peripubertal window. Significant interactions were observed for all transcripts, including both maintenance DNA methyltransferase (Figure 7A; $pI < 0.05$, two-way ANOVA) and de novo DNA methyltransferases (Figure 7B and 7C; $pI < 0.05$ for both, two-way ANOVA). *Dnmt1* mRNA expression was significantly lower in LP males than in NP males at PN 35 (-57%, Figure 7A; **NP:** 1.073 ± 0.14 vs. **LP:** 0.465 ± 0.075; $p < 0.05$) and PN 55 (-77%, Figure 7A; **NP:** 0.622 ± 0.054 vs. **LP:** 0.141 ± 0.019; $p < 0.05$). Similarly, LP males presented lower relative expression of

Dnmt3a at PN 35 (-30.7%, Figure 7B; **NP:** 1.014 ± 0.062 vs. **LP:** 0.701 ± 0.087; $p < 0.01$) and PN 55 (-51%, Figure 7B; **NP:** 0.494 ± 0.039 vs. **LP:** 0.245 ± 0.031; $p < 0.05$). However, a significant decrease in *Dnmt3b* expression in LP males was observed only at PN 55 (-43.4%, Figure 7C; **NP:** 0.761 ± 0.041 vs. **LP:** 0.435 ± 0.053; $p < 0.05$).

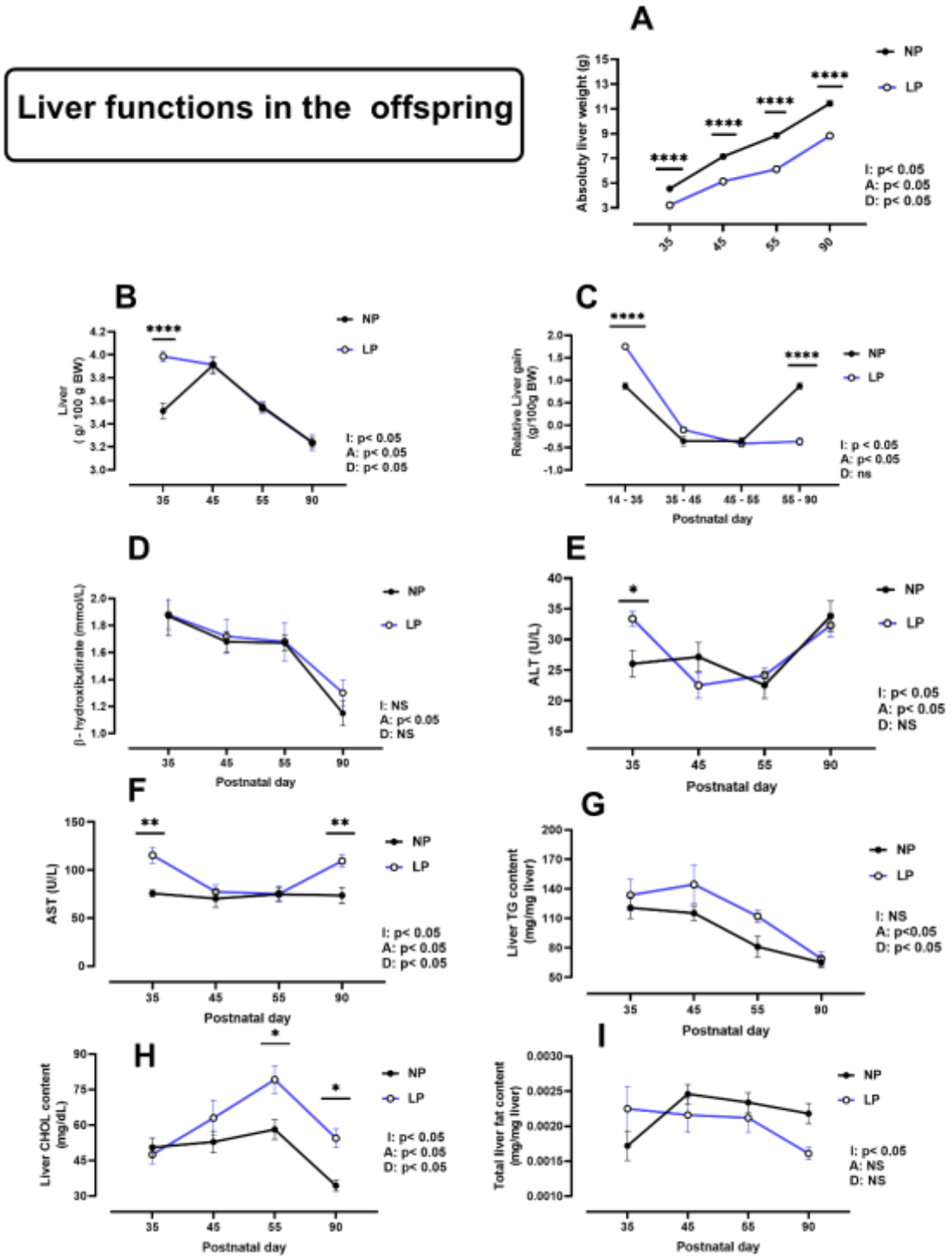


Figure 8: Effects of exposing dams to protein restriction during breastfeeding on hepatic metabolism in offspring from the peripubertal window to adulthood. **(A)** Absolute liver weight. (**B)** Relative liver weight. **(C)** Relative liver gain. **(D)** β-Hydroxybutyrate. **(E)** ALT. **(F)** AST. **(G)** Liver TG content. **(H)** Liver CHOL content. **(I)** Total liver fat content. The data are expressed as the mean ± S.E.M. Data are expressed as the mean ± SEM. Differences were evaluated by two-way ANOVA followed by the Bonferroni post hoc correction, with significance set at $p < 0.05$. The factors considered included D (LP diet), A (age), and I (interaction between diet and age). **NP:** offspring of dams fed an NP diet (n: 8--20); **LP:** offspring of dams fed an LP diet (n: 8--20).

Maternal exposure to protein malnutrition caused alterations in the hepatic metabolism of offspring from peripuberty through adulthood. LP offspring during peripuberty showed an interaction effect between diet and developmental age for absolute liver weight, relative liver weight, and hepatic weight gain (Figure 8A–C; $pI < 0.05$, two-way ANOVA). *Post hoc* analysis revealed that absolute liver weight was lower at all ages during development: PN 35 (**NP**: 4.55 ± 0.12 vs. **LP**: 3.21 ± 0.08, $p < 0.05$), PN 45 (**NP**: 7.15 ± 0.17 vs. **LP:** 5.13 ± 0.13, $p < 0.05$), PN 55 (**NP:** 8.84 ± 0.17 vs. **LP:** 6.13 ±0.12, $p < 0.05$), and PN 90 (**NP:** 11.44 ± 0.29 vs. **LP:** 8.825 ± 0.17, $p < 0.05$). In contrast, the relative liver weight was lower only at PN 35 (**NP:** 3.512± 0.06 vs. **LP:** 3.98 ± 0.04, $p < 0.05$). Additionally, hepatic mass gain was greater in the LP group from PN 14 to PN 35 (**NP:** 0.87 ± 0.06 vs. **LP:** 1.75 ± 0.05, $p < 0.05$) but lower from PN 55 to PN 90 (**NP:** + 0. 85 ± 0.06 vs. **LP:** - 0.361 ± 0.08, $p < 0.05$). The concentration of β-hydroxybutyrate was affected by age (Figure 8D; $pI < 0.05$, two-way ANOVA), with no differences observed between the NP and LP groups according to the Bonferroni *post hoc* correction.

Hepatic function was assessed by measuring the aminotransferase enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which showed an interaction effect between the factors analyzed (Figure 8E and F; $pI < 0.05$, two-way ANOVA). LP animals presented elevated ALT levels at PN 35 (NP: 26.05 ± 2.16 vs. LP:

33.39 ± 1.20, $p < 0.05$) and elevated AST levels at PN 35 (NP: 75.80 ± 3.48 vs. LP: 115.55 ± 8.25, $p < 0.05$) and PN 90 (NP: 73.71 ± 8.47 vs. LP: 109.49 ± 6.80, $p < 0.05$).

The effects of age (Figure 8G; $pA < 0.05$) and diet (Figure 8G; $pD < 0.05$) on hepatic triglyceride levels were observed. However, no differences were observed between the NP and LP groups according to the Bonferroni post hoc correction. In contrast, both the hepatic cholesterol content and total liver fat content had interaction effects (Figure 8 H and I; $pI < 0.05$, two-way ANOVA). LP rodents during peripuberty presented increased hepatic cholesterol at PN 55 (NP: 58.12 ± 4.24 vs. LP: 79.25 ± 5.81, $p < 0.05$) and PN 90 (NP: 34.37 ± 2.32 vs. LP: 54.34 ± 3.88, $p < 0.05$). Although the LP group had a lower threshold for total hepatic fat content, analysis of variance did not reveal significant differences between the offspring of dams fed a normal protein diet and those fed a low protein diet.

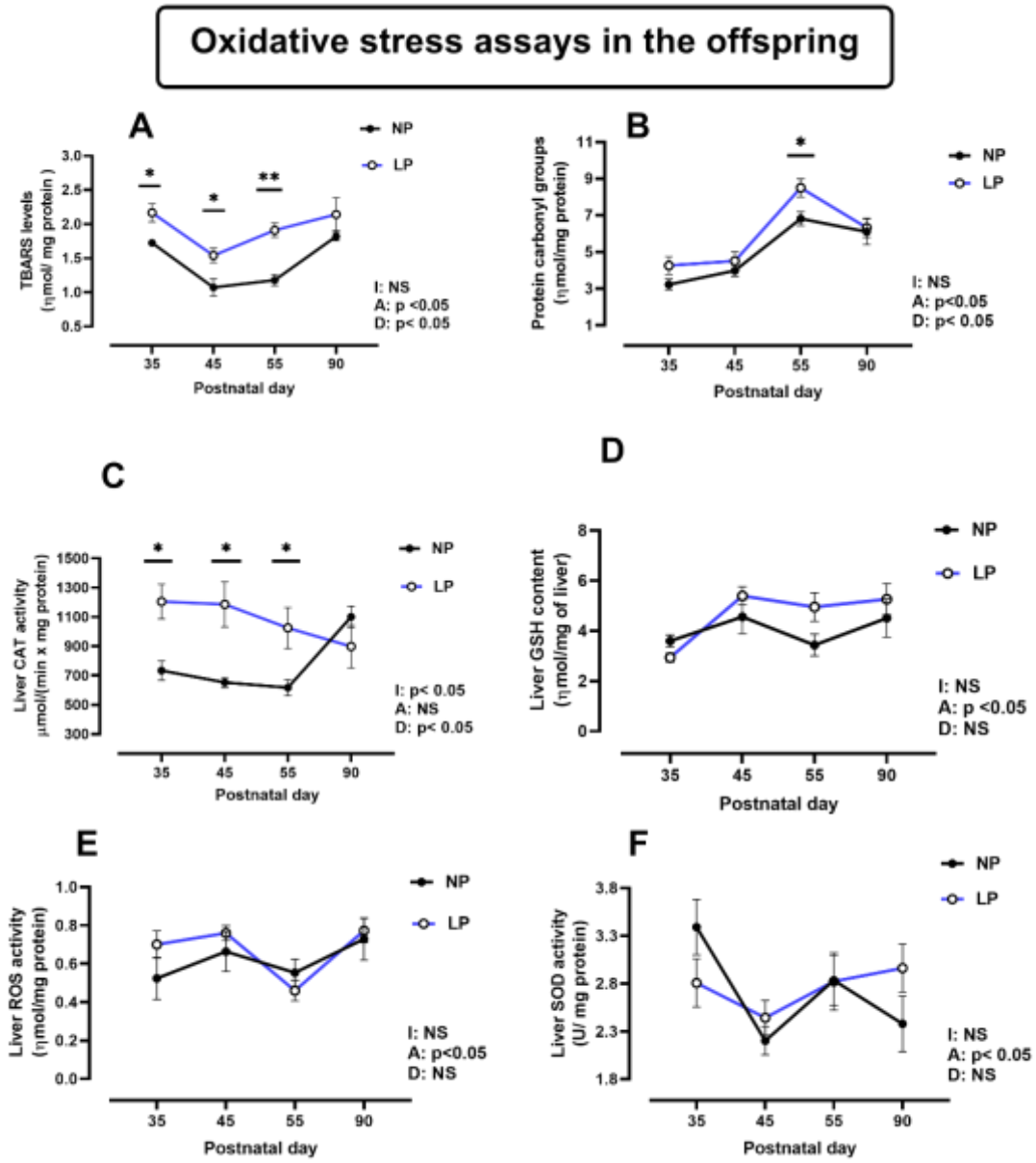


Figure 9: Hepatic oxidative stress assays were performed on offspring from dams fed either a normal-protein diet or a protein-caloric restriction diet for two-thirds of the breastfeeding period. The levels of redox states were measured for the following parameters: TBARS (A), protein carbonyl groups (B), catalase (C), liver GSH (D), liver ROS activity (E), and liver SOD activity (F). The data are expressed as the means ± SEMs. Differences between groups were analyzed via two-way ANOVA followed by the Bonferroni post hoc correction, with significance set at $p < 0.05$. The factors considered included D (LP diet), A (age), and I (interaction between diet and age). **NP:** offspring of dams fed an NP diet (n: 8); **LP:** offspring of dams fed an LP diet (n: 8).

Figure 9 shows the effects of maternal malnutrition on hepatic oxidative stress in offspring from the onset of peripuberty to young adulthood. The levels of TBARS and protein carbonyl groups in the male offspring of dams fed a low-protein diet were affected by both maternal diet (Figure 9A and B, $pD < 0.05$, two-way ANOVA) and age (Figure 9A and B, $pA < 0.05$, two-way ANOVA). LP animals presented increased TBARS concentrations at PN 35 **(NP:** 1.72 ± 0.04 vs. **LP**: 2.16 ± 0.13, $p < 0.05$), PN 45 (**NP:** 1.072 ± 0.12 vs. **LP:** 1.54 ± 0.10, $p < 0.05$), and PN 55 (**NP:** 1.18 ± 0.82 vs. **LP:** 1.91 ± 0.10, $p < 0.05$), with no differences in adulthood at PN 90. Protein carbonyl group levels were significantly greater only at PN 55 (**NP:** 6.81 ± 0.38 vs. **LP:** 8.50 ± 0.49, $p < 0.05$). Interestingly, catalase activity, an antioxidant parameter, was significantly affected by factors (Figure 9C, $pI < 0.05$, two-way ANOVA). Group comparisons revealed that this enzyme activity was approximately 50% greater in the livers of LP rats at PN 35 (**NP:** 733.83 ± 66.95 vs. **LP:** 1205.08 ± 118.90, $p < 0.05$), PN 45 (**NP:** 652.31± 36.08 vs. **LP:** 1185.90 ±153.26, $p < 0.05$), and PN 55 (**NP:** 617.34 ± 54.46 vs. **LP:** 1024.56 ± 140.26, $p < 0.05$) but not at PN 90. The GSH, ROS, and SOD activities were affected only by age (Figure 9E, F, and G, $pI < 0.05$, two-way ANOVA). The results of the Bonferroni post hoc correction revealed that the levels of GSH, ROS, and SOD activity in the liver did not differ between the NP and LP offspring at any of the ages evaluated.

DISCUSSION

The current findings reveal that maternal exposure to a low-protein diet during the initial two-thirds of lactation has a pronounced influence on the reprogramming of intermediary metabolism in offspring. The aforementioned maternal nutritional insult results in delayed weight gain in offspring, which in turn compromises their growth during childhood and leads to increased accumulation of lipids in the liver. Furthermore, animals with a lean phenotype display hormonal alterations that may be associated with

impaired intermediary metabolism regulation at various stages of their ontogenetic development. LP offspring exhibit alterations in hypothalamic methylation patterns, particularly during the peripubertal window, indicating that maternal malnutrition may epigenetically reprogram the hypothalamus of offspring, likely through breast milk.

Previous research has shown elevated corticosterone in the blood and milk of the male offspring of stressed dams (Martins *et al.* 2023a). Insulin counterregulatory hormones facilitate proteolysis and lipolysis, contributing to the hyperglycemic states observed in LPs with a cachectic phenotype and attenuating the anabolic effects of insulin dependent on energy storage. Our results, presented in Figure 1, show that LP offspring exhibit hypoinsulinemia at the end of the maternal insult, suggesting impaired pancreatic beta-cell function during lactation. Furthermore, hypoinsulinemic conditions impair anabolic functions, leading to slower growth and developmental delays. Furthermore, hypoinsulinemia in LP offspring suggests increased glycogenolysis and gluconeogenesis during early life, facilitating the release of glucose from the liver to meet the metabolic needs of these rodents and ensure their survival. Studies have shown that undernourished dams produce less milk because of their mammary gland function and low prolactin concentration (Moretto *et al.* 2011; Bautista *et al.* 2019). Despite the increased caloric density of the dams' milk, there was no significant increase in the offspring's body weight gain, which may explain the observed hypertriglyceridemia and heightened insulin sensitivity during early life. The poor health of the pups is due to a decrease in milk intake and insulin levels, which hinders growth. Despite this, LP offspring display elevated glycemia, and the normalization of endogenous corticosterone (Martins *et al.* 2023a) indicates that increased insulin sensitivity is insufficient to maintain normal glycemic homeostasis.

Low insulin levels in LP animals may increase gluconeogenesis, thereby promoting lipolysis and mobilizing nonesterified fatty acids from adipocytes. This results in increased uptake of free fatty acids by the liver, which in turn enhances hepatic glucose production and ketogenesis. Moreover, hyperglycemia may inhibit glycogenolysis (Hatting *et al.* 2018). Thus, both β-hydroxybutyrate and glucose intolerance in infancy are crucial for insulin-independent tissues such as those of the central nervous system, which have high energy expenditure and require substrates during neurogenesis. This phenomenon is relevant to the DOHaD (Developmental Origins of Health and Disease) concept, which suggests that poor nutrition in childhood can affect the health and development of offspring, increasing the risk of chronic diseases later in life.

Interestingly, early childhood exposure to famine in China is linked to increased risks of obesity, hypertension, hyperglycemia, and nonalcoholic fatty liver disease in adulthood (Chen *et al.* 2016). Hypoalbuminemia and inflammation are related to undernutrition status (Eckart *et al.* 2020). LP animals display hypoalbuminemia, potentially due to a reduced liver mass resulting from maternal undernutrition, a factor that may contribute to hepatoinflammation without evidence of systemic inflammation, as inferred from the albumin–globulin ratio, indicating possible tissue-specific inflammation. Furthermore, there is an increase in triglycerides and cholesterol in the liver, contributing to elevated hepatic fat content during infancy in males following maternal insult, which may be attributed to increased insulin secretion in response to elevated energy demand at the end of the first week (Martins *et al.* 2023a). Liver fat levels are elevated in LP offspring, which is associated with increased levels of fatty acid synthase (*Fasn*) in their livers (Vargas *et al.* 2023). These offspring may exhibit increased *Fasn* transcript expression during breastfeeding due to the substantial energy demands of maternal milk, which may increase de novo lipogenesis. Malnutrition is associated with

disruptions in hepatic lipid metabolism, including reduced tricarboxylic acid cycle activity, oxidative phosphorylation, and fatty acid oxidation (van Zutphen *et al.* 2016). Furthermore, hypoalbuminemia and low plasma levels of lipoproteins and triglycerides are linked to an increased incidence of hepatic steatosis (Truswell & Hansen 1969). Thus, the small liver in the LP phenotype may be caused by a lower number of liver cells, which affects protein synthesis and fat storage in the liver.

Our findings also revealed that the maternal LP diet altered hepatic triglyceride and cholesterol levels in LP offspring, whereas the total fat content in the liver during peripuberty was lower than that in NP offspring. These findings suggest that these animals do not develop hepatic steatosis during peripuberty, despite oxidative stress and elevated transaminases, likely due to the high energy demands of this developmental stage, which may increase liver inflammation. However, the enhanced antioxidant capacity and improved peripheral insulin sensitivity seem to help prevent fat accumulation in the liver.

In our study, LP offspring presented a hyperphagic phenotype throughout their ontogenetic development, which was essential for growth after weaning. However, their body weight gain never exceeded that of the NP animals until early adulthood, confirming that protein restriction during lactation contributes to the programming of body growth. Despite the dietary insult affecting maternal body weight, the offspring maintained a healthy phenotype even when exposed to a nutrient-restricted environment after weaning. LP rodents exhibited high glucose tolerance at PN35; however, by PN45, glucose tolerance remained unchanged, but they exhibited high insulin sensitivity. These findings suggest that the lean phenotype has well-regulated glucose metabolism during a vulnerable stage of intense cellular proliferation necessary for the growth of glucose-dependent tissues. In addition, maternal malnutrition resulted in reduced visceral fat

accumulation in LP offspring, a trait that persisted into adulthood. The proportion of adipose tissue gain varied by region and time period. Interestingly, mesenteric fat gain did not differ between the two phenotypes into adulthood, although both diet and age are known to influence its distribution. Furthermore, periepididymal fat mass gain was lower after the peripubertal period, whereas retroperitoneal fat showed the lowest rate of gain from middle to late peripuberty. It was unexpected that, despite having lower visceral adiposity, LP offspring exhibited a decline in β-hydroxybutyrate levels from the end of puberty, similar to the NP phenotype. Furthermore, despite hyperphagia, these animals appear to be in a hypermetabolic state, particularly during the peripubertal period. This likely contributes to their lower accumulation of visceral adipocytes, which are metabolically more active fat. Notably, the reduction in β-hydroxybutyrate levels may be attributed to its role as an energy source for organs such as the brain, heart, and muscles, which could explain the observed decrease in serum levels. This suggests a complex metabolic adaptation in LP offspring, where the increased energy demand from key organs might drive the reduction in β-hydroxybutyrate levels, even in the presence of a reduction in fat reserves.

In addition, LP animals presented lower glucose and triglyceride levels from peripuberty onward, likely due to increased insulin sensitivity. The elevated albumin/globulin ratio may be attributed to the effect of the maternal diet on reducing albumin production while maintaining globulin levels, suggesting a negative impact on plasma colloid osmotic pressure, which could lead to symptoms such as hypotension or edema in the LP phenotype. Both transaminases are elevated at the onset of peripuberty, but only AST remains high in adulthood. We did not observe excessive hepatic fat accumulation, but this does not rule out injuries caused by increased oxidative stress or changes in adiponectin levels, which could enhance inflammatory processes without

necessarily leading to fibrosis or chronic liver damage. Previous studies have shown that maternal protein restriction to 8% does not program offspring for liver steatosis dysfunction (Bertasso *et al.* 2022), and protein deficiency results in mild hepatic steatosis (Warren *et al.* 2023). Therefore, regardless of the source of protein, protein deficiency can lead to hepatic steatosis.

During the peripubertal period, cholesterol levels decreased in both phenotypes, with a more pronounced reduction observed in LP animals. In adolescents, total serum cholesterol levels decline in conjunction with accelerated growth and height gain during puberty. Conversely, higher body weight and shorter stature have been linked to elevated serum lipids (Kouda *et al.* 2003). We hypothesized that LP animals demonstrate enhanced utilization of cholesterol by peripheral tissues, which is attributed to the accelerated growth observed in these lean-phenotype rodents. This is particularly relevant given the essential role that cholesterol plays in the formation of structures, such as the biological membranes of cells undergoing intense cellular proliferation.

While LP animals have low insulin levels in adulthood, consistent with previous studies (Gravena *et al.* 2007; de Oliveira *et al.* 2011, 2014), corticosterone levels do not decrease until the late peripubertal period, indicating reduced responsiveness of the HPA axis. A previous study revealed that prepubertal rats exhibit high corticosterone levels during puberty, which plateau in animals between 30 and 40 days of age, whereas stress-induced ACTH reactivity occurs in rats between PN 50 and 60 (Foilb *et al.* 2011), suggesting that the adaptive stress response in LP animals may reach full maturation by the end of puberty. The offspring of protein-restricted mothers show lower corticosterone levels with aging, suggesting that HPA axis reactivity varies at different stages of life,

such as during the transition from the peripubertal stage until adult life (Zambrano *et al.* 2023).

Interestingly, LP offspring presented altered hypothalamic DNA methylation patterns from peripuberty to adulthood. Both *Dnmt1* and *Dnmt3a* transcription decreased at the beginning and end of this period, whereas *Dnmt3b* transcription decreased only at the end of peripuberty, suggesting altered temporal regulation of DNA methyltransferases in LP offspring. The *Dnmt3* family is implicated in maternal imprinting in mice (Hata *et al.* 2002), suggesting that epigenetic early postnatal programming of LP offspring may originate from dams through breastfeeding or behavioral changes. Paternal consumption of an obesogenic diet in rodents causes POMC hypermethylation in sperm and in the arcuate nucleus in male offspring, demonstrating the impact of paternal nutrition on transgenerational epigenetic transmission (Haberman *et al.* 2024). On the other hand, nutritional deficits can disrupt one-carbon metabolism and induce DNA hypomethylation by increasing S-adenosylhomocysteine (James *et al.* 2002). Hypomethylation in malnourished children can be reversed through the administration of dietary methyl donors, including betaine, folate, and methionine (Schulze *et al.* 2019). Independent of S-adenosylmethionine (SAM) levels, dietary methyl donor deficiency reduces *Dnmt1* levels in the livers of mice (Nohara *et al.* 2011), and high-intake low-quality proteins alter DNA transmethylation and the expression pattern of DNA methyltransferases in liver tissue (Akyol *et al.* 2018). In addition, increased hepatic synthesis of SAM has been associated with a hepatoprotective effect against oxidative stress associated with aging (Castillo *et al.* 2005). Hypothalamic epigenetic programming, which is influenced by maternal imprinting during lactation, demonstrates how altered metabolic signals can affect gene regulation during peripuberty, a period of significant changes in body composition,

reproduction, and behavior. However, these methylation differences in the hypothalamus decrease over time, becoming indistinguishable in adulthood. It remains to be investigated whether the observed hypomethylation is due to a reduction in methyl donors, decreased DNMT levels, or the accumulation of S-adenosylhomocysteine (SAH).

These results indicate that during peripuberty, the LP offspring presented increased oxidative stress, as evidenced by elevated levels of lipoperoxidation products and carbonylated proteins in their liver tissue. This oxidative damage appears to stem from increased production of reactive species, suggesting an activated pro-oxidant system, despite unchanged levels of reactive oxygen species (ROS). Furthermore, superoxide dismutase (SOD) activity remained normal, whereas the levels of catalase, which is responsible for the elimination of H_2O_2, were elevated in the livers of underweight animals. Although these animals presented increased levels of pro-oxidant markers associated with elevated hepatic transaminases, indicating liver damage, the metabolic response seems to enhance antioxidant capacity as a strategy to maintain homeostasis. In obese boys, oxidative stress is elevated, and antioxidants are diminished. Both normal-weight and obese boys show increased antioxidant capacity during puberty, especially in response to stress (Paltoglou *et al.* 2015). Additionally, hormones released during peripuberty may modulate these altered metabolic responses, altering antioxidant capacity. The unchanged production of ROS may indicate neutralization or diversion toward the generation of other reactive species, such as peroxynitrite or hydroxyl radicals, the latter of which likely contribute to the oxidation of proteins and lipids in the liver, compromising its homeostasis. Moreover, the female LP offspring in this study presented improvements in the hepatic antioxidant system in adulthood (VARGAS *et al.* 2024). This study confirmed that the oxidative capacity of male LP offspring, driven by catalase,

remains high until puberty. However, other pro- and antioxidant products and metabolites may also be produced.

Conclusion

In conclusion, protein restriction during lactation disrupts intermediary metabolism and induces epigenetic pattern changes in male rats with underweight phenotypes throughout their lifespan, which may have long-term health implications.

Supplementary data

Supplementary Table 1 – Primers used for RT–qPCR analyses and GenBank accession numbers of target genes.

Gene	NCBI Reference Sequence	Primer sequence (5' – 3´)
Dnmt1 DNA methyltransferase 1	NM_053354.3	F: TAGTTCGGTGGCTACGAGGA R: CGTTTAGCGGGACCCTTGAA
Dnmt3a DNA methyltransferase 3 alpha	NM_001003958.1	F: ACGACCAGGAATTTGACCCC R: CATGCCCACTGTGATGGAGT
Dnmt3b DNA methyltransferase 3 beta	NM_001396349.1	F: ACAACCATTGACTTTGCCGC R: TCAGAAGCCATCCGTTCTCG
Rpl19 (Ribosomal protein L19)	NM_031103.1	F: CAATGAAACCAACGAAATCG R: TCAGGCCATCTTTGATCAGCT

F forward; R reverse

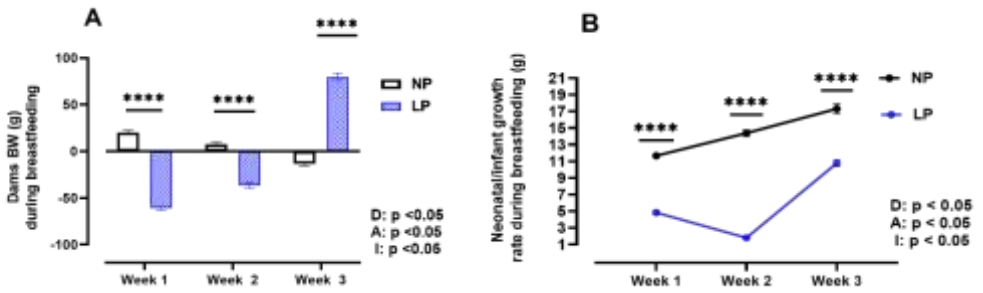


Figure 10: Dams' body weight changes and offspring neonatal/infant growth during breastfeeding were analyzed. (A) Maternal body weight during the lactation period and

(B) neonatal/infant growth rates during the breastfeeding phase are presented. The data are expressed as the means ± SEMs. Statistical differences between groups were assessed via two-way ANOVA followed by the Bonferroni post hoc correction, with significance set at $p < 0.05$. The factors considered included D (LP diet), A (age), and I (interaction between diet and age). NP: dams (n = 20) and offspring (n = 19) fed a normal protein diet; LP: dams (n = 19) and offspring (n = 20) fed a low-protein diet.

Figure 10 A illustrates how the diet of the dam during lactation affected both maternal and pup body weights while the pups remained under maternal care. The LP diet during lactation resulted in a significant decrease in maternal body weight (Figure 10 A, $pI < 0.05$). Interestingly, weight loss was more pronounced in the first week (**NP:** +20.47 ± 2.19 vs. **LP:** -61.23 ± 1.89, $p < 0.05$) than in the second week (**NP:** +7.75 ± 1.94 vs. **LP**: -36.63 ± 3.22, $p < 0.05$). After switching to a standard diet (NP), maternal weight was restored during the third week (**NP:** -13.37 ± 1.94 vs. **LP:** +79.92 ± 3.94, $p < 0.05$).

Maternal malnutrition during lactation also affected the offspring, resulting in lower body weights during infancy (Figure 10 B, $pI < 0.05$). In the first week, weight gain was reduced (**NP:** +11.66 ± 0.32 vs. **LP:** +4.82 ± 0.21, $p< 0.05$), and this reduction was even more pronounced in the second week (**NP:** +14.37 ± 0.40 vs. **LP**: +1.78 ± 0.13, $p< 0.05$). Although there was a slight recovery in the third week (**NP**: +17.29 ± 0.61 vs. **LP**: +10.77 ± 0.41, $p< 0.05$), the effects of early malnutrition persisted.

Finding sources

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A low-protein diet during breastfeeding delays puberty by altering reproductive physiology in underweight male rats in the peripubertal window

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Short title: An LP diet during breastfeeding delays puberty in underweight male rats.

ABSTRACT

The survival and development of newborn mammals depend on postnatal (PN) breastfeeding. It was investigated whether exposure to a low maternal protein (LP) diet during the first two weeks of lactation could alter the phenotype of their male offspring in the prepubertal, pubertal, and postpubertal periods, from the peripubertal window to adulthood. The experimental groups were designated NP and LP, and the subsequent batches were named NP-35, LP-35, NP-45, LP-45, NP-55, LP-55, NP-90, and LP-90. The assessments included age and weight at preputial separation, murinometric measurements, hormone assays, and gene expression determination via reverse transcription followed by real-time quantitative PCR (RT–qPCR) of the whole

hypothalamus (HPT modulators) and the testes (genes encoding genes encoding genes involved in steroidogenic and spermatogenic functions). Maternal exposure to a maternal LP diet in offspring delays puberty, which is associated with low body weight and low adiposity into adulthood. In the prepubertal phase, aromatase activity is high, contributing to a decrease in testosterone and its receptor (*Ar*) transcript, as well as the reduced activity of aromatase itself. Despite the increase in *Kiss1* in LP offspring, this increase was not sufficient to alter *Gnrh1* mRNA. Although the aromatase gene was altered during puberty, it did not affect the signaling of the sex hormone biosynthesis pathway. The *Fshr* transcript level was reduced in the postpubertal window, when testosterone levels returned to normal concentrations. The dietary insult altered the mRNA levels of Sertoli cells and spermatogenic genes, such as *Lgals1, Kit, Rhcg,* and *Lrrc34*, but *Ndrg4*, which is expressed in undifferentiated spermatogonia, was not affected by the LP offspring. In brief, the maternal LP diet caused delayed development in offspring, not only preventing the onset of puberty but also delaying it. Additionally, the regulation of the HPT axis, steroidogenesis enzymes, and germ cells was impacted, indicating that energy balance can influence the timing of puberty due to nutritional deficiencies transmitted through maternal milk.

Keywords: Maternal protein caloric restriction (LP); lean phenotype; low adiposity; peripubertal; delayed timing of puberty; HPT modulation; steroidogenic and spermatogenic genes.

INTRODUCTION

It is widely accepted that breastfeeding is a critical phase sensitive to nutrient disturbances, and stressors such as a maternal low-protein (LP) diet during lactation can induce the remodeling of brain circuits, which affects brain function; metabolic dysfunctions; hypotrophy of organs and tissues; and the reprogramming of offspring LPs into having a low adiposity phenotype, even after adequate postnatal food supply (Zambrano *et al.* 2005a; Fagundes *et al.* 2007, 2009b; Guzmán *et al.* 2014a; Martins *et al.* 2023b). Mammals exhibit altricial characteristics, requiring adult care for nourishment and locomotion. This phase of pronounced phenotypic malleability is analogous to the third trimester of gestation in humans because they are born with immature organs(Lesage

et al. 2006b; Clancy *et al.* 2007; Dutta & Sengupta 2016); thus, breastfeeding is susceptible to programming by LP nutrition (Zohdi *et al.* 2014). However, the thrifty phenotype hypothesis proposed by Hales and Barker involves a link between early nutritional behaviors and elevated rates of adult metabolic disorders, including obesity, diabetes, and hypertension, which affect people worldwide (Vaag *et al.* 2012b; Lacagnina 2020)The intake of the LP diet during the first two weeks of lactation promotes predictive adaptations in LP rats, maintaining a lean phenotype in peripuberty (Martins *et al.* 2018a).

Additionally, a maternal LP diet during breastfeeding provoked both low body weight and fat accumulation in offspring adults, a consequence of hypercortisolism and elevated catecholamine release that stimulates lipolysis ((Fagundes *et al.* 2007, 2009b). Furthermore, when offspring adults are offered an obesogenic diet in early adulthood, they become resistant to obesity later in life (Martins *et al.* 2023b), indicating that a hypercaloric diet increases energy expenditure in lean young-phenotype rodents. However, the onset of puberty in male rodents is a life transition between the immature reproductive organs and reproductive capacity, a period of rapid growth and high energy expenditure. Modifications in the maternal nutritional score (including both overnutrition and undernutrition) throughout breastfeeding affect hypothalamic circuits that control sexual differentiation and competence. (Caron *et al.* 2012), potentially causing permanent alterations in both the maternal phenotype and that of her offspring. The onset of puberty occurs between postnatal days 35 and 55, during the peripubertal window. This is associated with an increase in the pulsatile secretion of GnRH, which is essential for testicular growth, sex steroid biosynthesis, and maintenance of the spermatogenesis cycle (Bell 2018) later in life. Nevertheless, a causal link between the energy balance of LP offspring and reproductive functions during peripubertal windows, particularly regarding

the role of the kisspeptin peptide in the regulation of the hypothalamic–pituitary–testicular (HPT) axis (Harter *et al.* 2018; Navarro 2020), remains under investigation.

Puberty is initiated as a result of peripheral metabolic signals that stimulate the release of kisspeptin by neurons situated within the arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV). This, in turn, leads to an increase in the pulsatile secretion of hypothalamic gonadotropin-releasing hormone (GnRH). GnRH stimulates the anterior pituitary gland to release luteinizing hormone (LH), which binds to its receptor (LHcgr) in Leydig cells (LCs) to promote several functions related to steroidogenesis. GnRH also promotes the secretion of follicle-stimulating hormone (FSH), which binds to its receptor (FSHr) in Sertoli cells (SCs), stimulating the spermatogenic process (Nagirnaja *et al.* 2010; Casarini & Simoni 2021). Moreover, ARC neurons coexpress peptides such as kisspeptin (KISS), neurokinin B (NKB), and dynorphin A (DYN), which are called KNDy neurons, whereas KISS and NKB are involved in stimulatory actions, and dynorphin (DYN) negatively regulates GnRH release(True *et al.* 2011; Rønnekleiv & Kelly 2013a, b; Stamatiades & Kaiser 2018; Uenoyama *et al.* 2021; Xie *et al.* 2022). During childhood, makorin ring finger protein 3 (*Mkrn3*) released by the hypothalamus (HPT) exerts direct and indirect inhibitory effects on GnRH release, and its polymorphism causes precocious puberty(Maione *et al.* 2020; Seraphim *et al.* 2021). Treatment with GnRH analogs, such as leuprorelin and triptorelin, is considered the most effective and safe option for treating central precocious puberty (Brito *et al.* 2016).

The release of GnRH frequently occurs via sex steroid signaling in hypothalamic neurons, which in turn modulates gonadal functions. In the CNS, the modulation of behaviors, including libido, aggression and hedonic behavior, may be mediated by the

central aromatization of neurosteroids. The negative feedback of testosterone and estrogen hormones occurs in the hypothalamic nucleus and is mediated by androgen receptor (Ar) and estradiol receptors 1 and 2 (*Esr1* and *Esr2*) (Smith *et al.* 2005; Hrabovszky *et al.* 2007). While *Ar* and *Esr1* mRNAs are associated with the inhibition of GnRH secretion, Esr2 is associated with Esr1 stimulation (Hu *et al.* 2008a; Krsmanovic *et al.* 2009). Additionally, G protein-coupled receptor 30 (GPR30), which is encoded by *Gper1* and stimulated by 17β-estradiol, is expressed in GnRH neurons and plays a role in the rapid release of the GnRH neuropeptide (Kenealy & Terasawa 2012).

In male rats, an external marker of pubertal maturation is preputial separation (PPS) from the glans penis, which is stimulated mainly by testosterone (Korenbrot *et al.* 1977b; Bell 2018). During puberty, the concentration of testosterone is maintained by stimulating the LH hormone to bind to the LHcgr receptor in the LC, the site of steroid biosynthesis. Testosterone is the majority of male sex hormones produced during puberty by Leydig cells in the testes. It can be aromatized into estradiol by enzymes of the cytochrome P450 family (Zirkin & Papadopoulos 2018a), playing a role in the modulation of germ cells. Low protein intake during pregnancy and lactation leads to offspring with hypotestosteronemia (Chamson-Reig *et al.* 2009); additionally, pubertal protein-energy deficiency is associated with both low testosterone levels and reduced androgen receptor content (de Oliveira *et al.* 2018). Thus, the LP diet may affect testosterone homeostasis.

Therefore, we investigated the expression of the aromatase enzyme (Cyp19a1) in the hypothalamus and testes, which is involved in sex steroid synthesis. Initially, free cholesterol is transferred from the cytosol to the mitochondria via steroidogenic acute regulatory protein (STAR) (Miller 2007); subsequently, CYP11A1 converts cholesterol into pregnenolone, and androstenedione and testosterone are transformed into estrone and

estradiol, respectively, by the action of aromatase (Payne & Hales 2004; Zirkin & Papadopoulos 2018b). Maternal undernutrition during the perinatal period alters estrogen and androgen receptors after prepuberty in offspring, impairing folliculogenesis, ovarian steroidogenesis and ovulation (da Silva Faria *et al.* 2008, 2010; da Silveira Cavalcante *et al.* 2009; Guzmán *et al.* 2014b) associated with delayed vaginal opening (da Silva Faria *et al.* 2004). Although estrogen receptors are predominantly associated with female reproductive functions, in the testes, their presence is crucial for the regulation of spermatogenesis and germ cell viability (Dumasia *et al.* 2016, 2017). In addition to FSH, sperm production is also regulated by testosterone derived from steroidogenesis, and its actions depend on Sertoli cells with functional androgen receptors (Christin-Maitre & Young 2022).

During gonadarche, *Ar* mRNA is predominantly expressed in Sertoli cells, where it regulates cell proliferation and differentiation; moreover, these receptors facilitate the maintenance of the blood–testis barrier (BTB), providing structural support to differentiating germ cells (Wang *et al.* 2022a). The anterior hypothalamus and preoptic area contain high concentrations of aromatase and estrogen receptors, which regulate sexual arousal. Testosterone and estrogen interact in Leydig, Sertoli, and germ cells. A decrease in testosterone, accompanied by an increase in estrogen, can lead to testicular atrophy and reduced sperm production, which are often associated with erectile dysfunction (Schulster *et al.* 2016a). Moreover, the distribution of the estrogen receptors ESR1 and ESR2 and GPER1 in the testicular parenchyma is heterogeneous, with expression observed in both germ cells and somatic cells. In these cells, these receptors play intrinsic roles in spermatogenesis and in the regulation of cell proliferation and apoptosis (Chimento *et al.* 2014).

In the peripubertal stage, FSH binds to its receptor FSHr in SCs to promote several actions in spermatogenesis (Oduwole *et al.* 2018). Moreover, FSH and testosterone signaling mediate the differentiation of sperm cell subtypes (Walker & Cheng 2005; Wang *et al.* 2022b). Sertoli cells in the basal compartment guide germ cells toward the adluminal compartment. Undifferentiated spermatogonia divide to become differentiated spermatogonia, which become primary spermatocytes and then undergo meiosis I. Secondary spermatocytes quickly undergo meiosis II, becoming round or elongated haploid spermatids. These cells are called spermatozoa when they mature (de Kretser *et al.* 1998; O'Shaughnessy 2014; Prevot 2015; Oduwole *et al.* 2018). Maternal insults during lactation did not affect the expression of *Lgals1* or Ndrg4. However, they impair the mRNA levels of specific spermatogenic genes in different germ cell subtypes, including *Kit* (differentiating spermatogonia), *Rhcg* (spermatocytes), and *Lrrc34* (spermatids)(Ajayi & Akhigbe 2020; Akhigbe *et al.* 2023).

Indeed, maternal malnutrition during periods of high phenotypic plasticity in offspring has been associated with impairments in the formation and differentiation of testicles, as well as reductions in fertility and fecundity in rats (Oliveira *et al.* 2015; Ghasemi *et al.* 2024). A meta-analysis revealed that malnutrition during critical developmental periods is linked to diminished testicular size, reduced epididymal sperm count, decreased Sertoli cell number, and lower luteinizing hormone and testosterone levels in offspring, leading to reproductive impairments. These effects of malnutrition on reproductive health are influenced by the timing of the intervention and seasonal factors (Oliveira *et al.* 2015; Ajuogu *et al.* 2021).

There is a gap in the understanding of the relationships between maternal undernutrition and its impacts on the lean phenotype of offspring during the acquisition

of reproductive capability. We hypothesize that a low-protein diet during the first 2 weeks of breastfeeding causes a lean phenotype in the peripubertal period, altering the timing of puberty onset in male rats by directly affecting the activity of the HPG axis. To unravel this enigma, the present study objectively investigated body weight until weaning, the timing of puberty, body weight during puberty, adiposity INDEX, testicular weight, and the assessment of serum testosterone. Furthermore, gene expression in the whole hypothalamus (*Kiss, Tac3, Mkn3, Gnrh1, Cyp19α1, Ar, Gper, Esr1 and Esr2)* and key gene expression in steroidogenesis (*Lhcgr, Star, Cyp11α1, Cyp19α1)* and spermatogenesis (*Fshr, Lgals, Ndrg4, Kit, Rhcg, Lrrc34, Ar, Gper, Esr1 and Esr2)* in the testis were evaluated.

Materials and methods

Ethical approval

The handling of the animals and all the experimental procedures were in accordance with the rules of the ARRIVE Guidelines 2.0 for experiments involving animals (Percie du Sert *et al.* 2020) and the Brazilian Association for Animal Experimentation (COBEA) standards. These studies were approved by the Ethics Committee on Animal Research of the State University of Maringá (protocol number 8620070222/2022).

Experimental design

Handling and distributing the animals in the experiments

For the present study, 20 male Wistar rats (*Rattus norvegicus*) aged 80 days and 60 female rats aged 70 days were used at a ratio of 3 females:1 male. The animals were housed in separate cages and acclimated for a period of five days. After the five-day acclimation period, the males were introduced to the females for breeding. Pregnant

females were transferred to individual cages (n=6 dams/group). All animals were maintained under light-controlled conditions with a 12-h light–dark cycle (07:00 AM–7:00 PM) and temperature (22.0°C ± 2°C), with *ad libitum* access to standard chow and water. At birth, the puppies size was adjusted to eight pups (50% female and 50% male), and the dams were fed either a normal protein diet (NP; 20.5% protein (Nuvital; Curitiba/PR, Brazil, n= 6 dams, 3 weeks) or a low-protein diet (LP; 4% protein; n=6 dams per group, 2 weeks), followed by standard chow in the third week as previously described(Malta *et al.* 2016; Martins *et al.* 2018b). At weaning (at the 21st postnatal day (PN)21), the female offspring were previously evaluated by our research group (VARGAS *et al.* 2024); therefore, male offspring of the NP and LP groups were separated and housed (4 rats/cage), and they were fed standard chow for the experimental assays. After a 12-hour overnight fast, the NP and LP rodents were weighed. They were then euthanized at PN35, PN45, PN55 (peripubertal) and PN90 (adulthood). The experimental design is shown in **Figure 1.**

Age and weight at preputial separation

After weaning, the offspring NP or LP were monitored daily by determining the timing of puberty, assessing preputial membrane separation and externalization from the penile glands (Korenbrot *et al.* 1977c). This method was carried out from the end of the PN30 juvenile period, continuously once a day throughout the period preceding preputial opening, by gentle manipulation of the reproductive organ. On the day of puberty onset, the body weights of all the animals in the NP 55 and NP 90 groups, as well as those in the LP 55 and LP 90 groups, were recorded.

Murinometric assessment

At PN35, PN45, PN 55 and PND 90, after an overnight fast, a batch of rats from all groups (n = 18 from 5 litters for NP35, n=18 from 5 litters for LP35; n = 19 from 5 litters for NP45, n= 18 from 5 litters for LP45; n = 16 from 5 litters for NP55, n = 16 from 5 litters for LP55; n = 19 from 4 litters for NP90, n = 20 from 5 litters for the LP90 group) were weighed and euthanized via quick decapitation under deep anesthesia with sodium thiopental (45 mg/kg BW, i.p., Thiopentax, Cristália, Itapira, São Paulo, Brazil). The body weight gain was calculated as the difference between the final and baseline weights (BW final minus - BW initial) for the periods between PN25 and PN35, PN35 and PN45, PN45 and PN55, and PN55 and PN90.

The organs and tissues were collected from all the animals (n= 16–20 male offspring). To prevent biodegradation, the whole hypothalamus was not weighed during the harvesting process. The testes were harvested, weighed, frozen in liquid nitrogen, and stored at -80°C until subsequent analyses. To prevent biodegradation, the whole hypothalamus was not weighed during the harvesting process. The testis was weighed in grams (g), absolute values (g) were calculated and subsequently transformed to relative weights as grams per 100 grams of body weight (g 100 g of BW) at PN35, PN45, PN55, and PN90 days. The retroperitoneal, periepididymal and mesenteric adipose tissues were excised and subsequently weighed. The adiposity index was calculated in accordance with a previously established methodology and was calculated as follows: adiposity index = [(retroperitoneal (g) + periepidydymal (g) + mesenteric (g))/body weight] * 100 (Leopoldo *et al.* 2016).

Assays for measurement of testosterone

Blood was collected from the trunks of the plants after decapitation between 08:00 a.m. and 09:00 a.m. The samples were subsequently centrifuged at 2400 ×g for 20 minutes

(4°C), after which the plasma was separated and stored at −20°C until the assays were conducted. Testosterone levels were determined via a competitive enzyme-linked immunosorbent assay (ELISA) kit (Elabscience®, Houston, Texas, USA) in accordance with the manufacturer's instructions. The intraassay and interass coefficients of variation were 5.36% and 4.74%, respectively, according to the manufacturer's specifications. A total of 10 animals from 5 different litters were used per group.

Total RNA purification and relative gene expression by reverse transcription followed by real-time quantitative PCR (RT–qPCR)

The whole hypothalamus and testes were pulverized in liquid nitrogen via gral and pistil, transferred to 1.5 ml microtubes and stored at -80°C. Total RNA was extracted via the guanidine-phenol–chloroform method (Chomczynski & Sacchi 1987)with TRIzol reagent (Life Technologies, Carlsbad, USA) according to the manufacturer´s instructions. The total RNA concentration was estimated by the optical density (OD) of the solution via nanospectrometry (KASVI model k23-002). The absorbance was measured at 260 nm, and the degree of purity was determined by the A260/280 nm ratio. RNA integrity was analyzed by electrophoresis on a 1.2% agarose gel in TBE buffer. Two-half micrograms of total RNA was reverse transcribed with a GoScript reverse transcription system kit (Promega, Madison, USA). Real-time PCR from the product of reverse transcription (RT–qPCR) was performed via the PowerUp SYBR Green Master Mix Kit (Life Technologies, Carlsbad, CA, USA), and the amplification conditions for the genes were performed with the resources Applied Biosystems StepOnePlusTM real-time PCR system (Applied Biosystems, Singapore) according to the manufacturer´s instructions. The cycle sequence was as follows: 50°C/2 min, 95°C/2 min, and 40 cycles of 95°C/15 s

and 60°C/30 s. At the end of the reaction, a dissociation curve was generated to confirm the specificity of the reaction. The average values of the cycle threshold (Ct) were automatically determined by StepOneTM Software v2.3 (Applied Biosystems). Quantification was performed via the $2^{-\Delta\Delta Ct}$ method via relative quantification analysis, as previously described (Livak & Schmittgen 2001b). The ribosomal protein L19 (*Rpl19*) gene was utilized as a reference gene. The primer sequences and GenBank accession numbers of genes for each tissue are shown in **Table 1**. Batches of samples (n = 6–8 animals per group from 6 different litters) from all the groups were analyzed.

Statistical analysis

The results are presented as the mean ± standard error of the mean (SEM) and were subsequently subjected to a normality test, which was performed via the D'Agostino Pearson test. The statistical significance of the data was evaluated via Student's t tests or two-way ANOVA, followed by the Bonferroni *post hoc correction*. A statistically significant difference was indicated by a value of $p < 0.05$. This study considers the effects of maternal protein-caloric restriction (D), age (A), and the interaction between these two factors (I). The tests were carried out via GraphPad Prism version 8.0 for Windows (GraphPad Software, Inc., San Diego, CA, USA).

Results

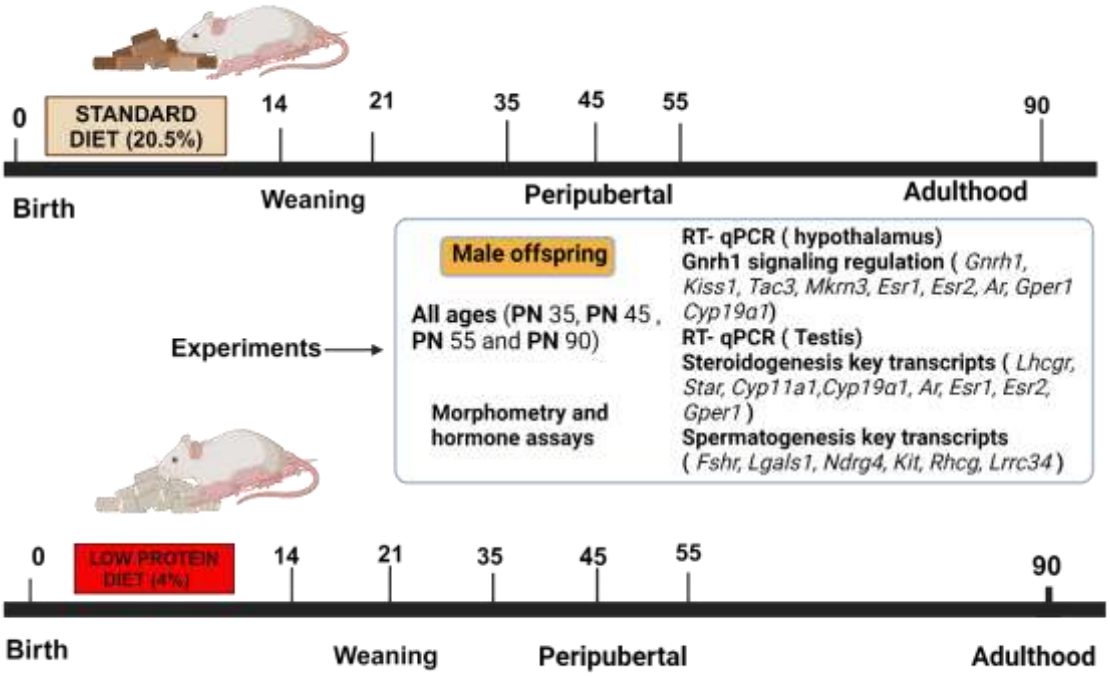

Figure 1: This figure shows two factors: **diet** (standard diet with 20.5% protein [NP] and low-protein diet with 4% protein [LP]) and age (postnatal days [PN] 35, 45, 55, and 90). A litter adjustment of 8 pups per dam was made. The critical time windows include a low-protein diet (LP) during the first 2 weeks of breastfeeding (PN 0--14), **weaning** (PN 21), **juvenile** (PN 21--35), **peripubertal** (PN 35--55), and **adulthood** (PN 90).

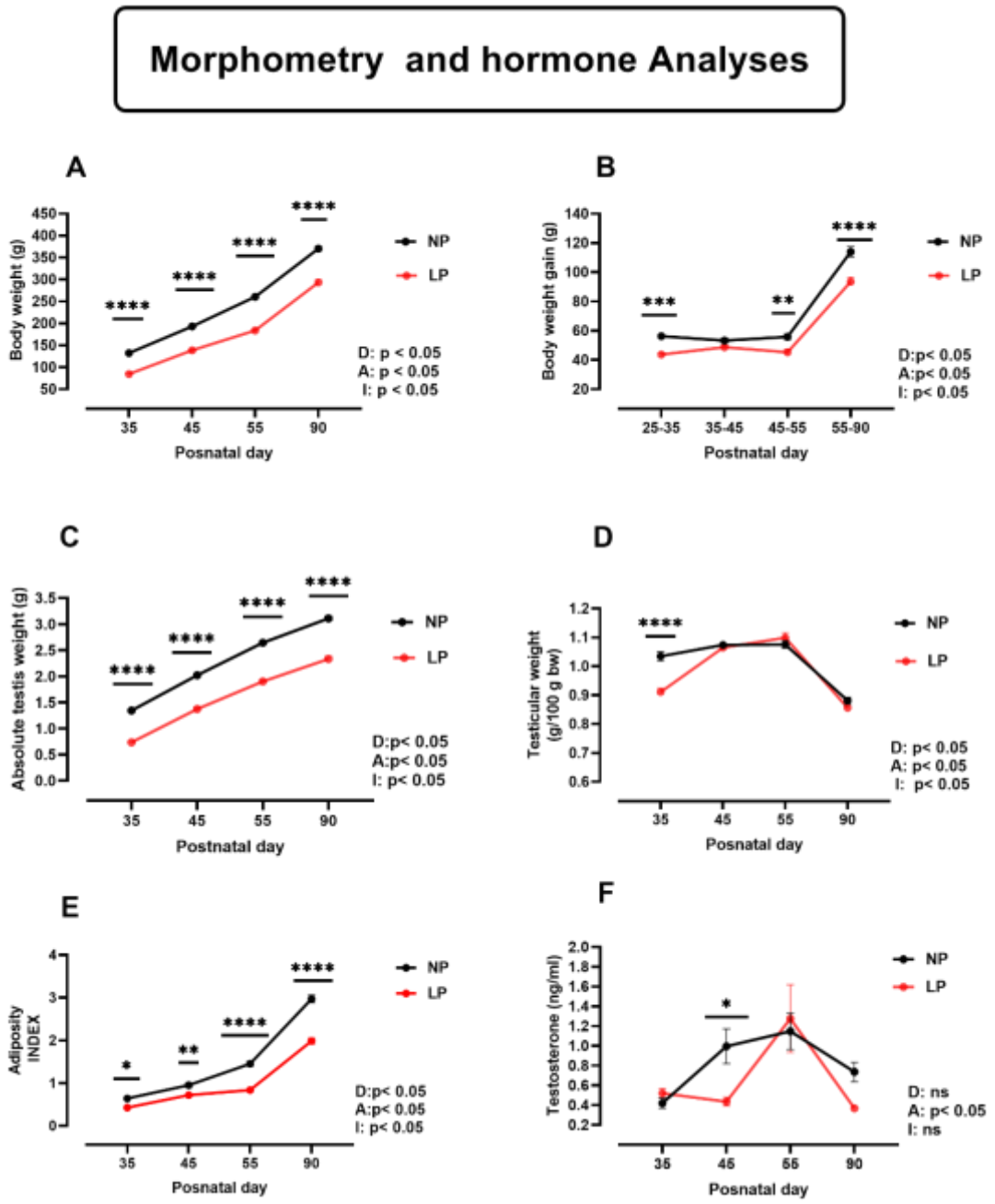

Figure 2: Consequences of maternal malnutrition on morphometric and testosterone analyses of offspring from the peripubertal window to adulthood. Final BW (A) BW gain (B) Absolute testis weight (C) Relative testicular weight (D) Adiposity INDEX (E) and testosterone hormone concentration (F). The data are expressed as the mean ± standard error of the mean (SEM). The animals for morphometry (n = 16–20 per group, from four to six different litters) and (n = 8–10) per group, from four to six different litters) *were used* for testosterone concentration analysis. Significant differences (two-way ANOVA) were followed by the Bonferroni *post hoc* correction. Consider **D**, diet factor; **A**, age factor; and **I**, interaction between factors. The results were considered significant when $p < 0.05$. Abbreviations: **NP**, normal protein diet; **LP**, low-protein diet.

Figure 2 (A-F) illustrates the effects of the LP diet on body weight, body weight gain, testicular weight, adiposity, and serum testosterone levels in male offspring from the peripubertal window to adulthood. Both the LP diet and offspring age reduced body weight ($pI < 0.05$, Fig. 2, two-way ANOVA), body weight gain ($pI < 0.05$, Fig. 2, two-way ANOVA), absolute testicular weight ($pI< 0.05$, Fig. 2, two-way ANOVA), and relative testicular weight ($pI < 0.05$, Fig. 2, two-way ANOVA). LP offspring presented lower body weights on PN days 35 (Fig. 2A; $p < 0.05$), 45 (Fig. 2A; $p < 0.05$), 55 (Fig. 2A; $p < 0.05$), and 90 (Fig. 2A; $p < 0.05$) and lower body weight gains on PN 35 (Fig. 2B; $p < 0.05$), 45 (Fig. 2B; $p < 0.05$), 55 (Fig. 2B; $p < 0.05$), and 90 (Fig. 2B; $p < 0.05$). The absolute testicular weight was reduced on PN 35 (Fig. 2C; $p < 0.05$), 45 (Fig. 2C; $p < 0.05$), 55 (Fig. 2C; $p < 0.05$), and 90 (Fig. 2C; $p < 0.05$), whereas the relative testicular weight was reduced only on PN 35 (Fig. 2D; $p < 0.05$), with no significant differences on PN 45 (Fig. 2D; ns), 55 (Fig. 2D; ns), and 90 (Fig. 2D; ns). Visceral adiposity was lower on PN 35 (Fig. 2E; $p < 0.05$), 45 (Fig. 2E; $p < 0.05$), 55 (Fig. 2E; $p < 0.05$), and 90 (Fig. 2E; $p < 0.05$). Only age affected serum testosterone levels ($pA < 0.05$, Fig. 2, two-way ANOVA), with a reduction observed only on PN 45 (Fig. 2F; $p < 0.05$), and no significant differences were detected on PN 35 (Fig. 2F; ns), 55 (Fig. 2F; ns), or 90 (Fig. 2F; ns).

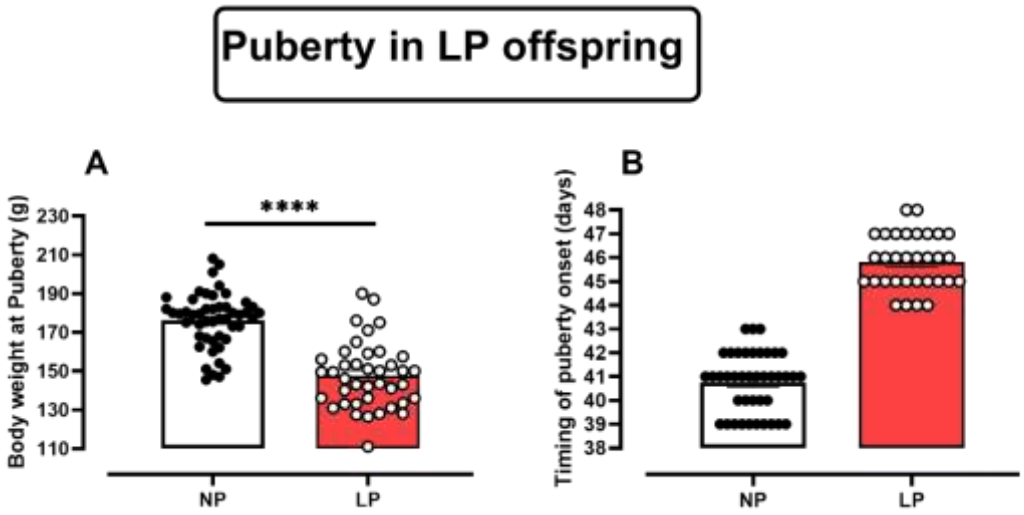

Figure 3: Effects of the maternal LP diet on body weight (A) and preputial separation (B) during the peripubertal window. The values are expressed as the means ± S.E.M.s. Data were analyzed via Student's t test for comparisons between experimental groups (n = 24–32 of eight different litters). Significance was set at $p < 0.05$. Abbreviations: **NP:** normal protein offspring; **LP:** low protein offspring.

Figure 3 shows that compared with the NP offspring, the LP offspring presented a lower BW (- 17.5%; Fig. 3A; $p< 0.05$) and a delayed onset of puberty (Fig. 3B; $p< 0.05$). The median and interquartile range of age at puberty were 45 [45, 48].

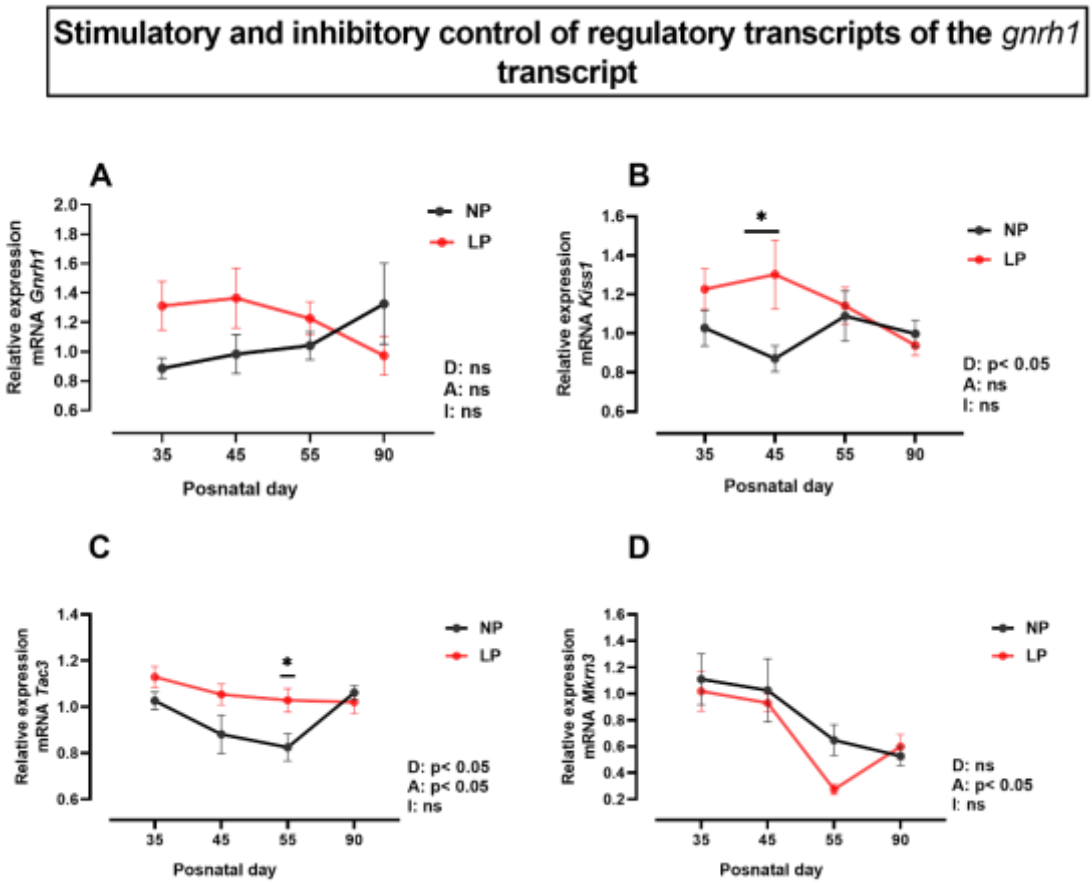

Figure 4: Stimulatory and inhibitory signaling of the hypothalamic *Gnrh1* transcript: (A) *Gnrh1* mRNA, (B) *Kiss1* mRNA (C), *Tac3* mRNA (D), *and Mkrn3* mRNA. The data are presented as the means ± SEMs of 7–8 rats from four to six different litters in each group. Asterisks represent significant differences according to two-way ANOVA followed by the Bonferroni correction. Consider **D,** diet factor; **A,** age factor; and **I,** interaction between

factors. *P*<0.05 indicated statistical significance. Abbreviations: **NP:** offspring of dams fed a normal-protein diet; **LP:** offspring of dams fed a low-protein diet.

Figure 4 shows the stimulators and inhibitors of the *Gnrh1* transcript in the hypothalamus. No factor affected the expression of the *Gnrh1* transcript, which remained unchanged compared with that in NP animals (Fig. 4A, $p < 0.05$). Dietary factors positively affected the *Kiss1* transcript level (Fig. 4B; $pD < 0.05$, two-way ANOVA), whereas both diet and age affected the *Tac3* transcript level (Fig. 4C; $pD < 0.05$; $pA < 0.05$, two-way ANOVA). LP animals presented increased relative expression of *Kiss1* at PN 45 (Fig. 4B; $p < 0.05$) and *Tac3* at PN 55 (Fig. 4C; $p < 0.05$). In contrast, there was an age-related reduction in Mkrn3 transcript expression (Fig. 4D; $pA < 0.05$, two-way ANOVA), although this change remained unaltered compared with that in the NP offspring.

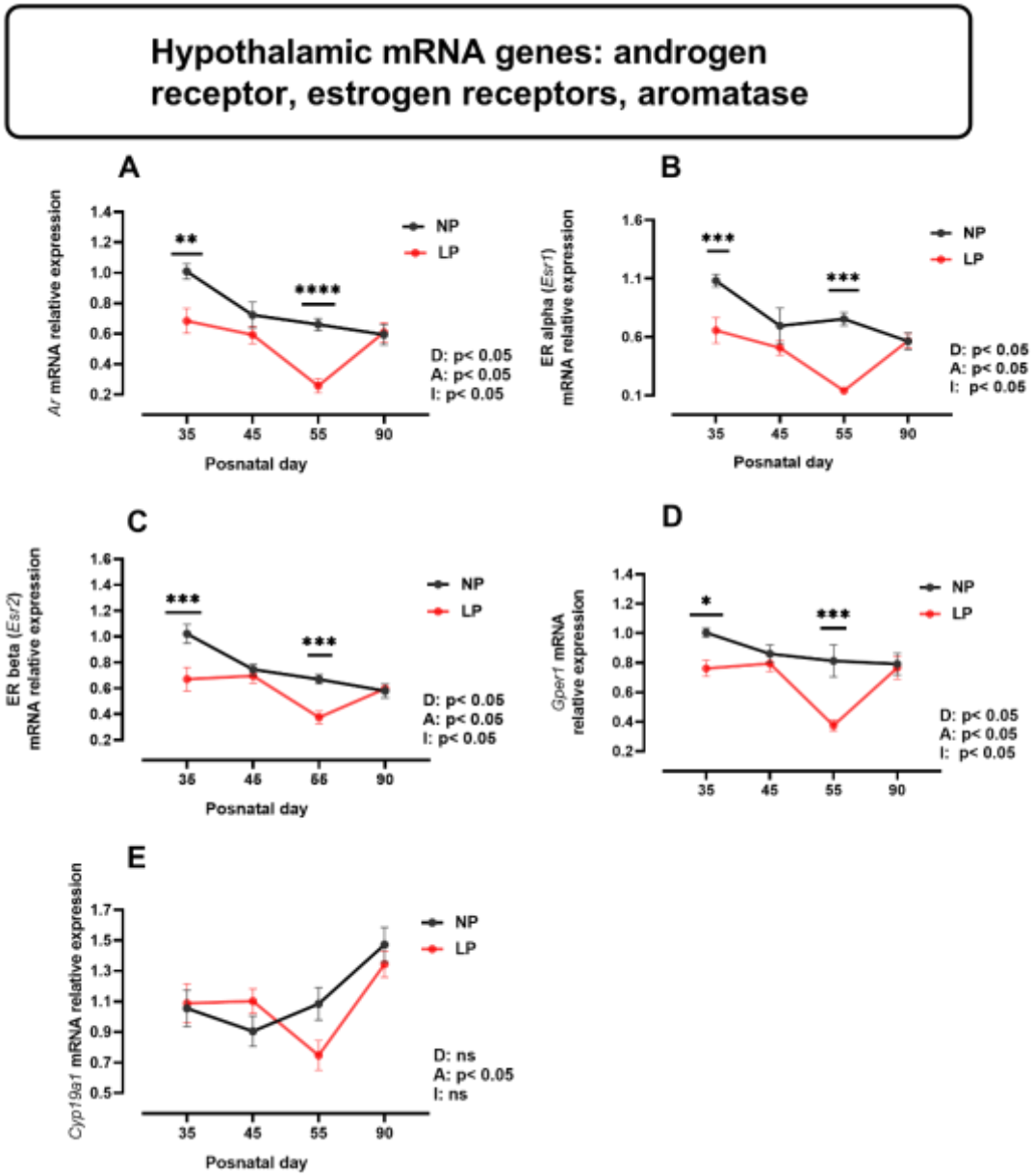

Figure 5: Role of androgen and estrogen receptors in hypothalamic tissue: (A) *Ar* mRNA, (B) *Esr1* mRNA (C), *Esr2* mRNA (D), *Gper1* mRNA (E) and Cyp19α1 mRNA. The data are presented as the means ± SEMs of 7–8 rats from four to six different litters in each group. Asterisks represent significant differences according to two-way ANOVA followed by the Bonferroni correction. Consider **D,** diet factor; **A,** age factor; and **I,** interaction between factors. *P*<0.05 indicated statistical significance. Abbreviations: **NP:** offspring of dams fed a normal-protein diet; **LP:** offspring of dams fed a low-protein diet.

LP offspring presented changes in receptors that regulate the HPT axis. A significant interaction effect between diet and age was observed, resulting in a reduction in the mRNA levels of *Ar* (pI < 0.05; Fig. 5A; two-way ANOVA), *Esr1* (pI < 0.05; Fig.

5B; two-way ANOVA), *Esr2* (pI < 0.05; Fig. 5C; two-way ANOVA), and *Gper1* transcripts (pI < 0.05; Fig. 5D; two-way ANOVA) in LP animals. Moreover, age influenced the transcription of *Cyp19α1* (Fig. 5E; pA < 0.05, two-way ANOVA), with elevated mRNA levels observed in adulthood. The relative expression of *Ar* was reduced at PN 35 (Fig. 5A; $p < 0.05$) and PN 55 (Fig. 5A; $p < 0.05$). Similarly, *Esr1* expression was lower at PN 35 (Fig. 5B; $p < 0.05$) and PN 55 (Fig. 5B; $p < 0.05$), as was *Esr2* expression at PN 35 (Fig. 5C; $p < 0.05$) and PN 55 (Fig. 5C; $p < 0.05$). *Gper1* transcript levels also decreased at PN 35 (Fig. 5D; $p < 0.05$) and PN 55 (Fig. 5D; $p < 0.05$). In contrast, the relative expression of *Cyp19α1* (Fig. 5E) did not significantly differ between LP and NP offspring throughout development.

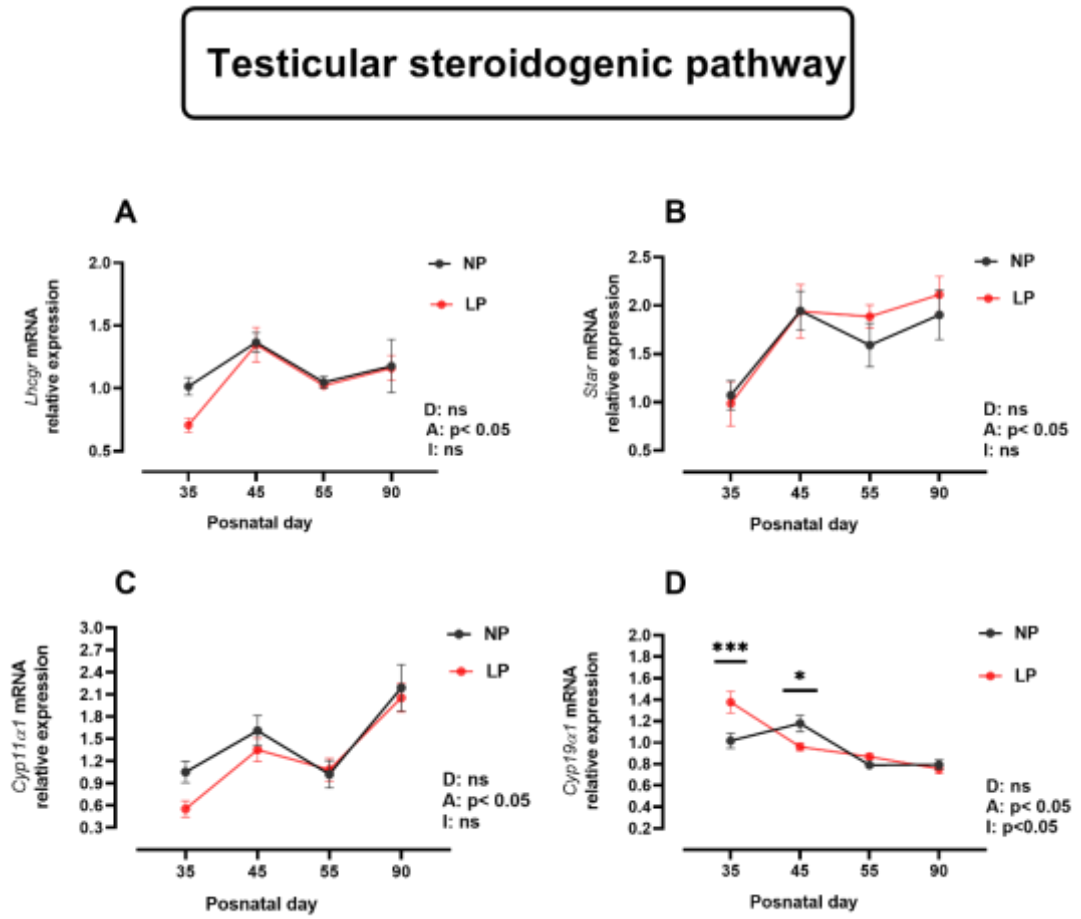

Figure 6: Relative expression of transcripts involved in testicular steroid biosynthesis: *Lhcgr* (A), *Star* (B), *Cyp11a1* (C) and *Cyp19a1* (D) mRNAs. The data are presented as the means ± SEMs of 7–8 rats from four to six different litters in each group. Asterisks represent significant differences according to two-way ANOVA followed by the Bonferroni correction. Consider **D,** diet factor; **A,** age factor; and **I,** interaction between factors. P<0.05 indicated statistical significance. Abbreviations: **NP:** offspring of dams fed a normal-protein diet; **LP:** offspring of dams fed a low-protein diet.

Figure 6 shows the expression of genes involved in testicular steroidogenesis from peripuberty to adulthood. Age was observed to affect the relative expression of *Lhcgr* (Fig. 6A; pA < 0.05, two-way ANOVA), *Star* (Fig. 6B; pA < 0.05, two-way ANOVA), and *Cyp11α1* (Fig. 6C; pA < 0.05, two-way ANOVA) mRNAs. However, no significant interaction between these factors was detected. Furthermore, no significant differences in *Lhcgr*, *Star*, or *Cyp11α1* expression were observed among the experimental groups at any age. In contrast, the expression of *Cyp19α1* was significantly affected by the LP diet and

age (Fig. 6D; pI < 0.05; two-way ANOVA). Specifically, the *Cyp19α1* transcript level in the testes was significantly greater at PN 35 ($p < 0.05$) and lower at PN 45 ($p < 0.05$) than in the NP offspring.

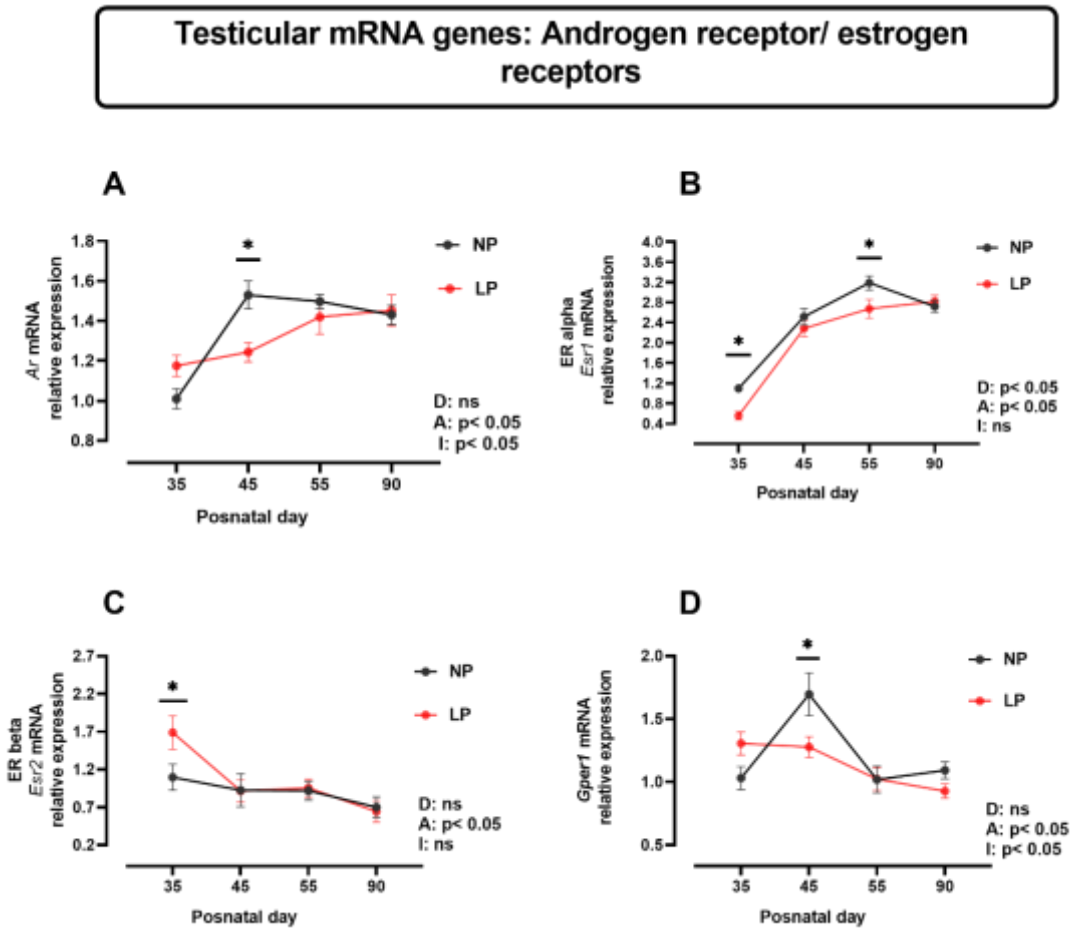

Figure 7: Role of androgen and estrogen receptors in the testis: mRNA *Ar* (A), *Esr1* (B), *Esr2* (C), and *Gper* (D). The data are presented as the means ± SEMs of 7–8 rats from four to six different litters in each group. Asterisks represent significant differences according to two-way ANOVA followed by the Bonferroni correction. Consider **D,** diet factor; **A,** age factor; **I,** interaction between factors. P<0.05 indicated statistical significance. Abbreviations: **NP:** offspring of dams fed a normal-protein diet; **LP:** offspring of dams fed a low-protein diet.

Figure 7 shows the mRNA transcripts of the androgen receptor and estrogen receptor in the testicular organ from the peripubertal phase to adulthood. The expression of *Ar* mRNA in the testes was affected by the interaction between factors (Fig. 7A; $pI < 0.05$; two-way ANOVA), whereas the expression of *Esr1* transcripts was altered by both the LP diet (Fig. 7B; $pD < 0.05$; two-way ANOVA) and age (Fig. 7B; $pA < 0.05$; two-way ANOVA). *Esr2* expression was influenced by age (Fig. 7C; $pA < 0.05$, two-way ANOVA). With respect to *Gper1*, there was an interaction effect (Fig. 7D; $pI < 0.05$; two-way ANOVA). LP offspring presented a significant reduction in the mRNA levels of the *Ar* receptor at PN 45 (Fig. 7A; $p < 0.05$). Additionally, *Esr1* expression was reduced at both PN 35 (Fig. 7B; $p < 0.05$) and PN 55 (Fig. 7B; $p < 0.05$). In contrast, *Esr2* expression was significantly greater in PN 35 offspring (Fig. 7C; $p < 0.05$) than in NP offspring. The relative expression of *Gper1* in LP offspring was significantly lower at PN 45 (Fig. 7D; $p < 0.05$) than in NP offspring.

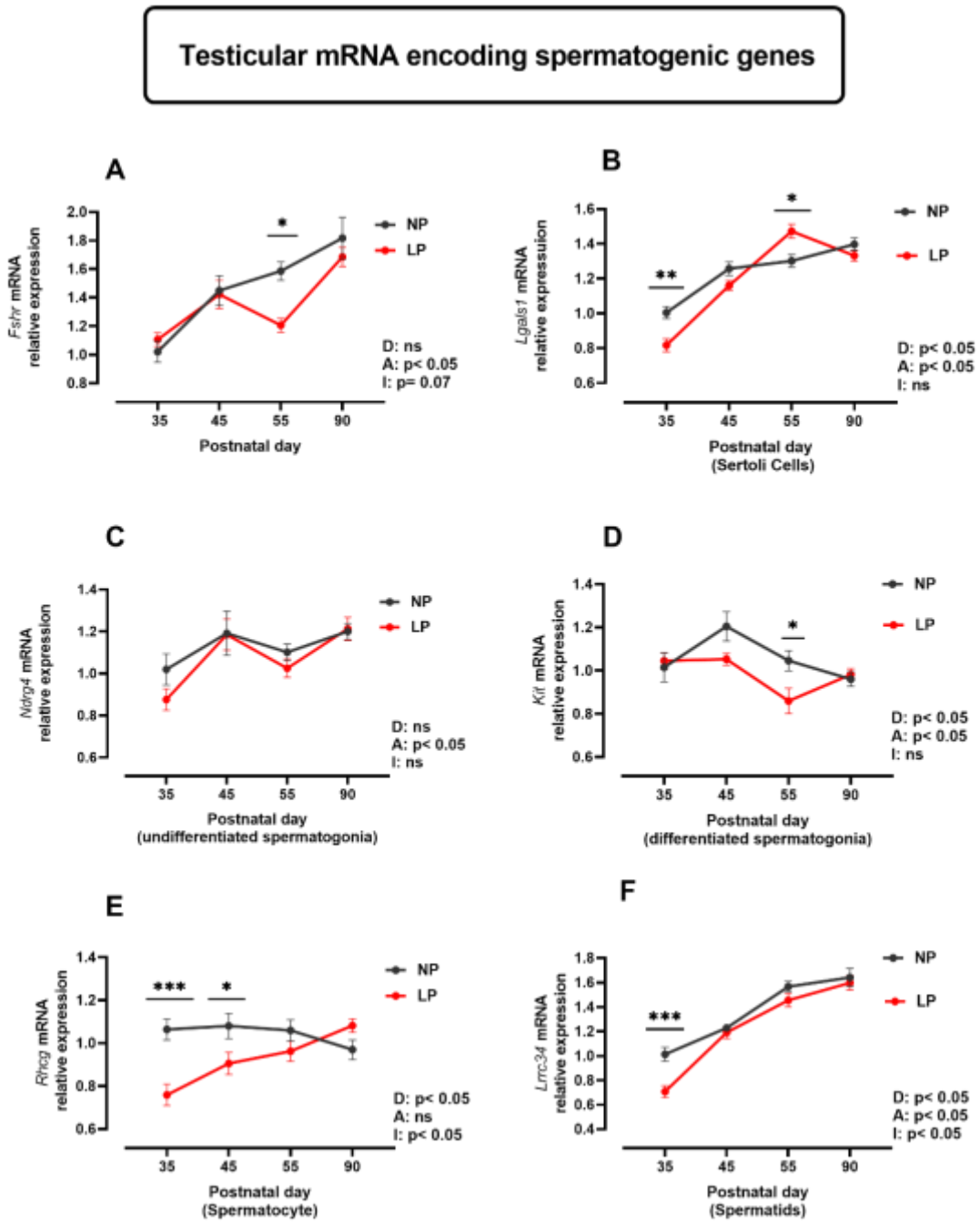

Figure 8: Effects of the LP diet and age on the mRNA levels of spermatogenic genes from puberty to adulthood. : *Fshr* (A) mRNA *Lgals1* (B*)* mRNA *Ndrg4* (C) mRNA *Kit* (D) mRNA *Rhcg* (E) mRNA *Lrrc34* (F). The data are presented as the means ± SEMs of 7–8 rats from four to six different litters in each group. Asterisks represent significant differences according to two-way ANOVA followed by the Bonferroni correction. Consider **D,** diet factor; **A,** age factor; **and I,** interaction between factors. P<0.05 indicated statistical significance. Abbreviations: **NP:** offspring of dams fed a normal-protein diet; **LP:** offspring of dams fed a low-protein diet.

Figure 8 shows how the expression of spermatogenic genes changes from puberty to adulthood. Only age affected the levels of *Fshr* mRNA (Fig. 8A; $pA < 0.05$, two-way ANOVA), although there was a trend toward interaction between factors (Fig. 8B; $p = 0.07$; two-way ANOVA). LP offspring presented lower *Fshr* levels than did NP offspring at PND 55 (Fig. 8, $p < 0.05$). There was also a main effect of diet (Fig. 8A; $pD < 0.05$, two-way ANOVA) and age (Fig. 8A; $pA < 0.05$, two-way ANOVA) on the levels of mRNAs encoding *Lgals1*. Post hoc comparisons revealed that the LP offspring presented lower and greater *Lgals1* expression than did the NP offspring at PN 35 (Fig. 8B, $p<0.05$) and PN 55 (Fig. 8B, $p<0.05$), respectively. There was an effect of age on the mRNA levels of *Ndrg4* (Fig. 8C; $pA < 0.05$, two-way ANOVA). Moreover, no differences were detected between the LP and NP offspring. Furthermore, both dietary factors (Fig. 8D; $pD < 0.05$, two-way ANOVA) and age (Fig. 8D; $pA < 0.05$, two-way ANOVA) significantly affected the levels of mRNAs encoding *Kit*. Specifically, LP offspring presented a reduction in *Kit* expression at PN 55 (Fig. 8B, $p<0.05$). A significant interaction effect on the expression of *Rhcg* (Fig. 8E; $pI < 0.05$, two-way ANOVA) and *Lrrc34* (Fig. 8F; $pI < 0.05$, two-way ANOVA) was detected. The *Rhcg* transcript presented significantly lower mRNA levels at PN 35 (Fig. 8E; $p < 0.001$) and PN 45 (Fig. 8E; $p < 0.05$). In contrast, *Lrrc34* mRNA levels were lower in LP offspring than in NP offspring only at PN 35 (Fig. 8F; $p < 0.05$).

Discussion

This study shows, for the first time, that a low-protein diet during the first two weeks of breastfeeding led to a delay in the timing of puberty onset in male offspring with the LP phenotype during peripubertal windows. Furthermore, the delay was linked to a

reduction in testosterone levels, which was attributed to an increase in testicular aromatization and elevated *Kiss1* mRNA expression in the hypothalamus. These effects may be associated with alterations in the mRNA expression patterns of genes that regulate the hypothalamic–pituitary–testicular (HPT) axis, particularly those that are intrinsically linked to sex steroid biosynthesis and sperm cell subtypes. Furthermore, the delay in the expression of transcripts involved in the development of germ cells that eventually form sperm may contribute to the reprogramming of the HPT axis from the peripubertal window into adulthood.

The LP group exhibited a delay in the initiation of puberty, with an average time difference of approximately five days, and exhibited a body weight that was approximately 30 grams less than that of the control group, which entered puberty at a weight of approximately 180 grams. The mean age at which puberty commenced was 41 days for the NP offspring and 46 days for the LP offspring, and testosterone was lower during this stage. In the prepubertal window, protein is a key macronutrient that determines the timing of puberty (Günther *et al.* 2010); furthermore, alternate-day fasting reduces body weight and delays puberty in normal-weight female mice and those fed a high-calorie diet during prepuberty, with effects varying on the basis of the duration of dietary restriction and the offspring phenotype (Ullah *et al.* 2024). Thus, nutritional insufficiency during vulnerable stages in rats impairs body growth and contributes to adverse reproductive health outcomes, such as oligospermia, and behavioral changes that may culminate in impaired reproductive competence (Zambrano *et al.* 2005b; Ohishi *et al.* 2012). Zambrano *et al*. (2005) reported that malnourished offspring exhibited a reduced size at puberty; however, this reduction was not accompanied by a delay in puberty when the dams consumed a diet containing 10% protein (Zambrano *et al.* 2005b). The protein content of the maternal diet in the cohort of malnourished dams was 4%,

which resulted in a body weight that was 55% lower at the time of weaning. This provides further evidence in support of the hypothesis that the timing of puberty is influenced by growth during infancy and the juvenile period and that a lack of protein can alter body composition during the final sexual differentiation process.

Furthermore, a reduction in testicular weight was observed at PN35, accompanied by a catch-up in testicular growth until the time of puberty. A delayed pubertal growth spurt in LP animals results in the individual attaining a height that is closer to the target height. Although body weight remained lower during the onset of puberty, the relative testicular weight at PN45 remained similar in the NP offspring until PN90. The offspring of LPs presented a lower body weight throughout the course of their ontogenetic development. Furthermore, the ratio of body mass to testicular size peaks during the period of peripuberty, which is characterized by a pronounced increase in fat deposits and a consistent production of sex hormones and spermatogenesis differentiation. Restricting food intake during the juvenile and pubertal phases in female rodents resulted in lower leptin levels, which is linked to less fat and a delay in the start of puberty (Zeinoaldini *et al.* 2006), demonstrating that leptin plays a role in energy sufficiency during puberty.

While circulating estrogen levels were not assessed in the offspring of malnourished dams, LP offspring clearly presented decreased expression of all estrogen and androgen receptor transcripts at both PN35 and PN55 during the peripubertal window, with no changes observed in these transcripts during puberty (PN 45) or adult life. The evidence of direct participation of estradiol in the control of pulsatile GnRH release is the time- and dose-dependent activation of both isoforms of receptors (ERα and ERβ)(Hu *et al.* 2008b), given that both transcripts are reduced at the beginning and end of puberty, and given their opposing effects on gonadotropin-releasing hormone (GnRH)

secretion, the overall secretion of GnRH would likely remain unchanged. This could explain the unaltered relative expression of *Gnrh1* mRNA, even though we did not measure the pulsatile secretion pattern of this hormone or the levels of pituitary hormones.

Furthermore, the mRNA transcripts of *Esr1* and *Esr2* in the CNS modulate energy balance, inducing hypophagia and increasing energy expenditure (López & Tena-Sempere 2015). LP offspring are hyperphagic and show lower expression of *Esr1* and *Esr2* throughout the hypothalamic structure. LP rats are hyperphagic and have lower body weight gain from weaning until the transition of adolescence (Bell 2018; Martins *et al.* 2018b). Furthermore, *Esr1* knockout is associated with both obesity and metabolic disorders (Frank *et al.* 2014). In addition, blocking estrogen receptors with injections of their antagonists from PN 5 to PN 13 reduced the body weight of adult male rats (Carrillo *et al.* 2020), suggesting an estrogen-mediated effect on early phenotype programming that regulates the energy homeostasis of ARC neurons. Furthermore, after puberty, these animals still have lower body weight and adiposity, suggesting that this reduction in transcription is likely to protect against obesity, especially during peripuberty, a time of increased body weight gain and high energy expenditure in LP animals. The lower expression of these receptors at the beginning and end of peripuberty indicates an increase in basal metabolism due to increased energy demands, which explains the lean phenotype and delayed puberty. These conditions are essential for stimulating the rapid growth of organs and tissues, brain development, and sexual maturation, particularly during the transition from PN35 to PN45. Our findings revealed no differences in aromatase mRNA expression in the hypothalamus. However, aromatase expression increases during the transition from peripuberty to adulthood. A decrease in aromatase during puberty in the CNS decreases sensitivity to steroid negative feedback with the onset of puberty (Lephart & Ojeda 1990). The administration of estradiol to adult male rats was observed to enhance

their copulatory behavior. Conversely, the use of aromatase inhibitors was found to abolish this effect (Roselli & Resko 1993). Maternal malnutrition does not affect the transcription of the aromatase enzyme during critical development after peripuberty.

The present study revealed that the modulation of hypothalamic brain circuits during peripuberty, particularly with respect to the molecular regulation of the HPT axis, is affected. The data revealed that neurons expressing transcripts that positively regulate the onset of puberty, as well as those involved in the regulation of the HPT axis during the peripubertal window, exhibited differential expression patterns in both the NP and LP groups. Kisspeptin neurons serve as an interface between energy balance and reproductive functions, directly regulating the release of GnRH (Rønnekleiv & Kelly 2013a; Harter *et al.* 2018; Xie *et al.* 2022). The knockout of both insulin and leptin receptors in the *kiss1* neurons of genetically modified mice delays the timing of puberty (Qiu *et al.* 2015). LP offspring show increased insulin peripheric sensitivity in adulthood and dysglycemia during fasting (Martins *et al.* 2018a). *Kiss1* during puberty may be modulated by insulin sensitivity, which is associated with greater body weight gain and influenced by the metabolic signals produced by the LP phenotype during the prepuberty window. In both in vivo and in vitro beta cell experiments, kisspeptin injections stimulated glucose-induced insulin secretion without affecting appetite or food intake in healthy males (Izzi-Engbeaya *et al.* 2018). Improved insulin sensitivity during puberty may positively regulate kisspeptin expression, thereby linking peripheral metabolism and reproductive competence in LP offspring. Maternal malnourishment did not impact the expression of the hypothalamic *Gnrh1* transcript throughout pubertal development or young adulthood.

The mean preputial separation occurred near an increase in *Kiss1* mRNA in the hypothalamus at PN45, which was positively correlated with hypotestosteronemia and

may have contributed to the reduction in negative feedback in hypothalamic neurons expressing Kiss1 mRNA, even without altering *Gnrh1* expression. Interestingly, maternal malnutrition did not affect Mrnk3 gene expression; moreover, a decrease in *Mkrn3* expression occurred with the progression of peripuberty, indicating that the absence of this inhibitory factor may be involved in the increased expression of *Kiss1* at PN 45 and *Tac3* at PN 55. *Mkrn3* expression is known to decrease in rodents during pubertal progression, and females are more affected than males when this gene is polymorphic (Maione *et al.* 2020). Although the reduced expression of *Kiss1* is associated with fasting conditions, the administration of exogenous kisspeptin restores the timing of puberty onset (Castellano *et al.* 2005). Our data revealed an increase in *Kiss1* transcription at puberty, which may be attributed to improvements in both energy and nutritional status after weaning, which are necessary for reproductive competence. Thus, our data show that maternal malnutrition during lactation does not impair *Gnrh1* mRNA gene transcription involved in GnRH synthesis; however, it alters upstream modulators of this complex neuronal network, such as kisspeptin and KNDy, which are expressed in *Tac3* neurons during peripuberty. These findings suggest that dynorphin (Dyn) levels are reduced in LP animals at the conclusion of the pubertal phase.

The major increase in *LHcgr* receptors in response to gonadotropins occurs between postnatal day 15 and PN38, occurring concurrently with testicular growth during prepuberty; these alterations are associated with increased Leydig cell numbers and testicular sensitivity to testosterone. (KETELSLEGERS *et al.* 1978; Stoker *et al.* 2000; Bhattacharya *et al.* 2019). The key enzymes involved in steroidogenesis typically increase from the peripubertal period to adulthood, which is essential for maintaining gonadal sex steroid synthesis. Maternal undernourishment did not affect the expression of *Lhcgr*, *Star*, or *Cyp11α1* mRNA transcripts in the testes of LP offspring. Testosterone is produced in

fat, muscle, and testes, where it binds with high affinity to the androgen receptor (Prevot 2015; Zirkin & Papadopoulos 2018c); the first increase occurs between PN25 and PN35. There is a production plateau between PN35 and PN55. This allows for a more accurate assessment of how maternal undernutrition affects the androgen concentration in offspring. The lower testosterone concentration observed in the lean phenotype is not due to a deficiency in cholesterol transport or to problems with the conversion of cholesterol to pregnenolone.

Testosterone serves as the substrate for the aromatase enzyme (Zirkin & Papadopoulos 2018c). Aromatase is present in the testes and various types of germ cells. Its expression is regulated by androgens, estrogens, glucocorticoids, and other substances. Aromatase plays a crucial role in testicular and sperm synthesis, where different factors interact to ensure the optimal production of hormones in the testicles (Lambard *et al.* 2005). LP offspring exhibit an increase in testicular aromatase mRNA transcription prior to the onset of puberty. However, this increase is followed by a decrease in aromatase enzyme mRNA near the onset of puberty. This reduction in testicular aromatization is accompanied by decreases in both the testosterone concentration and the *Ar* receptor mRNA, with levels returning to baseline by the end of peripuberty and into young adulthood. These findings indicate that the increased demand for testosterone during reproductive maturation may be associated with a reduction in aromatase enzyme transcripts in the testis. In men with hypogonadism, high aromatization is linked to more body fat, less muscle and an increase in bone density (Aguirre *et al.* 2015). Aromatase knockout mice exhibit an increase in adipose tissue without changes in food intake or caloric expenditure (Jones *et al.* 2000).

The oral administration of letrozole, an aromatase inhibitor nonsteroid, from infancy to adulthood in male rodents resulted in a reduction in food intake, diminished body growth, and a decrease in periosteal and endosteal circumference, which collectively contributed to bone fragility and delayed puberty (Pouliot *et al.* 2013). It remains uncertain whether the administration of aromatase inhibitors and dihydrotestosterone (a nonaromatizable androgen) affects the timing and progression of puberty in rats with a lean phenotype. The data indicate that the timing of puberty in these lean animals is unlikely to occur prematurely, given that they have sufficient nutrient levels throughout the juvenile period until sexual maturation. In rats, prolonged administration of anastrozole altered testicular weight in a dose-dependent manner, affected gonadotropins and impaired sexual behavior, although they remained fertile (Turner *et al.* 2000). The inhibition of aromatase with glyphosate during the perinatal window induces alterations in the socialization of adult rodents and affects sexual behavior, particularly in female rats (Ricci *et al.* 2022). Thus, aromatase inhibition in juvenile LP male offspring may provide an explanation for the absence of estrogens involved in hormonal signaling, influencing both the proper development of spermatogenesis and copulatory behavior during puberty (Schulster *et al.* 2016b). The changes in the pattern of aromatase expression, in the absence of prior alterations in gonadotropin signaling, indicate that gonadotropin signaling remains unimpaired in LP offspring. Estrogen replacement restored mating behavior and increased copulation success. The elevated expression of aromatase during prepuberty may indicate the physiological necessity of facilitating testicular growth, sustaining the spermatogenic cycle, and modulating secondary sexual characteristics. LP offspring may need more estrogen at PN35, which could affect testicular growth and maturation at the start of puberty. The increased demand for estrogen, followed by a decreased requirement as

sexual maturation progresses. A decrease in serum testosterone and aromatase expression occurs, particularly during the transition from prepuberty to puberty. These data indicate that reduced concentrations of testosterone and aromatase may be required to reduce intratesticular estrogen levels and restore testosterone from puberty through adulthood.

While the 17β-estradiol concentration was not quantified during ontogenetic development, a decrease in *Gper1* receptor transcription at puberty was detected. Furthermore, the increase in *Ar* expression in the LP groups occurred gradually, such that even with continuous growth, the levels remained below those of the NP animals. The mRNA level of *Gper1* transcripts was lower only at the pubertal peak in LP animals. In the NP offspring, the peak number of *Ar* and *Gper1* receptor transcripts occurred at PN45, which differs from the pattern observed in the LP offspring. The activation of the GPER-1 receptor in isolated Leydig cells from both humans and rodents has resulted in a reduction in testosterone synthesis of 20% to 30%, a process dependent on estrogen (Vaucher *et al.* 2014). Additionally, Gper plays a pivotal role in the rapid action of estrogen, which is essential for the function and maintenance of Sertoli cells, thereby regulating spermatogenesis (Lucas *et al.* 2010). In testicular biopsies, *Gper1* plays a pivotal role in spermatogenesis, regulating cell proliferation and apoptosis. Infertile men, there is a direct correlation between reduced protein levels and lower expression of *Gper1* transcripts, indicating that *Gper1* is intimately involved in testicular health and pathological conditions associated with infertility (Sandner *et al.* 2014).

Esr1 is expressed in Sertoli cells and spermatids in adult rodents, whereas *Esr2* is expressed in Leydig and Sertoli cells (Lucas *et al.* 2008). In contrast, the *Ar* receptor is predominantly expressed in spermatogonia (Berensztein *et al.* 2006). Moreover, *Esr1* expression levels are lower in LP offspring at the onset and end of the peripubertal

window. However, they demonstrated an exponential increase, comparable to that observed in NP offspring during adulthood. The Esr1 mRNA *level* decreased in vitro, and there were more apoptotic germ cells in the seminiferous tubules in testicular fragment cultures in response to ethinylestradiol (Nakamura *et al.* 2019). Rats in which the *Esr1* locus has been knocked out (KO) exhibit a reduction in testicular weight, a decrease in testosterone levels, and an inability to achieve fertility; thus, the *Esr1* gene mRNA is indispensable for the maintenance of fertility (Rumi *et al.* 2014). In contrast, LP rodents demonstrate greater *Esr2* expression exclusively during the prepubertal period (PN35), followed by a precipitous decline during puberty, which persists into young adulthood. In female rats, the *Esr2* locus KO is fertile and does not display any morphofunctional impairments in the male gonads (Rumi *et al.* 2017).

We also examined how the *Fshr* receptor and key genes that control sperm production are expressed in different types of germ cells. These findings help us understand whether poor nutrition during breastfeeding affects gene expression during puberty. We observed that age positively regulates the quantity of testicular *Fshr* receptor mRNAs. In LP offspring, these receptors are reduced by the time they finish puberty, when their testicles are the largest relative to their body weight. After this developmental stage, the expression of this receptor resembles that of NP offspring when they reach young adulthood. Testicular galectin-1 (*Lgals1*) is highly expressed in peritubular, Sertoli and germ cells (Lei *et al.* 2018). LP offspring presented lower *Lgals1* expression at PN35 of peripuberty. This may indicate that there were fewer Sertoli cells, a smaller area of seminiferous epithelium, or greater apoptosis of germ cells. LP offspring presented higher Lgals1 mRNA *levels* at PN55, which suggested that more Sertoli cells were present. Restoration of testosterone levels and a probable increase in inhibin B at the end of

puberty could reduce FSH release, which may lead to decreased *Fshr* mRNA expression (Stoker *et al.* 2000).

Maternal dietary problems during lactation did not affect the number of undifferentiated spermatogonia on the basal membrane of the seminiferous tubules in the offspring. However, protein-caloric restriction seems to affect some types of spermatogenic cells. The transcripts of *Kit, Rhcg*, and *Lrrc34* were reduced, indicating problems with the differentiation or count of these precursor cells of mature sperm. *Kit* expression decreased only at the end of puberty, suggesting that more cells died or that there were fewer mature sperm cells during early reproduction. The *Rhcg* transcript, which is specific to spermatocytes, was reduced from before puberty through puberty in this lean phenotype. *Lrrc34* was reduced exclusively during the prepubertal period, which coincides with delayed testicular growth. However, both groups demonstrated an exponential increase in the expression of this transcript, which is involved in the counting of spermatids, from puberty to adulthood. This finding indicates a significant increase in the number of these cells during the differentiation process.

Conclusion

In brief, exposure of dams to a low-protein caloric-restriction diet during two weeks of breastfeeding resulted in lean offspring phenotype rats characterized by low adiposity, growth retardation and difficulty in weight gain, which directly affected testicular development and alterations in the timing of puberty. This delay was not pathological but rather was due to an ontogenetic developmental delay. LP offspring presented with reduced body weight at puberty and delayed pubertal onset, suggesting that insulin sensitivity was due to increased hypothalamic *Kiss1* transcript expression, a key excitatory neural stimulus essential for Gnrh1 release, which remained unaltered. In

addition, the positive regulator *Tac3* was upregulated, whereas *Mkrn3* expression declined sharply after puberty, reflecting a reduction in the inhibitory signal present early in life.

Although key steroid biosynthetic enzymes were unaffected, aromatase transcript activity was elevated before puberty and decreased during puberty, which coincided with lower testosterone levels and decreased expression of androgen receptor transcription. Although estradiol levels were not measured, the decrease in aromatase activity likely reduced estradiol production, which may explain the decreased expression of the rapid-acting estrogen receptor *Gper1* during puberty, indicating a need to increase testosterone levels to maintain spermatogenic cycles and regulate somatic testis cells.

LP offspring presented alterations in the activity of both rapid and long estrogen and androgen receptors in the hypothalamus, with reduced expression before and after puberty. However, these receptors were upregulated at the onset of puberty, with no significant difference between the LP groups at this stage. Testicular estrogen receptor modulation is influenced by maternal malnutrition, resulting in reduced *FSHr* levels at the end of puberty. The expression of Sertoli cell genes decreased during peripuberty but increased later, possibly in response to the restoration of testosterone concentration. There was a decrease in the mRNA levels of genes encoding spermatogenic genes during the pubertal window, whereas the expression of the spermatogonial gene remained unchanged. These findings suggest that the maternal diet does not affect the number of undifferentiated cells in the testes of low birth weight animals. This study highlights potential epigenetic mechanisms underlying fertility and reproductive capacity in lean animals exposed to maternal nutritional deficiencies during lactation. In summary, this study opens the possibility of therapeutic interventions aimed at regulating energy balance and potentially advancing the timing of puberty, which warrants further investigation.

Supplementary data

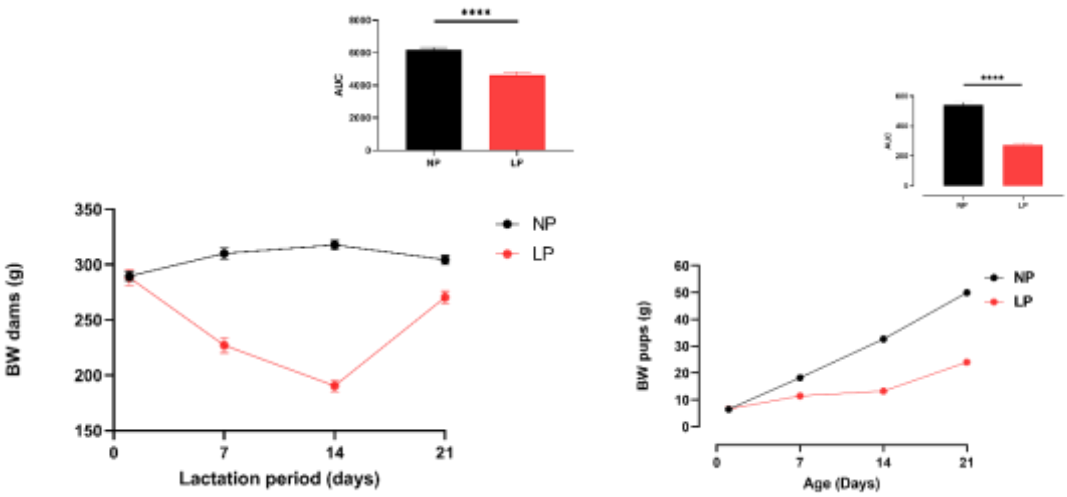

Figure 9: (**A**) Body weight of dams and (**B**) body weight of pups during the lactation period. The data are presented as the means ± SEMs of 16–20 dams and pups per experimental group. The lateral panels, as insets to figures A and B, depict the AUC. ****Significant differences in Student's t test between the NP and LP groups are represented by $P < 0.0001$. AUC, area under the curve; BW, body weight; LP, low protein; NP, normal protein.

As expected, the body weight of LP dams during lactation exhibited a notable decline, with a reduction of 25.1%, as indicated by the AUC ($P < 0.001$; Fig. 9A), resulting in a final body weight that was 11.2% lower at weaning. Over the course of the three-week lactation period, LP dams presented a 21.26% reduction in body weight during the first week, followed by an additional 16.12% decrease in the second week. In the third week, following refeeding on a standard diet, there was a + 41.96% gain in body weight, although this value remained lower than that of the NP dams during the same period. In contrast, NP dams gained + 6.8% of their body weight during the first week, followed by a further + 2.5% increase in the second week and a - 4.2% weight loss in the third week, indicating the maintenance of body composition.

As expected, the body weight of LP pups during lactation decreased by 49.7%, as indicated by the AUC ($P < 0.001$; 9B), and by 51.7% at weaning. Furthermore, the growth spurt pattern was assessed in both groups. In the LP pups, a significant increase in body

weight of 74.8% was observed at PN7 compared with PN1. At PN14, the gain was more modest, with a 15.4% increase relative to that at PN7. By PN21, the pups experienced the most significant growth, with an increase in body weight of 80.93% compared with that at the end of the second week. In contrast, the greatest body weight gain in the NP pups was observed at the conclusion of the first week, with an increase of 181.6% at PN7 relative to PN1. By PN14, which marks the end of the second week, the body weight gain was 78.5% in comparison to that at PN7. At the end of the third week, the NP offspring presented the smallest body weight gain, approximately 52.93%.

Declaration of interest

The authors declare that there are no conflicts of interest in this research.

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Supplementary Table 1A and 1B- Primers used for RT–qPCR analyses and GenBank accession numbers of target genes in whole hypothalamus and testis tissues.

1 A -Genes in whole Hypothalamus	NCBI Reference Sequence	Primer sequence (5' – 3´)
Gnrh1 (Gonadotropin-releasing hormone)	NM_012767.2	**F**: AGGAGCTCTGGAACGTCTGAT **R:** AGCGTCAATGTCACACTCGG
Kiss1 (KiSS-1 metastasis-suppressor)	NM_181692.1	**F:** GGAGCCACTGGCAAAAATGG **R:** GCCAGGCATTAACGAGTTCC
Tac3 (Tachykinin precursor 3)	NM_019162.2	**F:** TTGAAGAGAACACCCCCAGC **R:** GGTCTGAGAGTGGAGTGCTTT
Mkrn3 (Ring finger protein, 3)	NM_001399951.1	**F:** GACAGTGTTAGAGGCACGCT **R:** AGCCATAAGAAACGGTGCCA
Cyp19a1 (Aromatase)	NM_017085.2	**F:** CGTCATGTTGCTTCTCATCG **R:** TACCGCAGGCTCTCGTTAAT
Ar (Androgen receptor)	NM_012502.1	**F:** GCCATGGGTTGGCGGTCCTT **R:** AGGTGCCTCATCCTCACGCACT

Esr1 (Estradiol receptor 1)	NM_012689.1	**F:** CCATATCCGGCACATGAGTA **R:** TGAAGACGATGAGCATCCAG
Esr2 (Estradiol receptor 2)	NM_012754.1	**F:** CTCACGTCAGGCACATCAGT **R:** TGTGAGCATTCAGCATCTCC
Gper1 (G protein-coupled estrogen receptor 1)	NM_133573.1	**F:** CCCTTGACAGGCCACATAGT **R:** CTCCGTGCTGTCTGGTATGA
Rpl19 (Ribosomal protein L19)	NM_031103.1	**F:** CAATGAAACCAACGAAATCG **R:** TCAGGCCATCTTTGATCAGCT

F forward; R reverse

1 B - Genes in whole Testis	**NCBI Reference Sequence**	**Primer sequence (5' – 3´)**
Lhcgr (Luteinizing hormone/choriogonadotropin receptor)	NM_012978.1	**F:** AGTGGAGCCTTCCAGGGGGC **R:** AGGAAGACAGGGCGATGAGCGT
Fshr (Follicle stimulating hormone receptor)	NM_199237.1	**F:** TCACTGGCTGTGTCATTGCTC **R:** GAGATCTCTATTTTCTCCAGGTCTC
Cyp19a1 (Aromatase)	NM_017085.2	**F:** CGTCATGTTGCTTCTCATCG **R:** TACCGCAGGCTCTCGTTAAT
Cyp11a1 (Cytochrome P450, family 11, subfamily a, polypeptide 1)	NM_017286	**F:** GGAGCTGGTATCTCCTCTACCA **R:** TTGCCCAGCTTCTCCCTGTAA
Star (Steroidogenic acute regulatory protein)	NM_031558	**F:** ACCAAGCGTAGAGGTTCCAC **R:** TTCAGCTCTGATGACACCGC
Ar (Androgen receptor)	NM_012502.1	**F:** GCCATGGGTTGGCGGTCCTT **R:** AGGTGCCTCATCCTCACGCACT
Esr1 (Estradiol receptor 1)	NM_012689.1	**F:** CCATATCCGGCACATGAGTA **R:** TGAAGACGATGAGCATCCAG
Esr2 (Estradiol receptor 2)	NM_012754.1	**F:** CTCACGTCAGGCACATCAGT **R:** TGTGAGCATTCAGCATCTCC
Gper1 (G protein-coupled estrogen receptor 1)	NM_133573.1	**F:** CCCTTGACAGGCCACATAGT **R:** CTCCGTGCTGTCTGGTATGA
Ndrg4 (NDRG family member 4)	NM_ 001271091.1	**F:** GCCCAGCCATCCTTACCTAC **R:** GGCACACCACGAAGTGTTTG
Kit (KIT proto-oncogene receptor tyrosine kinase)	NM_ 022264.1	**F:** ATGGAAGATGACGAGCTGGC **R:** AATCTTTGTGATCCGCCCGT
Rhcg (Rh family, C glycoprotein)	NM_ 183053.1	**F:** AGCGCTGTAGGCTTCAACTT **R:** GTCGGCTTGGATGAGGTTCT
Lrrc34 (leucine rich repeat containing 34)	NM_001044696.1	**F:** CATCGGTGGTGTGGATGTGA **R:** GCCTTCAGGTCCAATGTCGT
Lgals1 (galectin 1)	NM_019904.1	**F:** TACACTTCAACCCCCGCTTC **R:** TGATGCACACCTCCGTGATG
Rpl19 (Ribosomal protein L19)	NM_031103.1	**F:** CAATGAAACCAACGAAATCG **R:** TCAGGCCATCTTTGATCAGCT

F forward; R reverse

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